# Investigations into the properties of mistletoe leaves, *Phoradendron spp.* (Viscaceae) and geophagic material consumed by *Ateles geoffroyi* (Atelidae) at sites within the Santa Rosa National Park, Costa Rica

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# ABSTRACT

This project arose because of the desire by the resident *Ateles* research observers, at Santa Rosa, to attempt to ascertain a function for what they deemed 'uncharacteristic' behaviours. The following were important considerations meriting further investigation.

- *Phoradendron* (mistletoe) consumption by *Ateles* spp. appeared to be present only at Santa Rosa
- Mistletoe selection was considered deliberate, as mature host leaf (which was available) was not selected
- More than one mistletoe was available in the normal home range of the monkey, but selection was dominated by one mistletoe/host combination
- Consumption was of relatively small volumes
- There was an apparent seasonality to the use of mistletoe
- Consumption of mature, mistletoe leaf occurred at times when there was no shortage of suitable fruit
- Limitations of the *Ateles* spp. digestive system made exploitation of mature leaf potentially problematic
- The monkeys were also seen consuming geophageous material; on occasion, this closely followed mistletoe consumption
- There were no published reports of *Ateles* spp. geophagy in Central America and/or tropical dry forest habitat

The aim of the subsequent investigation was to determine if there was any beneficial function that could be attributed to the materials and so provide a link to a self-mediation hypothesis for mistletoe and/or geophagy or to relate the determined geophagy properties to the other published functional hypotheses for geophagy.

The novel aspect of this project was the development of a specific 'gastric model' reflecting the differences between *Ateles* and human digestive systems. This modified model was used to investigate geophagic and *Phoradendron* samples. It was hoped that this approach would lead to the identification or constituents in the samples, which may have physiological significance.

Samples of the two species of mistletoe identified were collected from three *Phoradendron/*host tree combinations. Samples were extracted using the simulated gastric conditions and the extracts analysed. Analytical 'fingerprints' of the gastric extracts of the two species were obtained together with the antimicrobial activities of the extracts.

Species variation in Phoradendron constituents and antimicrobial activity was detected. The principal difference between the eaten and non-eaten Phoradendron species was identified using HPLC and LC-MS, as chlorogenic acid. Chlorogenic acid has antibacterial and antioxidant properties, stimulates the immune system and has activities related to regulation of blood sugar levels. *Phoradendron* consumption wass during the wet season, which may be a period of increased bacterial and parasite infection. It also coincides with a change in dietary fruits.

The geophagic samples were taken from sites previously used by *Ateles,* together with 'control' sites found in the home range of the study group. Analysis of the physical properties and characteristics was undertaken to attempt to identify the mineral content material. Further analyses then investigated the behaviour of the material in relation to the commonly accepted hypotheses for geophagy. Where possible these were investigated using the simulated gastric conditions.

The physical characteristics of the samples did not resemble the previously published reports for geophagic material used by humans or non- human primates. The results failed to detect the presence of montmorillonite and only a suggestion of the presence of kaolinite. The results do not suggest that it functions as an antacid, an anti-diarrhoeal or mineral supplement.

An increase in antibacterial activity was seen when geophagic material and *Phoradendron* samples were incubated together. A putative hypothesis for the mechanism of Fe limitation was suggested by the physical properties of the geophagic material and the Fe chelating potential of the chemical constituents of the *Phoradendron* leaf.

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I would like to dedicate this work to Chris,

my sons Colin and Andy

and my late mother.

They all encouraged me to follow my dream

# Table of Contents

## Chapter 1 Introduction and Literature review

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## 1.1 Project Background Introduction

For over 30 years, extensive studies have taken place of the behaviour and ecology of the three primate species found in the Santa Rosa National Park, Costa Rica: the black handed spider monkey (*Ateles geoffroyi*), the mantled howler monkey (*Alouatta palliata*) and the white-throated capuchin (*Cebus capucinus*) (Fedigan *et al.* 1985, Chapman 1987, 1988, Sorensen *et al.* 2000, Campbell *et al.* 2003, 2005, Asensio *et al.* 2012a,b). During the last 10 years, periodic observations were made of the consumption by *Ateles geoffroy*i (Figure 1.1) of small amounts (1-2 leaves) of **mature** mistletoe leaves, *Phoradendron* spp., genus Viscaceae and geophagy of dry material, defined as the deliberate consumption of soil, rock particles or clay materials. Observations of *Phoradendron* consumption and geophagy were made by the long-term research assistant Elvin Murillo-Chacon, together with visiting students and researchers (Filippo Aureli personal communication).

Figure 1.1 Male *Ateles geoffroy*i at Santa Rosa (courtesy of F. Aureli).

The mistletoe berries are the staple food for several species of neo-tropical birds present in Santa Rosa (Henderson 2010). Mistletoe leaves are consumed by several leaf eating (folivorous) mammals including deer (Gallina 1988) and gorilla (Watson 2001), consumption often linked to food scarcity. The omnivorous brown mouse lemur (*Microcebus rufus*) (Atsalis 2008) and the predominantly folivorous diadem sifaka (*Propithecus didema*) (Irwin 2008) both utilise a mistletoe species as a staple food in times of low fruit availability and in the latter case it is classified as a fallback resource (Irwin 2008). Fallback foods are defined as being of relatively poor nutritional quality, which become a significant dietary component during periods when preferred foods are scarce (Marshall *et al.* 2009). So far, there have not been reports in the literature for a predominantly fruit eating (frugivore) primate species consuming mistletoe leaves. *Phoradendron* leaf consumption has only been reported by *Ateles* at Santa Rosa as consumption was not observed in four other *Ateles geoffroyi* populations studied in Belize, Panama, Mexico and the Corcovado National Park, Costa Rica (Santorelli *et al.* 2011).

Young *et al*. (2011) extensively reviewed the literature on geophagy in human populations and 297 species of mammals, birds, and reptiles. Many common functions have been put forward for geophagy all suggesting a medicinal/beneficial use (Gilardi *et al.* 1999, Krishnamani *et al.* 2000, Dominy *et al.* 2004). Geophagy in Old World primates has been documented in gorillas (Mahaney *et al.* 1990), chimpanzees

(Aufreiter *et al.* 2001), baboons (Pebsworth *et al.* 2012a,b), macaques (Voros *et al.* 2001), lemurs (Jeannoda *et al.* 2003) and orangutans (Matsubayashi *et al.* 2011). In New World primates geophagy is reported in tamarins (Heymann *et al.* 1991), titi monkeys (Müller *et al.* 1997), howler monkeys (Bicca-Marques *et al.* 1994) and spider monkeys (Izawa 1993, Blake *et al.* 2010, Link *et al.* 2011a, b). Until recently, only one instance has been reported for *Ateles geoffroyi* in Central America, which was related to gnawing the limestone of a Mayan temple in Guatemala (Ferrari *et al.* 2008). As in the case of *Phoradendron* leaf consumption, the comparative study of five *Ateles geoffroyi* populations across Central America found that geophagy occurs only at Santa Rosa (Santorelli *et al*. 2011).

Commonly *Ateles* reported geophagy sites are adjacent to riverbanks or situated along small natural drains or intermittent stream courses and are also utilised by many non-primate species e.g. birds, bats, rodents and ungulates (Blake *et al.* 2011, Blake *et al.* 2013). Sites may also be wet mineral/salt licks (salados) which are long lasting seasonally stable sites, used over extended periods (Shimooka 2005). The sites utilised at Santa Rosa differed in that they were elevated, often on vertical faces and the material was dry and accessible to very few non-primate species (Filippo Aureli and Elvin Murillo-Chacon personal communication).

Both *Phoradendron* consumption and geophagy behaviours were considered by the established researchers at Santa Rosa to be sufficiently different from other reported primate behaviours to merit further investigation. An important further consideration was the significant amount of literature on the biologically active and potentially toxic constituents reported in mistletoes from the genus Viscaceae (Section 1.8) together with the various hypotheses for the functions of geophagy (Section 1.9).

## 1.2 Research Site

The Santa Rosa National Park, Costa Rica, established in 1971, is located in Guanacaste province on the NW Pacific coast close to the Nicaraguan border (Figure 1.2).


Figure 1.2 Map of Santa Elena Peninsula, showing the Santa Rosa Tropical Dry Forest Research station (Centro de Estudios Bosque Tropical Seco). http://www.costarica-nationalparks.com/santarosanationalpark.html (accessed Sept. 2012).

The park is part of the much larger Area de Conservación Guanacaste (ACG) which is a Natural World Heritage Site. The Santa Rosa National Park covers an area of 10,800 hectares, on the Santa Elena

Peninsula. The park lists over 115 species of mammals, over 10,000 species of insects, 250 species of birds, and around 100 species of amphibians and other reptiles (www.acguanacaste.ac.cr/1997, accessed Aug 2012). The park also contains one of the largest remaining areas of Tropical Dry Forest (TDF) habitat in the world.

## 1.3 Introduction to the spider monkey *Ateles geoffroyi*

Four candidate species of spider monkeys have been identified from morphological and chromosomal analyses: *Ateles geoffroyi*, *A. paniscus, A. belzebut*h and *A. hybridus* (Collins 2008). Populations are found from Yucatan in Mexico through Central America and from Ecuador in the west across to Guyana and Surinam in the north eastern region of South America and as far south as the Mato Grosso province in central Brazil (IUCN. 2012). *Ateles* commonly use low altitude humid rain forests below 800m (Collins 2008) however are also reported in tropical forests in the Andes, upland Amazonian forests, deciduous and mangrove forests (Wallace 2008). Until recently, *Ateles geoffroyi* was considered to be of low conservation concern because of their wide distribution, but in 2008 the species was reclassified as endangered due to extensive deforestation across Central America (The IUCN Red List of Threatened Species. Version 2014.2).

*Ateles* have a typical home range of 150-350 hectares with day journey lengths of 500 - 4500m, but do not use the ranges uniformly. Range use varies with season and study site (Wallace 2008). *Ateles* have skeletal adaptations to hand, forelimb, clavicle, pelvic girdle and hind limb that facilitate rapid movement high in the canopy. When venturing onto the ground, they often walk bipedally due to their skeletal morphology, which may make them vulnerable to attack on the ground (Campbell *et al.* 2005). *Ateles* consume ripe fruits in the upper canopy rarely visit the lower canopy and are rarely seen on the ground (Campbell *et al*. 2005). Avoidance of using the ground has been suggested as a mechanism to avoid predation (Cowlishaw 1997, Stanford 2002).

Anatomically and physiologically, the digestive system of *Ateles* is not adapted for leaf eating (folivory) (Chivers *et al.* 1980, Lambert 1998, Wallace 2005). *Ateles geoffroyi* and *Alouatta palliata* are similar sized and relatively closely related but have very different digestive strategies. *Alouatta palliata* have fermentative guts and a transit time of approx. 20 hours. This permits them to extract nutrients from leafy plant material (Milton 1981). *Ateles geoffroyi* lack the fermentative capacity and have a rapid gut transit time of 4-5 hours. This allows them to exploit a high-energy diet containing soluble sugars but limits the ability to obtain protein from dietary items (Lambert 1998). The majority of fruits consumed by *Ateles* are ripe and fleshy (Di Fiore *et al.* 2008) and 75–90% of foraging time is spent consuming ripe fruit (Chapman 1987, 1988, Symington 1988, Cant 1990, Castellanos *et al.* 1996, Dew 2002, Wallace 2005). Spider monkeys also consume unripe figs even when ripe figs are available (Felton *et al.* 2008).

### 1.3.1 *Ateles geoffroy*i diet at Santa Rosa

At Santa Rosa *Ateles geoffroyi* consume 73 species of fruit from 36 families (Filippo Aureli personal communication Table 1.1). However, 46 species are categorised as occasional, (defined as less than 5 independent observations per year; Filippo Aureli personal communication), with fruit consumed regularly from only 27 species (regularly was defined as more than 5 independent observations per year). In contrast, *Ateles*

*belzebuth belzebuth* in Ecuador consume more than 250 species of plant (Di Fiore *et al.* 2008). This difference may be a limitation imposed by habitat as the tropical dry forest of Santa Rosa contains only 30-50% of tree

Table 1.1 Fruits consumed at Santa Rosa, Occasional is defined as <5 independent observations/year.

| **PLANT TAXONOMY** | | | | | |
|---|---|---|---|---|---|
| **Family** | **Species** | **Fruit** | **Family** | **Species** | **Fruit** |
| Anacardiaceae | *Spondias mombin* | regular | | *Ficus goldmanii* | occasional |
| | *Spondias purpurea* | occasional | | *Ficus hondurensis* | regular |
| | *Spondias radlkoferi* | regular | | *Ficus morazaniana* | regular |
| Annonaceae | *Annona purpurea* | occasional | | *Ficus obtusifolia* | regular |
| | *Annona reticulata* | occasional | | *Ficus ovalis* | regular |
| Apocynaceae | *Stemmadenia obovata* | occasional | Musaceae | *Maclura tinctoria* | regular |
| Araliaceae | *Sciadodendron excelsum* | regular | Myrtaceae | *Musa cavendishii* | occasional |
| Asclepiadaceae | *Marsdenia engleriana* | occasional | | *Eugenia acapulcensis* | occasional |
| Bombacaceae | *Bombacopsis quinata* | occasional | Papilionaceae | *Eugenia salamensis* | occasional |
| | *Pseudobombax septenatum* | occasional | Passifloraceae | *Diphysa americana* | occasional |
| Boraginaceae | *Cordia panamensis* | occasional | | *Passiflora platyloba* | occasional |
| Burseraceae | *Bursera simaruba* | occasional | Polygonaceae | *Passiflora vitifolia* | occasional |
| Caricaceae | *Jacaratia dolichaula* | occasional | Rhamnaceae | *Coccoloba guanacastensis* | regular |
| Clusiacea | *Garcinia intermedia* | occasional | | *Karwinskia calderoni* | regular |
| Dilleniaceae | *Doliocarpus dentatus* | regular | Rubiaceae | *Krugiodendron ferreum* | regular |
| Ebenaceae | *Diospyros salicifolia* | regular | | *Alibertia edulis* | occasional |
| Elaeocarpaceae | *Sloanea terniflora* | occasional | | *Calycophyllum candidissimum* | occasional |
| Euphorbiaceae | *Sapium glandulosum* | occasional | | *Chomelia spinosa* | occasional |
| Fabaceae | *Inga vera* | occasional | | *Genipa americana* | regular |
| | *Samanea samon* | occasional | | *Guettarda macrosperma* | regular |
| | *Swartzia cubensis* | occasional | | *Ixora floribuna* | occasional |
| | *Quercus oleoides* | occasional | Sapindaceae | *Randia monantha* | occasional |
| | *Vachellia collinsii* | occasional | | *Allophylus occidentalis* | regular |
| Flacourtiaceae | *Casearia arguta* | occasional | Sapotaceae | *Dipterodendron costaricense* | regular |
| | *Muntingia calabura* | occasional | | *Chrysophyllum brenesii* | occasional |
| | *Prockia crucis* | occasional | | *Manilkara chicle* | regular |
| | *Zuelania guidonia* | occasional | Simaroubaceae | *Sideroxylon capiri* | regular |
| Lauraceae | *Ocotea veraguensis* | occasional | Sterculiaceae | *Simarouba glauca* | regular |
| Malpighiaceae | *Bunchosia ocellata* | regular | | *Guazuma ulmifolia* | regular |
| Melastomataceae | *Mouriri myrtilloides* | occasional | Styracaceae | *Sterculia apetala* | occasional |
| Meliaceae | *Trichilia americana* | occasional | Tiliaceae | *Styrax argenteus* | occasional |
| | *Trichilia martiana* | occasional | | *Apeiba tibourbou* | occasional |
| Moraceae | *Brosimum alicastrum* | regular | | *Luehea candida* | occasional |
| | *Castilla elastica* | regular | Urticaceae | *Luehea speciosa* | occasional |
| | *Ficus bullenei* | regular | Vitaceae | *Cecropia peltata* | regular |
| | *Ficus citrifolia* | occasional | | *Cissus pseudosicyoides* | occasional |
| | *Ficus cotinifolia* | regular | | | |

species diversity compared to tropical rainforests in Costa Rica (Hubbell 1979). In the low diversity sub-tropical

habitat at Tikal National Park, Guatemala a similarly low number of 30-40 fruit species are consumed by *Ateles* (Chapman 1987, Cant 1990).

Leaf consumption was limited to material from 10 families and 13 species, (Table 1.2). Regular consumption of mature leaves was limited to *Phoradendron quadrangulare*, with consumption consisting of a few leaves and not occurring during periods of food scarcity (Filippo Aureli personal communication). The distinction between young (small, pale green) and mature *Phoradendron quadrangulare* leaves (large, fully developed, darker green) is illustrated in Figure 1.3. Mistletoes are very slow growing and only produce their first leaves after two years; the leaf axils send out just one or two stalks with two leaves each year which continue to grow for a further 1-2 years and commonly mistletoes do not start flowering until aged 5 to 7 years (Calder 1983).

Table 1.2 Species from which leaves are consumed by *Ateles* at Santa Rosa, (courtesy of Filippo Aureli).

| **Plant taxonomy** | | **Plant Material** |
|---|---|---|
| **Family** | **Species** | **Leaf type / frequency*** |
| Anacardiaceae | *Astronium graveolens* | young – occasional |
| Apocynaceae | *Forsteronia spicata* | young - occasional |
| Apocynaceae | *Marsdenia engleriana* | mature - ccasional |
| Araceae | *Syngonium angustatum* | mature - ccasional |
| Boraginaceae | *Cordia panamensis* | mature - ccasional |
| Fabaceae | *Diphysa americana* | young - occasional |
| Fabaceae | *Hymenaea courbaril* | young - **regular** |
| Fabaceae | *Samanea saman* | mature - ccasional |
| Lauraceae | *Ocotea veraguense* | young - occasional |
| Moraceae | *Brosimum alicastrum* | young - occasional |
| | *Ficus cotinifolia* | young - occasional |
| | *Ficus hondurensis* | young - occasional |
| Malvaceae | *Apeiba tibourbou* | mature -occasional |
| Urticaceae | *Cecropia peltata* | young - occasional |
| Viscaceae | *Phoradendron quadrangulare* | mature - **regular** |

*occasional defined as less than 5 independent observations per year; regular defined as more than 5 independent observations per year.

The four species of host tree parasitised by *Phoradendron* are distributed throughout the Santa Rosa area and are present within the spider monkey home ranges. Deliberate selection of the mistletoe leaf, while **not** eating the host leaf was clear to the observers (Elvin Murillo-Chacon personal communication).

Figure 1.3 Example of young and mature *Phoradendron quadrangulare* leaves; mature shows signs of biting. (Author's own photograph).

Taxonomy and images of host tree leaf and *Phoradendron* leaf are presented in Appendix 1.1, Table 1.1. The differences in appearance between the host tree leaf and the *Phoradendron* leaves can clearly be seen.

The monkeys made route adjustments or stops to consume the *Phoradendron* (Filippo Aureli personal communication). In addition, there was seasonal variation in its consumption, which was predominantly during the September-November period. This deliberate consumption of limited amounts of **mature** *Phoradendron* leaves, in the context of the gastro intestinal morphology and transit time limitations (Section 2.1) suggests that consumption may serve some function for the well-being of the monkeys.

## 1.4 *Phoradendron* Identification

During the initial observation of the *Phoradendron* consumption, the behaviour was linked to three host tree species. These were *Luehea speciosa* Willd. (LS); *Manilkara chicle* (Pittier) (MC), *Tabebuia ochracea (*Cham) (TO). *Phoradendron* consumption was rare from *Guazuma ulmifolia* Lam (GU). Over 95% of observed *Phoradendron* consumption was from *Manilkara chicle*. Subsequently, the resident botanist identified two mistletoe species, based on flower morphology *Phoradendron quadrangulare* (Kunth) Krug & Urb growing on the host trees *Manilkara chicle* (MC) and *Guazuma ulmifolia* Lam (GU) and *Phoradendron robustissimum* Eichler growing on *Luehea speciosa*, (LS) and *Tabebuia ochracea* (TO).

Specimens were deposited at Instituto Nacional de Biodiversidad (INBio), where the identities were confirmed in 2009, and voucher specimens lodged, as shown:

*Phoradendron robustissimum* Eichler

| | | | |
|---|---|---|---|
| R. Aguilar 3191 | 1586137; | L. González 736 | 2845349 |

*Phoradendron quadrangulare* (Kunth) Krug & Urb

| | | | |
|---|---|---|---|
| L. González 3519 | 3975549; | R. Espinoza | 9901589424 |

## 1.5 *Phoradendron* Consumption

Observations of *Phoradendron* consumption were carried out whilst collecting data as part of the long-term study (not by the thesis author). As a background to the authors' project, spider monkey data recorded for 31 consecutive months between June 2006 and December 2008 were made available.

Table 1.3 *Phoradendron* eating frequency data, (for identified individuals) during September 2006-August 2007 (Courtesy of Filippo Aureli).

| **Id.** | ***Phoradendron* eating episodes** | **Time in followed subgroup (minutes)** | ***Phoradendron* eating rate /100 hours** | **Id** | ***Phoradendron* eating episodes** | **Time in followed subgroup (minutes)** | ***Phoradendron* eating rate /100 hours** |
|---|---|---|---|---|---|---|---|
| An | 1 | 3404 | 1.8 | In | 10 | 23323 | 2.6 |
| Be | 5 | 25587 | 1.2 | Ju | 1 | 3625 | 1.7 |
| Bg | 7 | 9034 | 4.6 | Mc | 6 | 5285 | 6.8 |
| Cc | 5 | 12269 | 2.4 | Md | 8 | 39318 | 1.2 |
| Cn | 0 | 2129 | 0 | Ne | 0 | 2279 | 0 |
| Co | 7 | 6458 | 6.5 | Pp | 7 | 13797 | 3 |
| Cu | 6 | 7458 | 4.8 | Si | 8 | 22384 | 2.1 |
| Es | 11 | 34453 | 1.9 | Sy | 10 | 39373 | 1.5 |
| Fr | 0 | 4091 | 0 | Ta | 3 | 3690 | 4.9 |
| Gh | 8 | 27119 | 1.8 | To | 1 | 2462 | 2.4 |
| Gr | 12 | 40602 | 1.8 | Zr | 5 | 4727 | 6.3 |
| Hu | 11 | 26414 | 2.5 | | | | |

Subgroups were followed during the entire course of the daylight hours balancing observations between mornings and afternoons when whole day follows were not possible and subgroups were followed 3-5 days a week (Filippo Aureli personal communication). Tables 1.3 and 1.4 illustrate the relative frequency of *Phoradendron* consumption in order to provide context to the authors' project. *Phoradendron* eating rate data were not available for the period September 2007 - December 2008. However, Table 1.4 details the number of episodes for each individual and the number of individual days on which it was observed.

Table 1.4 Number of *Phoradendron* eating episodes and number of independent days in which such episodes were observed during September 2007-December 2008 (courtesy Filippo Aureli).

| Individual | *Phoradendron* eating episodes | number of individual days | Individual | *Phoradendron* eating episodes | number of individual days |
|---|---|---|---|---|---|
| Be | 12 | 9 | Ha | 13 | 11 |
| Bg | 14 | 12 | Hu | 19 | 15 |
| Bu | 15 | 12 | In | 19 | 17 |
| Cc | 8 | 7 | Md | 17 | 13 |
| Cl | 1 | 1 | Ne | 11 | 8 |
| Co | 13 | 9 | Pp | 5 | 5 |
| Cu | 12 | 10 | Pq | 7 | 6 |
| Es | 18 | 5 | Sb | 8 | 7 |
| Fr | 12 | 11 | Si | 15 | 14 |
| Gh | 14 | 12 | Sy | 18 | 16 |
| Gr | 20 | 17 | Ta | 4 | 4 |

Leaf consumption would reduce the amount of fruit able to be consumed and constitutes extra mass to be transported therefore it would be expected that a frugivore would avoid leaf consumption during the main feeding/foraging periods. Fruit consumption is generally reported as occurring mainly during the morning and a little around mid-day – and in some populations there may be a peak in late-afternoon or prior to a resting period (Chapman *et al.* 1991, Di Fiore *et al.* 2008). The use of leaves, often considered a protein source (Milton 1979, 1980 in Milton 1999) would be expected to occur in late afternoon, to avoid filling up the gut in place of fruits and to permit the required longer leaf digestion time. This separation of time of consumption of fruits and leaves does not seem to apply in the case of *Phoradendron* leaves as prior to eating them the monkeys had been observed foraging and consuming fruits of *Ficus* spp., *Castilla elastic, Spondias mombin, Spondias radlkoferi* and *Maclura tinctoria* (Filippo Aureli personal communication).

## 1.6 Geophagy Observations

In order to provide context to the project data on geophagy episodes (not collected by the author) were made available for the period Sept. 2006-Dec 2008, (Tables 1.5-1.6).

Table 1.5 Geophagy rates September 2006 (courtesy of Filippo Aureli).Data relates to four independent episodes in 2006.

| Id | Geophagy episodes | Time in followed subgroup (minutes) | Geophagy rate per 100 hours | Id | Geophagy episodes | Time in followed subgroup (minutes) | Geophagy rate per 100 hours |
|---|---|---|---|---|---|---|---|
| Be | 1 | 25587 | 0.2 | In | 1 | 23323 | 0.3 |
| Bg | 4 | 9034 | 2.7 | Mc | 4 | 5285 | 4.5 |
| Co | 4 | 6458 | 3.7 | Md | 1 | 39318 | 0.2 |
| Cu | 2 | 7458 | 1.6 | Sy | 1 | 39373 | 0.2 |
| Es | 2 | 34453 | 0.3 | To | 1 | 2462 | 2.4 |
| Hu | 3 | 26414 | 0.7 | | | | |

No instances of geophagy were recorded for the period 10 November 2006-18 September 2008 i.e.no instances were observed in 2007.

Table 1.6 Number of geophagy episodes and number of independent days observed September 2008 - December 2008 (courtesy Filippo Aureli).

| Individual | Geophagy episodes | number of individual days | Individual | Geophagy episodes | number of individual days |
|---|---|---|---|---|---|
| Be | 1 | 1 | Hu | 2 | 2 |
| Bg | 4 | 3 | In | 2 | 2 |
| Bu | 2 | 2 | Ka | 1 | 1 |
| Cc | 1 | 1 | Lo | 2 | 2 |
| Co | 2 | 2 | Md | 2 | 2 |
| Cu | 5 | 3 | Ne | 2 | 2 |
| Es | 1 | 1 | Pp | 1 | 1 |
| Fr | 4 | 3 | Hu | 2 | 2 |
| Gh | 1 | 1 | In | 2 | 2 |
| Gr | 2 | 2 | Ka | 1 | 1 |
| Ha | 4 | 4 | Lo | 2 | 2 |

*No data on the time in the followed subgroup was made available to calculate rates.

*Phoradendron* consumption was recorded on 12 occasions between 9 June -14 December 2006. On three of the four occasions in 2006, *Phoradendron* consumption was followed by geophagy by a number of individuals (Table 1.7), typically occurring within 10-45 minutes. On one occasion (10 November) geophagy by five individuals was unrelated to *Phoradendron* consumption.

Table 1.7 Time of combined *Phoradendron* consumption and geophagy 2006 (courtesy of Filippo Aureli).

| Date | Id | Time *Phoradendron* consumption observed | Time geophagy observed | Date | Id | Time *Phoradendron* consumption observed | Time geophagy observed |
|---|---|---|---|---|---|---|---|
| 15/10/2006 | Bg | 11:24 | 11:34 | 25/10/2006 | Bg | 11.35 | 12.10 |
| | Es | 11:24 | 11:34 | | Bg | 13.41 | Not observed |
| | Hu | 11:24 | 11:33 | | Bu | 11.11 | 12.12 |
| | In | 11:24 | Not observed | | Co | 11.35 | Not observed |
| | Ju | 11:24 | Not observed | | Co | 13.37 | Not observed |
| | To | 11:24 | 11:34 | | Cu | 11.11 | Not observed |
| 19/10/2006 | Be | 10:23 | 11:01 | | Cu | 11.35 | 12.11 |
| | Bg | 10:23 | Not observed | | Cu | 13.41 | Not observed |
| | Cc | 10:23 | Not observed | | Ha | 11.40 | 12.12 |
| | Co | 10:23 | 11:01 | | Hu | 11.11 | Not observed |
| | Cu | 10:23 | 11:01 | | Hu | 11.35 | 12.12 |
| | Es | 10:23 | Not observed | | Mc | 11.35 | 12.09 |
| | Gh | 10:23 | Not observed | | | | |
| | Gr | 10:23 | Not observed | | | | |
| | Hu | 10:23 | 11:01 | | | | |
| | In | 10:23 | Not observed | | | | |
| | Mc | 10:23 | 11:01 | | | | |
| | Md | 10:23 | 11:01 | | | | |
| | Pp | 10:23 | Not observed | | | | |
| | Si | 10:23 | Not observed | | | | |
| | Sn | 10:23 | Not observed | | | | |
| | Sy | 10:23 | Not observed | | | | |
| | Zr | 10:23 | Not observed | | | | |

A similar juxtaposition of leaf eating and geophagy was reported by Klein (2008) who reported chimpanzees ingesting 'soil' shortly before or after consuming some plant parts, specifically the leaves of

*Trichilia rubescens*, which have *in vitro* antimalarial properties, significantly reporting soil ingestion increased the *in vitro* bioactivity of the bio-available gastric fraction from the leaves. Geophagy following *Phoradendron* consumption may act in a similar manner.

During 2008 geophagy was only observed on three occasions, and commonly followed *Phoradendron* consumption (Table 1.8) typically occurring within a relatively short time period of 10-45 mins. The numbers of additional individuals observed undertaking either behaviour has increased over the period 2006-2007 to 2008. *Phoradendron* consumption was seen in six new individuals and geophagy in nine new individuals.

Primates are usually selective in the choice of geophagy sites and only use a few (Krishnamani *et al.* 2000). There are three characteristics where geophagy at Santa Rosa differed from the majority of the literature: site choice, the number of incidences and seasonality. The site type utilised at Santa Rosa differs from what is reported in much of the literature for *Ateles* (Izawa 1993, Shimooka 2005, Blake *et al.* 2010, Link *et al.* 2011a,b) in that it was not at a salido. At Santa Rosa, dry material was taken from sites away from watercourses, commonly from vertical surfaces of low cliffs.

Table 1.8 Time of *Phoradendron* consumption and geophagy 2008 (courtesy of Filippo Aureli)

| Date | Id | *Time Phoradendron* Consumption observed | Time geophagy observed | Date | Id | *Time Phoradendron* Consumption observed | Time geophagy observed |
|---|---|---|---|---|---|---|---|
| 18/09/2008 | Bg | 08:53 | 09:14 | 06/10/2008 | Be | 09:32 | 10:03 |
| | Bg | 12:34 | Not observed | | Bg | 09:32 | 09:43 |
| | Co | 08:53 | Not observed | | Bu | 09:32 | 10:00 |
| | Cu | 08:53 | 09:13 | | Cc | 09:32 | 10:01 |
| | Cu | 12:34 | Not observed | | Co | 09:32 | 09:59 |
| | Fr | 08:53 | 09:13 | | Cu | 09:32 | 09:42 |
| | Ha | 08:53 | 09:14 | | Es | 09:32 | Not observed |
| | Ha | 12:34 | Not observed | | Fr | 09:32 | 09:43 |
| | Nc | 08:53 | Not observed | | Gh | 09:32 | 10:03 |
| | Ne | 08:53 | 09:17 | | Gr | 09:32 | 09:35 |
| 22/09/2008 | Bg | 11:42 | 11:59 | | Ha | 09:32 | 09:59 |
| | Bu | 11:35 | 11:59 | | Hu | 09:32 | 09:59 |
| | Bu | 11:43 | Not observed | | In | 09:32 | 09:59 |
| | Cc | 11:42 | Not observed | | Md | 09:32 | 10:03 |
| | Co | 11:35 | Not observed | | Pp | 09:32 | 10:03 |
| | Co | 11:43 | 12.04 | | Pq | 09:32 | 10:01 |
| | Cu | 11:43 | 11:58 | | Ru | 09:32 | 10:15 |
| | Es | 11:35 | Not observed | | Sb | 09:32 | 10:05 |
| | ES | 11:43 | 12:01 | | Si | 09:32 | 09:43 |
| | Fr | 11:43 | 12:00 | | Sy | 09:32 | 09:40 |
| | Gr | 11:43 | 12:01 | | | | |
| | Ha | 11:43 | Not observed | | | | |
| | Hu | 11:35 | 11:58 | | | | |
| | Hu | 11:42 | 11:59 | | | | |
| | In | 11:42 | 12:01 | | | | |
| | Md | 11:43 | 12:04 | | | | |
| | Ne | 11:35 | 12:04 | | | | |
| | Pq | 11:42 | 11:59 | | | | |
| | Sb | 11:42 | 12:05 | | | | |
| | Si | 11:42 | 12:00 | | | | |

A comparison of the incidences of geophagy observed at Santa Rosa (Tables 1.5 -1.6) highlights how infrequent it is compared with other sites. Blake *et al*. (2010) reported 66 days independent visits by *Ateles*

*belzebuth* during a 3 year period; Link *et al*. (2011a) reported *Ateles hybridus* making a monthly average of 21.7 +/- 7.2 visits per 100 days of camera trapping and Link *et al*. (2011b) reported *Ateles belzebuth* making 229 visits during 1612 days of observation. In contrast, at Santa Rosa geophagy is a rarely observed event, 7 days during the June 2006 – December 2008 study period.

Table 1 .9 25-year mean weather data for Liberia District (10º35 N; 85º32 W; 80m), Guanacaste Province (1977-2012). Costa Rica Weather Service (accessed Jan 2013).

| | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mean max temp $^0$C | 33.4 | 34.4 | 35.4 | 35.9 | 33.9 | 32.0 | 32.1 | 32.1 | 31.3 | 30.9 | 31.6 | 32.5 |
| Mean rainfall mm | 14 | 14 | 56 | 298 | 2168 | 2521 | 1600 | 2329 | 3725 | 3248 | 988 | 119 |
| Mean No. days with rain | 0 | 0 | 1 | 3 | 13 | 19 | 15 | 18 | 22 | 21 | 10 | 4 |
| Relative humidity (%) | 66 | 64 | 61 | 63 | 74 | 84 | 80 | 82 | 86 | 87 | 81 | 72 |

Regarding seasonality, *Ateles belzebuth* (Blake *et al*. 2010) and *Ateles hybridus* (Link *et al*. 2011b) showed a higher incidence of geophagy during dry sunny periods, or during the dry season. At Santa Rosa, the incidences of geophagy were during September-October i.e. the critical months of the wet season, as the two months have the highest rainfall (Table 1.9). Both *Phoradendron* consumption and geophagy occurred at a time when there was no scarcity of fruits as there are more fruits available in the wet than the dry season at Santa Rosa (Asensio *et al.* 2009).

*Ateles* social system is characterised by a high degree of fission–fusion dynamics where all community members are rarely together but split up and join in subgroups of variable size and composition (Aureli *et al.* 2008, Asensio *et al.* 2009). Consequently, individuals are not equally present in the followed subgroups. It is probable therefore, that instances of both behaviours were missed due to the limitations imposed by variation in sub-group composition (Filippo Aureli personal communication). As individuals cannot be observed continually this is addressed by the calculation of rates (frequency/time of observation). However, in a study of the same community of *Ateles geoffroyi* (during Jan 2005-Dec 2008), Asensio *et al.* (2012a) found that most community members contributed significantly to the data set. Thus, any level of bias due to the social dynamics would be expected to be similar for other spider monkey populations. However, the variability in subgroup composition and the related potential bias may make comparisons with non *Ateles* species problematic.

## 1.7 Self-Medication Hypotheses

Freelander *et al*. (1974) were among the first to consider the consequences of consumption of Plant Secondary Metabolites (PSM) on **herbivores**, whilst Dearing *et al.* (2005) provide a more recent review. Both discuss the classes of PSM compounds, the toxicology and detoxification and suggest strategies herbivores may use to reduce the detrimental impacts. More recent work focused on primates (Hart 2005, Huffman 2005).

Ingestion of PSM, at levels commonly present in plant material, may have serious physiological effects or may be fatal. Therefore, animals require mechanisms for detoxifying, excreting or avoiding PSM.

"*Every plant produces its own set of secondary chemical compounds, which to a greater extent are unique to it or its species*", (Freeland et al. 1974, p 284).

Production of enzymes for detoxification and excretion utilises resources (e.g. amino acids) diverting them from other physiological processes and so is costly to the animal. Selection of material with variable

levels of PSM has been documented for cattle, sheep, voles, rats and hyrax (McLean 1970, Freeland 1973, Franke *et al*. 1936, Hilker e*t al* 1967, Norman 1970, and Coe 1967 in Freelander *et al*.1974) and lemurs (Carrai *et al.* 2003, Negre *et al.* 2006).

The potential avoidance and/or exploitation of PSM by primates have been investigated by many researchers (e.g. Glander 1977, 1982, 1994, Mowery *et al.* 1997, Simmen *et al.* 2005). Janzen (1978) was the first to describe probable use by vertebrates of PSM as an antiparasitic, attributing the lack of protozoan parasites in *Colobus guereza* and *Colobus badius*, in Kibale, to regular consumption of PSM. Subsequently antiparasitic self-medication became the focus of much work (e.g. Jisaka *et al.* 1992, Coop *et al.* 1996, Huffman 1997, Lozano 1998, Hemmes *et al.* 2002, Hutchings *et al.* 2003, Min *et al.* 2003, Hoste *et al.* 2006, Krief *et al.* 2006, Negre *et al.* 2006, Singer *et al.* 2009). Other potential beneficial uses of PSM have been identified e.g. antimicrobial and antiviral (hydrolysable tannins) (Buzzini *et al.* 2008, Chirumbolo 2011); anti-inflammatory (phenylpropanoids) (Korkina *et al.* 2011) and antioxidant (phenylpropanoids, flavonoids) (Heim *et al.* 2002, Korkina 2007); cardio-protective (flavonoids) (Cook *et al.* 1996, Testai *et al.* 2013) and immunomodulation (several classes of PSM) (Andersen *et al.* 2010, Provenza *et al.* 2010).

Ethnomedical (the use by indigenous peoples) or veterinary examples and biological activity have been used to support animal self-medication hypotheses. There are however problems when extrapolating data between species. There may be gastrointestinal morphological differences across species, such as the number of stomachs in ruminants. In monogastric species, the stomach plays an important role in the disintegration and dissolution of drug formulations. This together with gastric emptying controls the rate of the 'drug' reaching the proximal gut and being absorbed (Reppas *et al.* 2010, Toutain *et al.* 2010). There are also inter-specific differences in the enzymes involved in function/metabolism/detoxification of 'medicinal' compounds; genetic differences may contribute to both qualitative and quantitative differences in drug metabolism between species (Martignoni *et al.* 2006).

Medicinal products may be used prophylactically or therapeutically; each situation has different criteria and characteristics.

### 1.7.1 Prophylaxis

Prophylaxis is the prevention or reduction in the probability of a future illness due to infection or ingestion of a toxin. In these cases, the self-medicating individual may be asymptomatic to the observer (Hart 2005) such as when the host has reached a level of tolerance of parasite load or may be infection free. The driver for self-medicating behaviour in animals may be hard to determine unequivocally in field situation (Hart 2005). Prophylaxis may be episodic where, for example, the risk factors vary (Hart 2005) e.g. the use of antimalarial or antiparasitic products where exposure risks are variable (Altizer *et al.* 2006). Seasonal changes may alter risks e.g. changes in host immunity (Nelson *et al.* 1996, Martin *et al.* 2008), increases in potential hosts due to aggregations at water or fruit sources or the clustering of births (newborn are more vulnerable to infections) (Hosseini *et al.* 2004) or increased incidence of infective forms of parasites in wet periods (Linthicum *et al.* 1999).

When the mechanisms for acquisition and transmission of self-medicating behaviour are reviewed in the context of the evolution of herbal medicine, one important question to be addressed is how organisms without

complex communication skills and/or high levels of cognitive abilities make 'therapeutic decisions' which can then be transmitted (Hart 2005, 2011). Kyriazakis *et al*. (1998) hypothesised that mammalian herbivores are 'hard-wired' to change dietary items in response to parasitism. A similar innate mechanism is suggested for medicinal compound use in moths, ants and fruit flies (de Roode *et al.* 2013) and caterpillars (Singer *et al.* 2009). The spontaneous swallowing of hairy leaves (smooth leaves were chewed) in healthy naive captive primates (two chimpanzees and one bonobo) is suggested as an example of such an innate behaviour (Menzel *et al.* 2013). Different observer chimpanzees subsequently copied the consumption of hairy leaves. In this situation, it is difficult to determine the driver and so hypothesise self-medication in the observer animals, as it is expected that captive animals would be healthy and have minimal parasite loads. Copying as a mechanism for transmission meets the criteria for a biological tradition namely:

"*a distinctive behaviour pattern shared by two or more individuals in a social unit, which persists over time and that new practitioners acquire in part through socially aided learning" (Fragaszy et al. 2003).*

An alternative mechanism of acquisition and transmission is through learned experience. Learned pre-ingestive cues of a post-ingestive event are used in selecting or avoiding foods (Yearsley *et al.* 2006, Favreaua *et al.* 2010). Taste, smell and visual clues enable animals to discriminate between foods and govern food choices. An associatively conditioned response based on the post-ingestion effects may arise, with such post ingestion feedback calibrating the sensory experiences 'like/dislike' in response to past or present experiences of the physiological effect of the food choice (Villalba *et al.* 2009). Lambs in a treatment group were conditioned to consume foods and toxins (grain, tannins, oxalic acid) that led to 'negative internal states' and then allowed to eat a 'medicinal substance' known to reduce the effects. Control lambs ate the same foods but were given the medicine at times unrelated to the 'negative internal state' and therefore experienced no close temporal effect of the benefits. After this conditioning, the treatment animals always chose to consume the 'medicinal substance' after eating one of the food/toxins. The control animals never chose the 'medicinal substance' (Villalba *et al.* 2006), illustrating the ability of naive animals to learn such an association.

Prophylaxis in animals is commonly linked to seasonal or experimentally induced changes in exposure to risk factors followed by observed consumption of plant material used in ethnomedicine and of known pharmacology (Hart 2005). Parasitic infections in wild populations of primates are likely to be chronic and sub-lethal (Nunn *et al.* 2006). Obvious symptoms of disease are rare in wild primate populations as:

"*infected individuals often mask weakness to maintain social position and avoid attacks by predators*" (Gillespie et al. 2008).

In the absence of obvious symptoms, such as diarrhoea, confirmation of parasitic infections requires data relating to parasite excretion before and following medication. Parasite excretion is variable according to the life cycle of the parasitic organism and there are inherent difficulties in sampling (Villanua *et al.* 2006) making confirmation of infection status difficult. Thus without a detailed knowledge of the current and future state of health of the individual, medicinal use can only be inferred and designation as prophylaxis or therapeutic use is problematic.

Many of the hypotheses of self-medication are based on either the local ethnomedical use or *in vitro* pharmacology of individual constituents of plant material e.g. the berries of *Phytolacc*a *dodecandra* eaten by chimpanzees in Kibale, Uganda. *Phytolacca dodecandra* is reported to have *in vitro* activities e.g. molluscicidal

(Kloos *et al.* 1982), antiviral (Koch *et al.* 1996), antibacterial (Taye *et al.* 2011) and antifertility activities (Stolzenberg *et al.* 1976). The pith and fruit of *Aframomum* species (wild ginger family) ingested by chimpanzees, bonobos and gorillas across Africa is sold in Uganda as a treatment for antibacterial, antifungal and anthelminthic actions (Huffman 1997). Several factors need to be considered in order to confirm a self-medicating function. Activity may be due to the activity of a single component or due to interactions between two or more components. Synergistic effects occurs where a single compound acts on several targets, several compounds act on a single target or multiple compounds act on several related targets (Wagner 1999, Bagetta *et al.* 2011). Investigations of synergy are rarely undertaken on multicomponent systems (Nelson *et al.* 1999).

Active compounds must be available in the situation in which they are required to exert either a local effect or to be absorbed for a systemic effect. The components extracted from plant material and hence the biological activity may vary with choice of extraction method and solvent used (El-Ghorab *et al.* 2004, Sajfrtova *et al.* 2013). The components and activity of organic solvent extracts may also differ from those obtained from simulated biological systems such as a gastric model. Nair *et al*. (2008) identified the need to have appropriate guidelines and methods for the testing of herbal/alternative pharmaceutical preparations and concluded that the use of different dissolution media and pH was required to monitor the release of active components with differing solubility profiles.

In the example of gastro-intestinal (GI) parasites, active compounds need to be released from the plant matrix in the GI tract. Therefore, active ingredients must be soluble in aqueous gastric media, resist pH and enzymatic degradation and retain activity at the stomach pH and possibly subsequently the pH of the intestines (Reppas *et al.* 2010). For a systemic effect, active compounds must be in a form capable of being absorbed. It is possible that compounds which have *in vitro* activity may become inactive or have increased activity due to digestion effects in the stomach (Söderlind *et al.* 2010).

A positive *in vitro* test does not automatically transfer to *in vivo* biological activity (Athanasiadou *et al.* 2004), nor is the reverse true that inactive *in vitro* is inactive *in vivo* (Villalba *et al.* 2010). The conditions of testing are also significant. For example *Vernonia amygdalina*, utilised by *Pan troglodytes schweinfurthii,* contains three vernonioside glycosides and four sesquiterpene lactones. The latter were found to have *in vitro* antischistosomal activity, as was one of the vernonioside glycosides, Vernonioside B1. In acidic *in vitro* conditions, a secondary aglycone produced from Vernonioside B1 was more potent than the parent compound (Jisaka *et al.* 1992). This is an example where the observation and the pharmacology may support the hypothesis for self-medication and highlights the importance of investigating the activity of compounds produced in the appropriate *in vivo* conditions. The vernonioside B1 aglycone would be produced in *in vivo* acidic stomach conditions.

Ideally, investigations of biochemical medicinal activity should utilise plant extracts produced in a manner closely replicating the uses by animals and not be limited to testing of organic solvent extracts (El-Ghorab *et al*. 2004, Sajfrtova *et al.* 2013) or single isolated compounds (Wagner 1999, Bagetta *et al.* 2011). The use of such simulated gastric models for testing of ingested potential toxins is well documented (e.g Ruby *et al.* 1993, Ruby *et al.* 1996, Ruby *et al.* 1999, Oomen *et al.* 2003, Dominy *et al.* 2004, Versantvoort *et al.* 2004, Versantvoort *et al.* 2005).

Self-medication where selection was based on the physical characteristics of plant material and not PSM

has been hypothesised e.g. the whole leaf-swallowing among wild chimpanzees (*Pan troglodytes)*, bonobos *(Pan paniscus*) and gorilla (*Gorilla gorilla*) (Huffman 2003). The leaves are folded one at a time between the tongue and palate. The leaves pass through the gastro-intestinal tract visibly unchanged (Wrangham 1995, Huffman *et al.* 1997). Infection by the flat worm *Oesophagostomum stephanostomum* was reported as being associated with 78% of instances of leaf-swallowing (Huffman *et al.* 1997). Whole leaf swallowing by a subspecies of chimpanzee, *Pan troglodytes vellerosus,* in Gashaka, Nigeria has been reported (Fowler *et al.* 2007). Unchewed rough-surfaced herbaceous leaves of *Desmodium gangeticum* and clumps of sharp edged grasses together with parasitic worms *Oesophagostomum stephanostomum* were recovered in faecal samples. Fowler *et al*. (2007) suggests that the sharp edges or rough texture would irritate the GI tract, induce diarrhoea and facilitate parasite expulsion. The behaviour, limited to the rainy season and presence of the worms, may be an example of driver and beneficial effect but could be considered both prophylactic, controlling parasite load, or a therapeutic response to increased parasite load.

### 1.7.2 Therapeutic

Therapeutic self-medication differs from prophylactic in that it would be a response to an immediate need and limited in duration to the event/illness. The use of herbal medicines in traditional human societies have been extensively reviewed, and the most common therapeutic use for plant material is for acute conditions such as acute infection, gastritis, vomiting, toothache and fever (Chevallier 2000). These therapeutic interventions are concerned with treating conditions that may have an immediate threat on the survival or fitness of an individual.

There is a lack of reported observations of therapeutic self-medication in primates and animals in general, probably due to the relative infrequency with which acutely sick animals are encountered in the wild (Adelman *et al.* 2009). There is a low probability of observing onset of an acute illness. Acutely ill animals exhibit changes in behaviours such as lethargy, somnolence and anorexia (Hart 1988, Kent et al. 1992b, Exton in Adelman *et al.* 2009). Such changes would potentially reduce encounter rates compared to healthy individuals. Therefore, this limits the potential observation of a sick animal using a medicinal plant response to an acute illness (Hart 2005). Observations of acute illness in animals are often limited to incidences of sudden death, for example eight sudden deaths were recorded in three studied chimpanzee communities, Taï National Park, Ivory Coast, between October 2001 and June 2002, and all of the individuals had been in apparent good health shortly before. In the case of several individuals there was less than 5 hours between observations and finding the dead animals (Leendertz *et al.* 2004). In Rwanda 20% of sudden deaths in the mountain gorilla are due to respiratory infections, transmitted from infected humans (Palacios *et al.* 2011). There are therefore few clear reports in the literature of such apparent acute therapeutic self-medication in animals. One such possible observation is that of an unwell *Pan troglodytes schweinfurthii* chewing the bitter pith of *Vernonia amygdalina* (Huffman *et al.* 1989). The symptoms were lethargy, dark urine and irregular bowel movements, characteristics that may/may not be related to infections. The inclusion of an unusual plant material in the diet may be a response to other than infection such as when red colobus monkeys (*Colobus badius*) and some howler monkeys (*Alouatta* spp.) select leaf material with high tannin content. Leaves with high protein/low tannin ratio were those usually selected (Glander 1994). High tannin levels are thought to precipitate alkaloids and reduce

bloating (Oates 1977). Intake of material considered potentially toxic might also have physiologically beneficial effects. For example tannin consumption increased in pregnant or lactating sifaka (*Propithecus verreauxi verreauxi*), and was associated with an increase in body weight and stimulation of milk secretion (Carrai *et al.* 2003). This may be considered to have direct benefit to the mother preventing loss of condition and the transgenerational benefit of higher offspring survival rates.

Self-medication hypotheses can also be applied to the use of geophageous material, such as clays e.g. acute/therapeutic when applied externally to treat wounds or sites of infection or symptoms of diarrhoea (Vondruskova *et al.* 2010, Otto *et al.* 2013). However, it may not be possible to place geophagy into such narrow categories. Geophagy for mineral supplementation (Johns *et al.* 1991, Holdo *et al.* 2002) might be considered either prophylactic or therapeutic to restore imbalances caused by seasonal changes in dietary composition (Ayotte *et al.* 2006). In this situation, analysis of the foods/geophagy material may point to deficiencies and therefore suggest the driver for medication and potential benefits.

There is some *in vivo* evidence for the efficacy of clay consumption in lambs, and the inclusion of clays in domestic livestock feeding regimes has become more common (Vondruskova *et al.* 2010). The listed beneficial effects following ingestion of clays include: immobilisation of antinutritional components in feeds, reduction in numbers of pathogenic gut bacteria, reduction in activity of bacterial enzymes in the small intestine which prevents irritation and damage and improvements in morphological characteristics of the mucosa (Vondruskova *et al.* 2010).

The difficulties in categorising prophylactic or acute therapeutic are thus illustrated. A theoretical framework has been suggested by Singer *et al*. (2009) identifying criteria for self-medication as a response to infection to be met:

*i)* *self-medication should improve the fitness of animals infected by parasites or pathogens*

*ii)* *self-medication in the absence of infection should decrease fitness*

*iii)* *infection should induce self-medication behavior*

To date most publications only have potential toxicity of PSM as evidence for the second criterion leading to an assumption that if consumption were not costly all individuals would exhibit it. Increased fitness may not be derived by the individual but by group members/siblings (social prophylaxis) and off-spring (transgenerational prophylaxis) (de Roode *et al.* 2013). There is little data on non-herbivores, who may respond in a different manner through different exposures to PSM. Data obtained under experimental conditions must relate to the usual habitat conditions and meet the three criteria to conclude a medicinal benefit as a response to infection.

## 1.8 Mistletoes – Introduction

Mistletoes belong to the Order Santalales with over 1600 species worldwide. The two largest families are the Loranthaceae (1000 species) and the Viscaceae (550 species). *Phoradendron* spp., are members of the Viscaceae Figure 1.4.

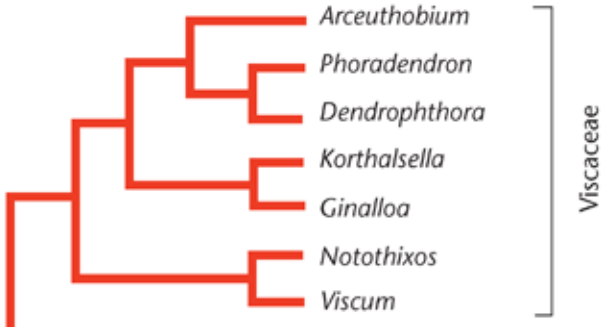



Figure 1.4 Phylogenetic tree showing relationships among genera in the Viscaceae modified from Nickrent (2011).

*Phoradendron* are the largest mistletoe genus found entirely in the New World. Their range extends from temperate North America, the Caribbean and Central America to temperate regions of South America with *Phoradendron* having the greatest diversity in the highlands of Mexico (Nickrent 2011). The Viscaceae are all obligate hemiparasites, capable of photosynthesis but obtaining water, minerals, nitrogen, carbon and other dissolved compounds from the host xylem fluid, through their direct connection with the host xylem vessels (Ehleringer *et al.* 1985b, Schulze *et al.* 1991, Richter *et al.* 1995, Glatzel *et al.* 2009). Xylem flow is unidirectional from root to leaf. Mistletoes are commonly succulent having high water content (Ehleringer *et al.* 1986). *Viscum album*, the European mistletoe, is the most widely studied species. Both Greek and Roman cultures used mistletoe as a medicinal plant.

### 1.8.1 Medicinal uses of mistletoes

There is an extensive literature on the potentially beneficial components in mistletoes and the medicinal uses. These include mistletoes from: Europe (Büssing 2000), Africa (Deeni *et al.* 2002), South America (Fernandez *et al.* 1998), Central America (Rivero-Cruz *et al.* 2005), the Indian sub-continent (Islam *et al.* 2004) and Asia (Lee *et al.* 1999, Lev *et al.* 2011). The biological effects of mistletoe extracts include cytotoxicity, apoptosis, tumour inhibition, induction of immune processes and antioxidant activity. Leaf extracts have been used in Europe for cancer treatment since 1920 (Bar-Sel 2011). There are ethnomedical reports from Europe, Africa and Asia of use in treating diabetes. In Mexican herbal medicine, aqueous extracts of *Phoradendron tomentosum* are used in for treating Type II diabetes mellitus (Calzado-Flores *et al.* 2002, Careaga-Olivares *et al.* 2006) whilst in Venezuela aqueous extracts from *Phoradendron piperoides* are used in treating diabetes mellitus (Rodríguez *et al.* 2008). In Brazil aqueous extract of *P. piperoide*s is used as a gastric antispasmodic (Marçal *et al.* 2007).

Digestion of mature leaf presents difficulties for herbivore/folivore species as the leaves are tougher, contain more fibre, condensed tannins, resins and silica (Rockwood *et al.* 1979, Glander 1982) and would consequently present an even greater difficulty for the frugivorous *Ateles*. This additional digestibility problem of **mature** mistletoe combined with the variety of potential active compounds present, the restricted period of consumption and the selectivity of choice of a single species (>95% of incidences) all combined to stimulate the interest in *Phoradendron* consumption and its potential use to *Ateles geoffroyi.*

The following classes of potentially important biologically active phytochemicals have been identified as being present in members of the Viscaceae, including mistletoe thionins, mistletoe lectins, polyphenolic

compounds, and other miscellaneous compounds (Pfüller 2000, Ochocka *et al.* 2002, Varela *et al.* 2004).

### 1.8.2 Mistletoe Thionins (viscotoxins, ligatoxins and phoratoxins)

The thionins are widely distributed classes of closely related cationic peptides containing 42-50 amino acids and 6-8 cysteine residues; thionins are considered part of the plant defence system (Florack *et al.* 1994, Castro *et al.* 2005). Thionins from different species have high levels of homologous sequences (Carrasco *et al.* 1981, Bohlmann *et al.* 1991, Li *et al.* 2002). Thionins modify membrane permeability, leading to depolarisation, mitochondrial damage and cell death and also modify immune responses (Carrasco *et al.* 1981, Florack *et al.* 1994, Stein *et al.* 1999).

In *Viscum album* thionin Type III genes are expressed in seeds and in leaves (García-Olmedo 1999, Larsson 2007). The cDNA encoding for mistletoe thionins have been identified (Schrader *et al.* 1991). cDNA is complementary DNA synthesised from mRNA (messenger RNA) and is used to locate genes in the double strand DNA from a cell and so subsequently initiate synthesis of mistletoe thionins. Mistletoe Type III thionins (45-46 amino acids) have been isolated from stems and leaves are shown Table 1.10.

Table 1.10 Summary of occurrence of identified mistletoe thionins

| **Species** | **thionin** | **References** |
|---|---|---|
| *Viscum album* | viscotoxins A1, A2, A3, B, B2, 1-PS, U-PS, C1 | Büssing (2000), Ochocka *et al.* (2002), Pal *et al.* (2008) Samuelsson *et al.* (1970, Schaller *et al.* (1996, 1998), Stein *et al.* (1999), Pfüller (2000), Romagnoli *et al.* (2003) |
| *Phoradendron tomentosum* | phoratoxins A, B | Samuelsson *et al.* (1967), Mellstrand *et al.* (1973), Mellstrand (1974) |
| | phoratoxins C-F | Johansson *et al.* (2003) |
| *Phoradendron liga* | ligatoxin A,B | Thunberg *et al.* (1977, 1982), Li *et al.* (2002) |
| *Dendrophtora clavata* | denclatoxin A, B | Samuelsson *et al.* (1997) |

Thionins are found in storage vesicles in the mistletoe leaf and may be part of a storage capacity for limited resources such as sulphur, nitrogen and phosphorus (Urech *et al.* 2011). Many perennial plants store and recycle such resources where environmental resources are limited (Aerts 1996). Thionins are found in the outer layer of the leaf and are part of the plant defense against microbial parasites (Broekaert *et al.* 1997).

Table 1.11 Examples of biological activity of mistletoe thionins

| **Biological activity** | **Reference** |
|---|---|
| modify membrane permeability, leading to depolarisation, mitochondrial damage and cell death | Carrasco *et al.* (1981), Florack *et al.* (1994), Giudici *et al.* (2003) |
| cytotoxic activity against a variety of cancer cell lines | Schaller *et al.* (1996), Bussing *et al.* (1999b), Li *et al.* (2002), Johansson *et al.* (2003) |
| Activity against bacteria, yeasts and insect larvae | Broekaert *et al.* (1997), Deeni *et al.* (2002), Giudici *et al.* (2004), Pelegrini *et al.* (2005) |
| strong immunomodulatory effects | Stein *et al.* (1999), Tabiasco *et al.* (2002), Lavastre *et al.* (2004), Elluru *et al.* (2006), Vasconcellos *et al.* (2009) |

*Viscum album* leaves, grown in Europe, showed seasonal variations of viscotoxin with a peak of maximum concentration in June (20 mg/g dry weight) (Urech *et al.* 2009). This variation in content is linked to leaf senescence in *Viscum album* and is closely correlated with the selective degradation of viscotoxins (Schrader-Fischer *et al.* 1993, Urech *et al.* 2011). This hypothesis is supported by the reduction in mistletoe

thionins levels found in senescing leaf (Schrader-Fischer *et al.* 1993) and this storage/recycling may contribute to seasonal variation in biological activity (Urech *et al*. 2011). Examples of reported biological activity are shown in Table 1.11.

## 1.8.3 Mistletoe lectins (ML)

Lectins are a widespread group of biologically active glycoproteins between 50-63KDa (KiloDaltons) molecular weight. Lectins are Type-2 Ribosome-inactivating-proteins (2-RIPs) which enzymatically damage ribosomes so inhibiting protein synthesis. The structure, properties and uses have been extensively reviewed (Doyle *et al.* 1984, Peumans *et al.* 1995, Van Damme *et al.* 1998, Sharon *et al.* 2004, 2007, Sharon 2008). Three genes responsible for production of the mistletoe specific lectins ML-1,2 and ML3 in genus Viscaceae, have been identified and characterised (Kourmanova *et al.* 2004) hence mistletoe lectins (ML1-3) are not host derived compounds. Mistletoe lectins ML1-3 have been isolated from *Viscum album* (Franz *et al.* 1981, Eifler *et al.* 1993, Blaschek *et al.* 2011) and *Phoradendron* (Endo *et al.* 1988, Endo *et al.* 1989, Lee *et al.* 1992, Lee *et al.* 1999, Varela *et al.* 2004). Examples of reported biological activity are shown in Table 1.12.

Table 1.12 Examples of biological activity of lectins

| **Biological activity** | **Reference** |
|---|---|
| antiviral, antibacterial, antifungal, insecticidal, antiparasitic | Pistole (1981), Llovo *et al.* (1993), Pusztai *et al.* (1995), Lee *et al.* (1998), Bussing *et al.* (1999a,) Carlini *et al.* (2002), Stein *et al.* (2006), Costa *et al.* (2010) |
| induction of apoptosis | Kim *et al.* (2004), Hoessli *et al.* (2008) |
| cytotoxic | de Mejia *et al.* (2005) |
| immunomodulatory effect | Hajto *et al.* (2003), Elluru *et al.* (2006), Lyu *et al.* (2006), Hoessli *et al.* (2008) |
| induce cellular repair of damaged DNA | Kovacs *et al.* (1991), Weissenstein *et al.* (2014) |
| stimulation of pancreatic growth | Pusztai *et al.* (1995,1998), Kelsall *et al.* (2002) |

There are also seasonal and tissue variations in lectin content similar to mistletoe thionins (Blaschek *et al.* 2011). Maximum levels in *Viscum album* (2mg/g dry weight) were detected in December (Urech *et al.* 2009). Lectins are very stable peptides with a high content of cysteine amino acids. This makes them protease resistant and so resistant to gastrointestinal digestion (Boettner *et al.* 2002, Kelsall *et al.* 2002). Mistletoe lectins have been shown to translocate across the gut wall and both ML-specific induced IgA and IgG immunoglobulins were detected in mice tissue/serum (Lavelle *et al.* 2000) following ingestion. Both of these immunoglobulins are involved with defence against pathogens.

## 1.8.4 Polyphenolic compounds

Several thousand plant polyphenols are known, containing at least one aromatic ring with one or more hydroxyl groups in addition to other substituents. The group include the flavonoids, tannins, phenylpropanoids and proanthocyanidins (Structures Sections 4.5.1.4-4.5.1.7). The metabolic pathway/genome structures for flavonoid and phenylpropanoid synthesis in *Phoradendron* are listed in the MetaCyc database (Caspi *et al.* 2010).

### 1.8.4.1 Flavonoids

Over 10,000 flavonoids of varying classes have been identified (Andersen *et al.* 2010, Dixon *et al.* 2010). Flavonoid PSM are found in the leaves, seeds, bark and flowers of plants and are part of the plant defence against pathogens, herbivores, ultra violet (UV) protectants and attractants to pollinators (Heim *et al.* 2002, Dixon *et al.* 2010). Flavonoid compounds have been identified in both the Loranthaceae and Viscaceae (Wagner *et al.* 1998, Orhan *et al.* 2002, Orhan *et al.* 2006) including *Phoradendron* spp. (Dossaji *et al.* 1983, López-Martínez *et al.* 2012, Jimenez-Estrada *et al.* 2013).

Flavonoids have antioxidant activity and there is a positive correlation between mistletoe antioxidant activity, and flavonoid content (Crozier *et al.* 2000, Crozier *et al.* 2008, Vicas *et al.* 2012, Jimenez-Estrada *et al.* 2013, Pietrzak *et al.* 2013). Flavonoid antioxidant activity is related to their free radical scavenging properties (Lobo *et al.* 2010) and this and their metal ion chelation properties vary with structure (Morel *et al.* 1994, Cook *et al.* 1996, van Acker *et al.* 1998, Pietta 2000, Heim *et al.* 2002, Ren *et al.* 2008). Examples of reported biological activity are shown in Table 1.13.

Varela *et al*. (2004) identified apigenin and luteolin c-glycosylflavones in *Phoradendron liga*. Varela *et al*. (2004) also identified a major difference between *Viscum* and *Phoradendron* spp. in the types of flavonoid present which in *Viscum* spp. are quercetin or its derivatives. The difference is due to the lack of a flavone-3-hydroxylase enzyme in the synthetic pathway in *Phoradendron* (Varela *et al*. 2004).

Table 1.13 Examples of biological activity of flavonoids

| **Biological activity** | **Reference** |
|---|---|
| antibacterial and antiviral | Cushnie *et al.* (2005), Ozçelik *et al.* (2006), Aron *et al.* (2008) |
| antiparasitic | Tasdemir *et al.* (2006a, 2006b), Kerboeuf *et al.* (2008), Bourjot *et al.* (2010) |
| antifungal | Sathiamoorthya *et al.* (2007) |
| carbohydrate metabolism | Cazarolli *et al.* (2008), Hanhineva *et al.* (2010) |
| anti-inflammatory and antinocioceptive | Fernandez *et al.* (1998), Orhan *et al.* (2006) |
| cardio-protective | Cook *et al.* (1996), Heim *et al.* (2002), Wen-Feng *et al.* (2006), Testai *et al.* (2013) |
| reduce plasma lipids | Jung *et al.* (2003), Onunogbo *et al.* (2012) |

Flavone–C glycosides have also been reported in *Phoradendron tomentosum* (Dossaji *et al*. 1983). Rivero-Cruz *et al.* (2005) isolated sakuranetin, a flavanone, from the Mexican mistletoe, *Phoradendron robinsonii*, which had anti-tubercular activity. The individual flavonoids present may vary with mistletoe/host combinations (Dossaji *et al*. 1983).

### 1.8.4.2 Phenylpropanoids

Phenylpropanoids are the largest single category of PSM produced by higher plants (Korkina 2007, Korkina *et al.* 2011). Phenylpropanoids are induced as a response to stresses such as pathogen attack, physical and UV damage, nutritional deficiencies and the regulation of development, growth and flowering (Solecka 1997).

Examples of reported biological activity are shown in Table 1.14. Three phenylpropanoid glycosides have been isolated in *Viscum album* (Deliorman *et al.* 1999) and also the phenylpropanoid cinnamic acid derivatives, caffeic, ferulic and sinapic acid and their degradation products (Pfüller 2000).

Table 1.14 Examples of biological activity of phenylpropanoids

| Biological activity | Reference |
|---|---|
| antioxidant | Kono *et al.* (1998), Korkina (2007), Ferreres *et al.* (2008), Vicas *et al.* (2012) |
| anticancer | Itoigawa *et al.* (2004) |
| antiviral | Kernan *et al.* (1998), Gálvez *et al.* (2006) |
| anti-inflammatory | Korkina *et al.* (2011), de Cassia da Silveira *et al.* (2014) |
| wound healing, and antibacterial | Didry *et al.* (1999), Gálvez *et al.* (2006), Hemaiswarya *et al.* (2011) |
| anti-diabetic | Nicasio *et al.* (2005), Thom (2007), Ong *et al.* (2013) |
| cardiovascular | Panossian *et al.* (1998), Mubarak *et al.* (2012) |
| anti-platelet activity | Panossian *et al.* (1998) |

## 1.8.5 Alkaloids

Over 5000 different alkaloid compounds have been identified. Many are confined to a single genus or sub family (Henry 1949, Evans 2009). Alkaloids are more common in annual rather than perennial plant species (Levin 1976) but when present are higher in new leaf than mature leaf (Rockwood *et al.* 1979).

Reports of the presence of alkaloids in mistletoes in the literature are rare and much of the work has not been confirmed by isolation in pure form or structure elucidation due to the extreme lability of possible types of alkaloid (Khwaja *et al.* 1980, Khwaja *et al.* 1986, Büssing 2000, Pfüller 2000). Khwaja *et al.* (1986) claim to have extracted alkaloids from *Viscum album* L. var. coloratum Ohwi, evaporating crude extract and producing a brown gummy residue which when subsequently tested no alkaloids were identified nor were the results of any alkaloid identification tests presented. Recently two novel amino alkaloids were detected and characterised in *Viscum album* (Amer *et al.* 2012). The alkaloid rubrine C (N-methylglycine hydroxide) was reported in *Phoradendron rubrum*, which is parasitic on the mahogany species (*Swietenia mahogani*) (West *et al.* 1967). *Swietenia mahogani* is reported as containing several different bitter tasting alkaloids (Sahgal *et al.* 2009). There are no other reports for alkaloids in *Phoradendron* spp.

Janzen *et al*. (1984) analysed the alkaloid content of three ages of leaf from 80 species trees at Santa Rosa including the 4 host tree species parasitised by *Phoradendron*. Alkaloids were only detected in young and middle aged leaves of *Tabebuia ochracea*. It is therefore unlikely that host derived alkaloids would be present in the consumed mistletoe, *Phoradendron quadrangulare*, growing on either host trees *Manilkara chicle* or *Guazuma ulmifolia*.

## 1.8.6 Miscellaneous constituents

Other classes of compounds identified in *Viscum* or *Phoradendron* include phytosterols and triterpenes (Wagner *et al.* 1998, Pfüller 2000, López-Martínez *et al.* 2012), polysaccharides and poly alcohols (Pfüller 2000). Mistletoe content of monosaccharides and polyols (Arda *et al.* 2003), carbohydrates (Escher *et al.* 2004a) and amino acids (Escher *et al.* 2004b) also vary with host and season.

Variations in mistletoe constituents and biological properties may be influenced by the resources available to the host tree, its habitat and its phenology. *Viscum album*, growing on the willow is used mainly as a sedative, whereas when growing on the pear is used as a cardiovascular medicine, and when grown on the hawthorn is used as a hypotensive drug (Panossian *et al.* 1998). Seasonal variations in the types of constituent or biological activity have been reported (Schrader-Fischer *et al.* 1993, Schaller *et al.* 1996, Schaller *et al.* 1998, Urech *et al.* 2011). These host and seasonal variations are exploited by manufacturers of mistletoe

medicinal extracts (Scheer *et al.* 1992). Seasonal variation has been reported in antioxidant activity (Onay-Ucar *et al.* 2006) and antidiabetic activity (Osadebe *et al.* 2010).

### 1.8.7 Nutrient supplementation

*Phoradendron* spp., as a parasitic plant, lacks a specific mechanism for uptake of nitrogen and minerals. Mistletoes are dependent upon diverting resources from the host (Calder 1983). Mistletoes are physiologically dependent on the host for water and inorganic nutrients e.g. nitrogen, which are accessed from the host xylem (Ehleringer *et al.* 1985b, Ehleringer *et al.* 1986). Mistletoes have no access to host phloem. Phloem tissues have bidirectional flow between leaf and root. Mistletoes must therefore compete with the host for water and adjust to seasonal variations in host physiology and xylem composition. Host plants cycle mobile nutrients such as potassium and phosphorus between xylem and phloem. However once acquired by a mistletoe there is no possible translocation back to the host, due to the lack of phloem connection between host and mistletoe (Türe *et al.* 2010). This leads to mineral accumulation in mistletoe leaf. Chemical analysis of mistletoe leaves showed higher concentrations of potassium and sodium by comparison with the host (Ehleringer *et al.* 1985a, Stewart *et al.* 1990, Glatzel *et al.* 2009). This dependence on host state creates seasonal variations in resources available to the mistletoe. The levels of both sodium and potassium may be exploited beneficially or may be toxic.

## 1.9 Geophagy Introduction

Geophagy is more common in the tropics (Wilson 2003a, Wilson 2003b). Geophagy may be considered a type of self-medication. Geophagy has been observed in many animals, as diverse as reptiles (tortoise), invertebrates (the isopod *Hemilepistus reaumuri*), mammals (elephants) (Holdo *et al.* 2002, Holdo *et al.* 2004) and birds (Brightsmith *et al.* 2004, Symes *et al.* 2006, Brightsmith *et al.* 2008). Geophagy specifically in primates was reviewed by Krishnamani *et al*. (2000) and more recently, Ferrari *et al*. (2008) reviewed geophagy in 23 species of New World primates.

Many hypotheses have been put forward for the practice in humans, non-human primates and other vertebrates (Young *et al*. 2011). The hypotheses have been divided into adaptive/beneficial such as detoxification, mineral nutrient support, anti-infective or antiparasitic, the mitigation of symptoms by behaving as an antidiarrhoeal or an antacid and as a non-adaptive aberrant behaviour.

### 1.9.1 Detoxification

Geophagy as a mitigation of potential PSM in dietary items has been suggested by many authors (Johns 1986, Johns *et al.* 1991, Klaus *et al.* 1998, Symes *et al.* 2006, Brightsmith *et al.* 2008). Detoxification is commonly linked to consumption of the PSM classes of alkaloids, phenols and tannins.

When ingested dietary toxins must survive the gastric pH, the potentially degrading enzymes and pass through the gastric mucosa to reach the blood stream in order to exert a systemic effect. The simplest mechanism for uptake of a molecule is by passive diffusion from a high to a lower concentration. Passive diffusion is dependent upon the size of the molecule, its lipid solubility and degree of ionisation and the mucosal surface area available for diffusion. Hence any property of geophageous material which alters one of

these may either increase/decrease toxin uptake. Detoxification is commonly related to the clay minerals present (Wilson 2003a,b).

The pharmacokinetics of clay-organic interactions has been studied under gastrointestinal conditions and adsorption is generally either through ion-exchange and/or physical adsorption (White et al. 1983, Theng 1979 in Bergaya *et al.* 2006b). Naturally occurring alkaloids have been shown to be effectively bound by clays (Bergaya *et al*. 2006). Sorption of high-molecular-weight cations has been shown to reach adsorption levels that exceed the cation-exchange capacity of the clays (Bergaya *et al.* 2006).

Exposure to classes of PSM is influenced by diet choices. Hladik (1977) conducted analyses of 382 species available to and the 38 foods eaten by chimpanzees in Gabon and reported only 15% as containing alkaloids. Hladik (1977) suggested that the alkaloids were either non-toxic to the chimpanzee, concentrations were insufficient to be toxic or the plant material was not eaten in a sufficient quantity to be toxic. The chimpanzees were often seen eating between 10-20g geophageous material twice daily. Hladik (1977) concluded that in this instance the function of geophagy was more likely to be related to tannin and phenol content. *Colobus guereza* also chose food items based on micronutrient and protein content and low fibre content and food items selected were low in alkaloids and tannins (Fashing *et al.* 2007). Acutely toxic alkaloids and cardiac-glycosides are found at high concentrations in new leaves whereas higher concentrations of the less toxic tannin/phenolics are found in mature leaf (Rockwood *et al*. 1979). Consumption by red leaf monkey (*Presbytis rubicunda*) of the highest amounts of soil coincided with seeds eaten in August, which had very low levels of tannins whilst at times when higher levels of tannins were consumed no soil was ingested. Davies *et al*. (1988) concluded that it was unlikely that soil was eaten to absorb tannins. The circumstances of the *Colobus* and the red leaf monkey make detoxification of tannins an unlikely hypothesis for the geophagy observed.

Schaefer *et al.* (2003) suggested that ripe fruit in a tropical forest belongs to a continuum ranging from high nutrient value and low levels of PSM to poor nutritional value and high PSM; reporting that fruits rapidly taken from the trees by vertebrates were selected for lipid and carbohydrate content. Fruits high in water and phenols were avoided and unripe fruit had high levels of phenolics (Schaefer *et al.* 2003). Cazetta *et al*. (2008) reporting similar results, showed that ripe fruits rapidly consumed by vertebrate seed dispersers are nutrient rich and low in tannins and phenols. Wendeln *et al.* (2000) analysed 14 species of *Ficus* in Panama (3 commonly eaten in Santa Rosa) and found tannin levels below the 2% deterrent levels. Wendeln *et al.* (2000) hypothesised that fruits chosen for protein content will be low in tannin as tannins bind to proteins making them unavailable or, where chosen for carbohydrate content, would have very low tannin levels, only sufficient to reduce fungal and insect attack. Iason *et al*. (2006) suggest that animals avoid or limit the consumption of PSM to levels that can be physiologically tolerated. This avoidance of PSM calls into question the need for frugivores to use geophagy for detoxification of PSM. Other types of toxin which may have health impacts are produced by bacteria (Fioramonti *et al.* 1987a, Fioramonti *et al.* 1987b, Brouillard *et al.* 1989) and fungi (Phillips 1999) and are adsorbed by geophageous material.

The relative rarity of the geophagy and the timing following ingestion of *Phoradendron* may be indicative of its use in detoxification of higher levels of PSM compounds or compounds rarely encountered by the *Ateles* or exploitation of a synergistic effect.

### 1.9.2 Mineral nutritional support

Geophagy material selected by birds had lower or similar levels of calcium, potassium, magnesium, manganese and copper, compared to plant materials consumed (Brightsmith *et al*. 2008). The mineral intake from a typical fruit-based diet was lower in calcium, iron, magnesium, sodium and zinc compared to a folivore diet (Rode *et al.* 2003, Fashing *et al.* 2007). Calcium supplementation by reproductive age mammals has been hypothesised (Schulkin 2000, Bravo *et al.* 2008). In humans geophagy during pregnancy has been linked to populations who do not consume milk and who have a diet of toxin rich plant food (Wiley *et al.* 1998). In this case calcium supplementation and/or the possible protection of the foetus from toxin exposure may be functions. *Ateles* in Columbia drank salido water with high levels of sodium, often a scarce resource (Izawa 1993).

Attempts to link geophagy to mineral deficiencies in the diet are problematic e.g. *Ficus* spp. are staple for many primate populations (Serio-Silva *et al.* 2002, Felton *et al.* 2008, Parr *et al.* 2011). Eight species of *Ficus* are eaten at Santa Rosa (Filippo Aureli personal comm.). Frugivorous bats of the subfamily *Stenodermatinae* who specialise on *Ficus* fruits visit sodium rich salt licks more than bats of the subfamily *Carolliinae* who supplement their diet with insects, potential sources of sodium (Bravo *et al.* 2012). Sodium levels have been found to vary significantly in the same *Ficus* spp. in different habitats with some Central American levels ~ 1000 times higher than those in the Peruvian Amazon, where the *Ficus* were very low in sodium (Bravo *et al.* 2012).Thus not only dietary items but local habitat make direct comparisons problematic.

### 1.9.3 Anti-infective and Antiparasitic

The Ebers Papyrus – recognised as the world's oldest medical text – recommended clay for ailments including diarrhoea, dysentery, tapeworm, hookworm, wounds and abscesses (Nunn 2002). Geophagy to reduce endoparasites was suggested following observation that black howler monkeys, eating a mud based bird nest, excreted high numbers of cestode worms in the faeces (Bicca-Marques *et al.* 1994). However, parasite excretion in faeces may be subject to variation due to endogenous or exogenous factors and the time of faecal production (Villanua *et al.* 2006) all of which may introduce a bias if only randomly encountered samples are examined.

Knezevich (1998) hypothesised that regular ingestion of soil permitted Rhesus macaques to tolerate some level of parasitic load. Thus whilst reporting that 89% of the study group had at least one species of GI parasite only 2% of the group exhibited diarrhoeal symptoms and there was no apparent deleterious effect such as any reduction in fecundity. A significant characteristic of this behaviour was the geophagy was often linked to consumption of processed monkey chow which was used to supplement the diet (Mahaney *et al.* 1995b). In this example, it remains difficult to use a lack of symptoms to support the hypothesis.

Clay particles reduce the viability of the fungal parasite *Histoplasma capsulatum* (Lavie *et al.* 1986a,b). The clays reduced the rate of respiration of *H. capsulatum* by adhering to the mycelial surface reducing the movement of nutrients, metabolites, and gases across the mycelial wall. Adhesion to the surface of a GI parasite would potentially have a similar effect (Thompson *et al.* 1995). Montmorillonites have been shown to increase the thickness of the mucous barrier layer by increasing polymerisation of mucous glycopeptides (Leonard *et al.* 1994). An increase in thickness would reduce the numbers of infective organisms able to attach

to the epithelial cell walls. Attachment is the critical primary stage establishing infections by bacteria and protozoan parasites (Nobuko *et al.* 2011). Any or all of these mechanisms may limit the infective load to a point where symptoms are reduced and deleterious effects are minimal.

Virulence for many of the pathogenic bacteria (Gaballa *et al.* 2010, Reniere *et al.* 2010), fungi (Johnson 2008) virus (Weinberg 1996) and protozoa (Wilson *et al* 1998, Weinberg 1999) is mediated by iron acquisition using specifically biosynthesised siderophore compounds, which scavenge iron from the environment. Preventing siderophore action would reduce virulence (Miethke *et al.* 2007). Siderophores can be bound to clay minerals, rendering them ineffective (Lavie *et al.* 1986b, Siebner-Freibach *et al.* 2004, Maurice *et al.* 2009). Many geophagy sites are sources of potential parasite infection (Geissler *et al.* 1998, Pebsworth *et al.* 2012a) particularly those frequented by other mammals. Geophagy material as a source of infection has been suggested; however this will be influenced by site choices. Analysis of 42 samples used for geophagy in Pemba found that none of the acceptable chosen earth samples contained any detectable helminth of risk to human health (Young *et al.* 2007).

The wet season use of both *Phoradendron* and geophagy may be driven by increases in rates of parasite infection and higher rates of infection during the wet season has been reported (Huffman 1997, Altizer *et al.* 2006, Gonzalez-Moreno *et al.* 2013). Parasitic disease transmission is density dependent and Donnelly *et al.* (2013) concluded that in more seasonal environments density dependent parasitic diseases are likely to be more virulent.

### 1.9.4 Mitigation of symptoms

#### 1.9.4.1 Anti diarrhoea

Knezevich (1998) suggested that geophagy and the lack of diarrhoea were linked. The geophagy material consumed by the *Rhesus macaque* contained both clay minerals kaolinite and smectite - a montmorillonite mineral. A characteristic of montmorillonites is the ability to adsorb large volumes of water (Baeshad 1955, Bergaya *et al.* 2006a). This potentially slows down passage of material through the intestines, permitting water to be absorbed preventing diarrhoea. Bacterial endotoxins and mycotoxins also cause diarrhoea. Montmorillonites adsorb the mycotoxin T2 and toxins produced by *Vibrio cholera* reducing increases in gastric motility induced by the toxins hence reducing diarrhoea (Fioramonti *et al.* 1987a, Fioramonti *et al.* 1987b).

Geophagy by grizzly bears to alleviate diarrhoea related to seasonal changes in diet is suggested by Mattson *et al.* (1999). Bears had emerged from hibernation and had consumed mushrooms and large amounts of ungulate meat. The geophagy material was also high in potassium, in which bears are deficient when emerging from hibernation (Mattson *et al.* 1999). Diarrhoea may also relate to a change in nature of the carbohydrate contained and increased dietary fibre and be unrelated to parasite infections. Mountain gorillas, (*Gorilla gorilla beringei*), in Rwanda suffer from diarrhoea when there is a seasonal change in the main constituent of the diet to >90% bamboos shoots (*Arundinaria alpina*). Symptoms appear to be relieved by consuming a halloysitic natural earth (Mahaney *et al.* 1995a). Bamboo shoots have high levels of potassium, carbohydrates and dietary fibre (Feleke 2013); a change to predominantly bamboo shoots may temporarily also cause diarrhoea due to the increased potassium load. This may be mitigated by the consumption of clay

mineral and its cation adsorption effect. Muscle weakness may occur as a result of low potassium levels following clay ingestion (Severance *et al.* 1988).

Kaolinite minerals such as halloysite differ from montmorillonites and are only able to bind a limited amount of water to their charged surfaces (Bergaya *et al.* 2006a). Davis *et al.* (1988) suggested antidiarrhoeal function for geophagy by red leaf monkeys (*Presbytis rubicunda*), reporting the main clay minerals present were kaolinitic and metahalloysitic minerals. Davis *et al.* (1988) compared it to a human medicinal product Kaopectate©, containing kaolinite previously available to treat diarrhoea, using this to support their hypothesis. The original Kaopectate© and a later modified product were withdrawn from market following failure of the efficacy testing by the USA Federal Drug Administration (FDA Safety Report 2004).

Kaolinite, similarly to montmorillonites, interacts with the intestinal mucosa, leading to changes in intestinal morphology and in the expression of several cytoskeleton proteins. Kaolinite ingestion slowed down gastric emptying and intestinal transit in rats *in vivo* (Voinot *et al.* 2012). 'Salido' wet material (containing clay minerals) consumed would have limited effectiveness due to mineral morphology and possible hydration state.

#### 1.9.4.2 Antacid - acid neutralisation

Geophagy in human pregnancy is often associated with nausea and vomiting of the first trimester and alleviation of heartburn and other symptoms in the later stages (Young *et al.* 2010). Antacid properties are dependent upon the chemical and mineralogical properties and sample pH. Above pH7 there is a strong likelihood of carbonate minerals being present, which have antacid properties. At higher pH geophageous material may contain soluble alkaline salts; hydrolysis of such salts would release alkali or alkaline earth cations e.g.Ca, Mg, Ba. Both carbonates and alkali salts under acidic stomach conditions would exhibit antacid properties. Acidic geophagy material might also have antacid properties depending upon the amount of aluminium oxide/hydroxides present. One such mineral is gibbsite, an aluminium hydroxide mineral, which occurs mainly in weathered tropical and subtropical habitats. The pH of samples is rarely presented in the literature and the presence of gibbsite uncommon (Wilson 2003a).

Rapid anaerobic fermentation of highly digestible foods in the enlarged forestomach of Colobine monkeys (analogous to that of ruminants) can lead to the production of large amounts of volatile fatty acids. The subsequent decrease in forestomach pH may cause fatal acidosis (Goltenboth, 1976 in Davies *et al*. 1988). Clay minerals are able to adsorb organic molecules, such as volatile fatty acids, (Davies *et al.* 1988). This property is unaffected by the pH of the clay/geophageous material.

### 1.9.5 Non-adaptive hypotheses

#### 1.9.5.1 Cultural

Geophagy has a long tradition in both Central America and West Africa. It has persisted as a cultural behaviour in migrant populations to North America, where the original function is no longer a significant issue (Hunter 1973, Hunter 1984). Children are the largest proportion of consumers regardless of nutritional, health state or socioeconomic status. Hunter suggests this is cultural transmission, (Hunter 1973).

#### 1.9.5.2 Famine food

Whilst not consumed as a food substitute it is associated with the consumption of wild varieties of potato, in Andean, Mexican and Native American populations (Johns 1986) and in the production of acorn bread by both Native Americans and Sicilians (Johns *et al.* 1991). These are examples where geophagy permits exploitation of a marginal food in food species, poor habitat or times of scarcity whilst mitigating the adverse effects of PSM associated with these practices (Dominy *et al.* 2004).

#### 1.9.5.3 Physiological imbalances

Micronutrient disturbances lead to disturbances in brain enzyme functions which may drive cravings or induce changes in taste sensitivity making geophagic material more palatable (von Bonsdorff 1977; Youdim *et al*. 1977; Chisholm *et al*. 1981 and Prasad 2001a,b in Young *et al*. 2011).

Voigt *et al*. (2008) concluded that the specific function of geophagy at a particular site is related to the physiological condition and requirements and the taxa of the animal visiting the site and there will be no single overriding function applicable to visitors to a site. Thus, interspecies and intersite comparisons are not necessarily appropriate when attempting to postulate a nutritional function.

## 1.10 Physiologically Based Extraction Techniques, PBET

Human PBET *in vitro* digestion models have been developed by many different organisations to investigate the availability of nutritional minerals and the toxicology of metals, pesticides, plastic residues and organic contaminants such as mycotoxins (Oomen *et al.* 2003, Oomen *et al.* 2004, Versantvoort *et al.* 2005). These models have also been used by the pharmaceutical industry for testing the release of drugs from different formulations. There is a difference between bioaccessibility and bioavailability. The amount of substance released from a potentially toxic source, pharmaceutical formulation, soil or leaf matrix into the digestive juices is defined as the bioaccessible fraction. Bioaccessible fractions can be variable and as little as 50% of the constituent present in the source matrix (Oomen *et al.* 2002). Oral bioavailability describes the proportion of the bioaccessible material that reaches the systemic circulation and so is available to interact with physiological systems either beneficially or as a toxin. Therefore, the bioavailable fraction is dependent upon the bioaccessible fraction (Ruby *et al.* 1996,1999).

Much of the work investigating the properties of geophagy materials has been criticised by Wilson (2003a) as not being under physiological conditions and this has led to the development and use of Physiological Based Extraction Tests (PBET) in the analysis of geophageous material. In addition, the majority of the published work on efficacy of plant extracts related to material not obtained under physiological digestion conditions. There were no publications using PBET conditions relating to plant related self-medication or geophagy in primates when this project commenced. The development and use of a tailored model was considered appropriate for the investigations undertaken in this project.

## 1.11 Aims and Objectives

The aim of this project is to investigate the possible functions of geophagy and the consumption of mature *Phoradendron* leaves by Geoffroyi's spider monkey (*Ateles geoffroyi)* living in Santa Rosa National Park, Costa Rica. Therefore the project has two main focuses. The first: to characterise the geophagy material consumed by *Ateles geoffroyi*. A determination of the physico-chemical characteristics of consumed geophagy materials will enable comparisons with published primate geophagy literature. There is currently no published work relating to geophagy by *Ateles geoffroyi* at Santa Rosa. The second is to undertake a preliminary screening of a simulated gastric digest of *Phoradendron* leaf eaten by *Ateles geoffroyi* in order to determine if the phytochemistry suggests a possible function for the *Phoradendron* consumption. The regular consumption of mistletoe leaves by *Ateles geoffroyi* at Santa Rosa is of particular interest as there are no previous reports in the literature of a primate consuming mistletoe leaves. The project has three specific aims.

**Specific Aim 1**: To determine a possible function for the consumption of the geophageous material. This will be achieved in the following manner:

**Objective 1**. To identify any clay or other minerals present in the samples using Munsell© colour characteristics, determination of Water content, Loss on Ignition, X-Ray Diffraction spectroscopy (XRD), Infra-Red spectroscopy (IR), X-Ray Fluorescence (XRF) and the pH of sample material.

The results from these techniques will permit some broad comparison with previously published primate data as many of the hypotheses for the function of geophagy are determined by the nature of the clay mineral content and pH (e.g. mineral supplementation). These properties also determine the adsorption of toxic and anti-nutritive toxins, gastro-protection and antibacterial and anti-parasitic activity.

**Objective 2.** To develop a suitable *Ateles* gastric model in order to investigate the behaviour of geophagy samples in physiological conditions.

The use of such a model provides information about the physical behaviour of geophagy material in simulated *Ateles* GI conditions; in particular pH, may influence the behaviour of colloidal and clay materials. Plant digests produced in such a model would also more accurately reflect plant bioaccessible extracts rather than those obtained from organic solvent extraction.

**Objective 3**. To identify the physico-chemical characteristics of the geophagy material using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS), Laser Diffraction Particle size analysis and Ultra Violet Spectrophotometry (UV).

Results from the ICP-MS techniques can provide information on the mineral composition of the material including the nature of any clay minerals present. In addition, results can provide an indication of the presence of any potential biologically important nutritional or toxic minerals and which would be potentially available for absorption. Results from the Laser particle size distribution, aggregation and interaction with the mucin can determine the potential of the geophagy material to behave as a GI protectant and the potential for adsorption

of classes of PSM. UV spectrometry will be used to investigate the adsorption by geophagy samples of sample classes of PSM. The adsorption of examples from potentially important classes of PSM compounds will indicate the potential for detoxification by the geophagy material of the PSM compounds in the gastric conditions.

**Objective 4**. To test the gastric digests for antibacterial activity against a range of bacterial species.

Several classes of antibacterial compounds have been isolated from soils. In addition clay leachates are known to have anti-bacterial properties and many of the mechanisms involved are common to antiparasitic and antiprotozoal activity.

**Specific Aim 2:** To determine whether differences in the mineral content and the phytochemistry of the *Phoradendron* influences the selection of the *Phoradendron* species consumed and if such a selection suggests a possible function for the *Phoradendron* consumption. This will be achieved in the following manner:

**Objective 5.** To determine the mineral content of the *Phoradendron* leaf using XRF. To identify the phytochemical characteristics of digest of the eaten and non-eaten *Phoradendron* species produced from the gastric model. This will be achieved using UV and standard phytochemical screening of the gastric digests and Reverse Phase - High Pressure Liquid Chromatography (RP-HPLC). Microbiological testing will be used as a proxy for biological activity. The gastric digests are to be tested against a variety of bacterial organisms.

The results of the XRF will indicate which minerals are present in the *Phoradendron* leaf. Mistletoe physiology creates concentrations of certain elements in the leaves. These concentrations may vary between *Phoradendron* species and host/*Phoradendron* combinations and may be a source of mineral supplementation

The results from these techniques applied to gastric digests can provide indicators of differences in classes of chemical components available to act either locally in the GI tract or potentially systemically. They may also permit differentiation between *Phoradendron* species. They may permit identification of major constituents present in eaten and non-eaten and confirm these using Certified Reference Materials, where available. Microbiology may indicate interspecies and temporal variations in activity of the digests.

**Specific Aim 3** - To determine whether there is any interaction when geophagy follows *Phoradendron* consumption. This will be achieved in the following manner:

**Objective 6.** To analyse the phytochemistry and biological activity of a combined *Phoradendron* and geophagy digest from the *Ateles* gastric model. The digests obtained by digestion of both materials together will be analysed using UV, RP-HPLC and subjected to microbiological testing.

The results of these analyses may suggest a possible function for the combined behaviours in the adsorption of phytochemicals and a possible change in biological activity.

# Chapter 2 Development of the *Ateles* Gastric model

This chapter relates to Specific Aim 1, Objective 2 (Section 1.11): To develop a suitable *Ateles* gastric model in order to investigate the behaviour of geophagy samples in physiological conditions.

## 2.1 Introduction to development of PBET modelling

In the field of drug development, pharmacological results led to the recognition that the total amount of an ingested product does not always reflect the amount that is measured in the body and hence the response to a drug (Oomen *et al.* 2002, Hund-Rinke *et al.* 2003, Le 2012). Pharmacokinetics (the disposition of a drug in the body) and the factors that influenced this and thus the pharmacology became important areas of research. This led to two important concepts bioaccessibility and bioavailability.

- the bioaccessible fraction is the amount of a pharmaceutical/nutrient that is released into solution from an ingested material and available for absorption.
- bioavailability describes the proportion of the bioaccessible fraction that reaches the systemic circulation and exerts an effect.

Bioavailability is governed by factors such as the degree of dissolution, physiology and the mechanism of uptake into the circulatory system.

Human gastrointestinal (GI) digestion/dissolution processes can be simulated, in a simplified manner, with *in vitro* digestion models permitting measurement of the bioaccessible fraction and extrapolation to potential bioavailability. Early investigations of the availability of vitamins and minerals relied upon the use of *in vitro* methods and animal models and were concerned with specific issues, such as the availability of items such as iron (Fe) from dietary items (Monsen *et al.* 1978) and the interaction of food constituents that may limit its absorption (Hallberg 1974, Hallberg *et al.* 1986). Initial simple *in vitro* methods were developed further as an alternative method to administering radio-nucleotides to humans and as a response to uncertainties in the validity of inter-species comparisons using animal models and subsequent extrapolation to humans due to differences in metabolic processes (Quinn *et al.* 1958, Hucker 1970, Smith 1991, Kararli 1995, Lin 1995).

An early *in vitro* model to determine iron release replaced mastication by homogenization, and subjected the homogenate to a pepsin-HCl digestion for one and a half hours at body temperature (Jacobs *et al.* 1969). A criticism of this model was that as most of the dietary iron is absorbed from the small intestine and not from the stomach, the simple pepsin/HCl method did not determine the true availability of iron (Narasinga Rao *et al.* 1978). At the alkaline pH of the intestine, solubility of iron decreases considerably and at this alkaline pH the proportion of ionisable iron is very low. Narasinga Rao *et al*. (1978) added a second stage to replicate the transition to intestinal conditions. Test foods were 'digested' with pepsin-HCl at pH 1.35 and subsequently the pH was adjusted to pH 7.5 then the digestate filtered and analysed. The work was extended further with the comparison of two different digestion methods (Lock *et al.* 1980):

- *In vitro* gastric digestion - Subjects presented themselves for gastric juice collection after an overnight fast. Duplicate 0.2 g portions of a range of foods were incubated in plastic centrifuge-tubes containing 5 ml harvested gastric juice at $37^0$C for 1.5 hr.
- Simulated gastric digestion - Portions of 0.2 g of the same foods were incubated at $37^0$C for 1-5 hours

with a range of pepsin solutions (5 ml, 5 g/l), adjusted with HCl to pH ranging from 1-4 to 2.8, at intervals of 0.2 pH units.

The results showed that the amount of Fe liberated from the same foods was greater with pepsin-HCl than that obtained using harvested gastric fluids. It was also higher than *in vivo* iron absorption of a similar dose measured in human subjects (Layrisse *et al*. (1969) in Lock *et al*. 1980). Lock *et al*. (1980) concluded that the Fe liberated by the pepsin-HCl system does not reflect the true bioavailable of Fe. The difference between pepsin-HCl and gastric juice bioaccessibility was not explained however the authors reported that it did not appear to be due to the proteins or the mucoproteins present in gastric juice.

Miller *et al*. (1981) adjusted the pepsin/HCl method, even though the results from these did not correspond to previously published human *in vivo* results. This further modification of the *in vitro* method took into account the effect of changes such as pH has on iron solubility. Using this modified method, Miller *et al.* (1981) showed that bioaccessibility was a function of the iron chemistry and solubility. Solubility could be improved by the presence of ascorbic acid and citric acid. Both were then included in the Miller model which also included the following modifications: the pH change from gastric to intestinal levels was gradual and reproducible and only soluble forms of iron were utilised.

Bioaccessibility and bioavailability were subsequently employed to investigate the potential toxicity of ingesting metal contaminants in soils and from toys by children. Ruby *et al.* (1993) attempted to develop an *in vitro* method to enable them to evaluate *in vivo* bioaccessibility of ingested lead (Pb) from contaminated waste soils material. This arose following several publications of *in vivo* animal studies, which suggested that Pb was not readily bioavailable from mine wastes. Ruby *et al.* (1993) compared *in vitro* availability using a pepsin/HCl method based on the works and references in Miller *et al*. (1981) and an *in vivo* method using New Zealand white rabbits (*Oryctoiagus cuniculus*). Ruby *et al.* (1993) concluded that the primary factors governing solubility and bioaccessibility were geochemical factors. The physiological factors i.e. GI tract pH, mixing and transit times, which influence bioavailability, were comparable for rabbits and humans, and so the rabbit model was appropriate. However, the GI tract of rabbits contains high levels of small carboxylic acids, e.g. acetate, lactate, malate and citrate due to the type of diet and digestion processes. These acids were added to the *in vitro* method. Pb chelates with simple organic acids and such complexes are more readily absorbed by the intestinal epithelium. These organic acids would not normally be present in similar concentrations in the human stomach.

Environmental toxicology researchers and regulatory bodies had been using *in vitro* methods or animal testing using rabbits and rats to investigate the toxicity of metal species in soil. Much of this work using rabbits and rats had serious limitations in that gastrointestinal morphology and digestion differed markedly from that of humans. From the drug industry it also had become apparent that there was wide interspecies variation in the metabolic pathways and between sexes (Smith 1991) and gastrophysiology (Martinez *et al.* 2002). Reddy *et al.* (1991) highlighted the problem of interspecies comparison as shown by variation in the effects of both known enhancers and inhibitors of iron adsorption in humans and rats. The work attempted to eliminate, where possible, any methodological factors that might account for differences in the effect of dietary factors on absorption in rats and humans. The results showed that non-heme iron absorption data in rats cannot be extrapolated to humans. The influence of dietary enhancers and inhibitors was much less, and certain dietary components such as meat or tea failed to influence absorption in rats but did so in humans.

A comparison of lead (Pb) bioavailability from *in vivo* animal data with those from *in vitro* US Environmental Protection Agency (USEPA) standard procedures showed lower availability from the GI animal data (Ruby *et al.* 1996), attributing this difference to much slower and lower solubility in the GI tract of the lead-bearing minerals. Additionally, once in the small intestine, any dissolved Pb could be bound to proteins and enzymes, in complexes with amino acids, carboxylic acids and tannins released from food. These interactions would also reduce the soluble portion available for absorption into the blood stream. (Ruby *et al.* 1996) stated that extrapolation from an animal model to children can be made if the toxin/contaminant behaves in the same manner in both circumstances. However, such data is limited to assessment of the exposure to (bioaccessible) toxin/contaminant but not the uptake of material across the intestinal wall into the blood/circulatory system i.e. the bioavailable fraction. In a further review of *in vivo* and *in vitro* data bases Ruby *et al.* (1999) stated that:

"*all of the animal models currently in use for bioavailability assessment, with the possible exception of monkeys, have substantial anatomical and physiological differences from humans, and none of these models has been validated against estimates of metal absorption in children or adults. Thus it is possible that the animal models used most extensively to date (rats and swine) may produce relative bioavailability estimates that are not equal to those in humans*" (p3703).

Even with these limitations, these animal models, with adaptations by other researchers, were used for several years in environmental toxicology, and had been used by the pharmaceutical industry for testing the release of drugs from different formulations. The issues of inter species differences and problems of validation of results were the drivers for developing specific human *in vitro* models, which more closely replicate the human digestive and absorption processes. Oomen *et al*. (2002) reviewed five of the most widely used *in vitro* digestion models:

(1) Simple Biological Extraction Test (SBET) method of the British Geological Survey, this is a single compartment (Static stomach model), without food. (The SBET method was based on (Ruby *et al*. 1996, 1993).
(2) the DIN 19738 Ruhr-Universität Bochum, a two-compartment model (Static GI model), stomach and intestine; with the addition of milk-powder.
(3) RIVM (The Netherlands), a three compartment model (Static GI model) and contains an oral compartment using simulated saliva; without any food.
(4) Simulator of human intestinal microbial systems of infants procedure - (SHIME) (LabMet/Vito, Belgium), a two-compartment model (Static GI model) with the inclusion of cream and baby milk.
(5) TIM method (The Netherlands Organisation for Applied Scientific Research TNO), this is a specially manufactured piece of equipment using a complex computer controlled system, (Dynamic GI model). The system is able to mimic peristalsis, has oral and gastric compartments with phased changes in gastric compartment pH together with separate duodenal, jujenal and ileal compartments, again with computer controlled pH and delivery of simulated secretions. There were also differences in the residence times, content and sources of secretion components.

The DIN and RIVM utilised simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) based on formulations developed by Rotard *et al*. (Rotard (1995) in Wijnands *et al.* 2003) The German DIN 19738:2004-

07 (Deutsches Institut für Normung e. V. Herausgeb Standard) method was developed for the investigation of *'Absorption of organic and inorganic pollutants from contaminated soil material*'.

The results obtained from a multi-lab round-robin assessment of these models utilising a Certified Reference Soil were analysed (Oomen *et al.* 2002). The results showed that, for metal contaminants, the single compartment – stomach only conditions gave higher bioaccessible values than did models with multiple gastric compartments, indicating that gastric pH was the main cause of the difference in stomach and extended GI model results. (Oomen *et al.* 2002) concluded that, in the case of metal released from soils, the fasted, low pH gastric model generated the 'worst case scenario' for exposure and that fed/semi-fed was more representative of 'average' cases.

The model developed in Oomen *et al.* (2002) was subsequently used for investigation of the effects of constituents and GI conditions on the bioavailability of potentially toxic metals found in soils (Oomen *et al.* 2003, Oomen *et al.* 2004). The use of this model was extended to other fields. The Oomen *et al.* (2002) model was used to investigate the availability of organic as opposed to metal contaminants in foods (Versantvoort *et al.* 2004, Versantvoort *et al.* 2005). The results reported the method as providing reproducible results for mycotoxins and suitable for using in risk assessment for exposure to organic food contaminants.

The major problem with data obtained from such models is that there are no Certified Reference Materials suitable for use because of the varieties of matrices that would be required. Consequently, there is almost no experimental *in vivo* data available against which to validate the *in vitro* methods. Usually animals with similar gastrointestinal tract characteristics to humans, such as immature swine, are preferred and have been shown to be reasonable analogues (Casteel *et al.* 1997, Casteel *et al.* 2006). Where animal trial models are carried out, there needs to be additional studies to show how the animal bioavailability can be related to human bioavailability. None of the *in vivo* testing regimes using animal models had been validated against estimates of metals absorption in either children or adults, due to the toxic nature of the metals of interest hence data from *in vitro* tests are difficult to interpret. Some validation of a stomach phase test used for the determination of lead in house dusts was obtained by correlation with blood lead levels of the children living in the houses where the dusts were sampled (Ruby *et al.* 1999).

The Bioaccessibility Research Group of Europe (BARGE) is a research network formed in 2012 to study human bioaccessibility of significant toxic contaminants in soils such as arsenic, lead and cadmium via the gastrointestinal tract. It has undertaken collaborative research and developed a harmonised *in vitro* physiologically based bioaccessibility procedure for soils, called the Unified BARGE Method (UBM). The UBM *in vitro* test has been validated for arsenic, cadmium and lead against a juvenile swine *in vivo* model (Wragg 2012). It is currently the recommended method in UK, France, Netherlands, Belgium, Portugal and Sweden and is under consideration in other European countries for these contaminants. It aims to standardise procedures worldwide.

In the field of pharmaceutical studies of bioavailability, the most frequently cited limitations of *in vitro* assays concerns the qualitative and quantitative deficiencies in the metabolism of test chemicals in comparison with *in vivo* assays. These and related deficiencies are particularly important considerations when testing for example endocrine active substances (EAS), since several hormonally active chemicals, including some that are derived from plants, are known to require bioactivation. There are currently calls to encourage the inclusion

of the assessment of metabolism, particularly human metabolism, in *in vitro* assays designed to evaluate chemicals for endocrine activity and bioactivation (Jacobs *et al.* 2013).

For geophagy studies Wilson (2003a) provides an overview of PBET models used to investigate geophagy and includes a survey of species and material characteristics. He states that many of the proposed hypotheses for the function:

"*relate to the exact nature, behaviour and the properties of the clay minerals in the geophageous material*" (p.1526).

The review however criticises several of the 17 publications reviewed for a lack of any attempt to determine bioaccessibility of nutrient elements in the relevant simulated physiological conditions. It highlights the fact that much of the extractions reported have used only 0.1M HCl or acid ammonium oxalate at pH2 and criticises these limitations. An example of such a simple PBET method containing simple organic acids and pepsin adjusted to pH2 was used to investigate the bioavailability of toxic elements in soils consumed by humans in Uganda (Smith *et al.* 2000). In acknowledgment of the limitations of their modelling Ruby *et al.* (1996) were not able to validate the *in vitro* model against data obtained from direct measurement in children stating that:

"*animal models were relied upon for comparison to the PBET results, based on the premise that data from appropriate animal models can be extrapolated to humans for the purpose of exposure assessment*" (p.423).

One response to these comments taking the issue of bioavailability further was Dominy *et al.* (2004) with investigation of the adsorption of tannic acid a (soluble tannin), quebracho (a condensed tannin), quinine (an alkaloid) and calcium oxalate by kaolin minerals in a multi-compartment simulated gastro intestinal (GI) tract environment. Between 20-30% reductions in the bioavailable fraction were observed when refined kaolin was included in the model digest.

The same criticisms can be levelled at investigations of bioaccessibility and bioavailability of active compounds present in plant material ingested by humans and animals. Much of the historic work on active ingredients in plant material used organic solvent extractions. This is not representative of GI conditions and may not reflect the bioaccessibility of compounds released from plant material under physiological conditions.

These considerations were the impetus for creating a tailored GI model, which was more appropriate for investigations of both geophagy and *Phoradendron* consumption by *Ateles geoffroyi.* There were several important factors, such as differences in morphology, diet, transit time and GI electrolyte differences, which had to be taken into consideration in the development of a model. The following sections present these factors in detail.

## 2.2 Comparative anatomy

Human PBET models are based on human anatomy and digestive physiology, Figure 2.1 shows the major structures of the human digestive system.

The principal function of the mammalian stomach is the storage of food and mixing the contents with gastric fluid to allow digestion to start. The mucosa lining the stomach secretes approx. 1.5 litres of gastric fluid per day. Gastric fluid contains varying amounts of the following: water, HCl, electrolytes (sodium, potassium,

calcium, phosphate, bicarbonate, and sulphates), mucus and pepsin. The structure of the stomach is variable from a simple single compartment in humans to the multiple compartment stomach of ruminant animals. The stomach contents empty into the small intestine, where further mixing, digestion and subsequently absorption takes place. The large intestine consists of three regions, the caecum, the colon, and the rectum terminating with the anus.

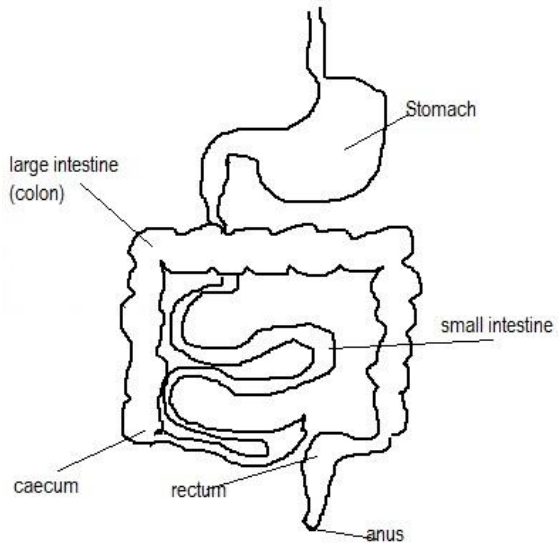



Figure 2.1 Diagram of principal structures of human GI tract (not to scale: drawn in Paint©).

The primary function of the large intestine is to absorb water and electrolytes that have already passed unabsorbed through the small intestine. There are major species differences between primates, and between humans and primates with respect to both anatomy and the time foods take to pass through the digestive system i.e. the transit times (Milton 1981, Lambert 2002).

Non-human primate species show anatomical specialisations to the stomach, caecum and colon compared to human structures (Chivers *et al.* 1980, Lambert 1998). These are considered a response primarily to their diet. Folivores require the capacity to deal with large amounts of structural carbohydrates and to detoxify plant PSM. In many of these species, the stomach and the large intestine have undergone the most extensive specialisation to enable carbohydrate fermentation and digestion of the structural carbohydrates.
This type of adaptation is seen in *Colobus* monkeys, which mainly eat leaf material (Chapman *et al.* 2002, Harris *et al.* 2010). *Colobus* monkeys differ from most primates in that they have a large multi-compartment stomach. The forestomach utilises microbial fermentation in an alkaline environment and is separated from a second acidic non-fermenting region (Lambert 1998). In *Ateles* spp., the stomach is an enlarged J-shape and the duodenum, the first section of the small intestine, is a shortened C-shape, in contrast to the elongated U-shape of other mammals including humans (Chivers *et al.* 1980). Frugivores, such as *Ateles*, with a bulky high volume carbohydrate rich diet and rapid transit times require a relatively large stomach surface area for rapid absorption to avoid loss of soluble components (Lambert 1998, Lambert 2002). In humans the high proportion contributed by the small intestine (≥ 56%) has been suggested as an adaptation to a diet that is nutritionally dense. The different relative proportions of the stomach and the small intestine in *Ateles* is suggestive of a bulky diet, relatively easily digested and absorbed by a large surface area (Milton 1999). There are considerable differences in the lengths and hence the volumes of the various components of the GI tract (Table

2.1). There are also considerable species differences in transit times. Milton (1981) used plastic markers placed in foods to determine the transit time for foods in *Ateles geoffroyi*. The time of first appearance averaged 4.4 ± 1.53 hours with a range of 2.75 – 7.75 hours. Transit time for the similar sized howler monkey *Alouatta seniculus*, a folivore, is 35 hours. Transit times for humans are considerably longer. Time to empty 50% of stomach and 50% of the small intestine is 2.5-3 hours each and large intestine 30-40 hours, and may be as long as 100 hours (O'Donnell *et al.* 1990).

Table 2.1 A comparison of the anatomical structures of the gastrointestinal tract. Values taken from (Milton 1981)[A], (Chivers *et al*. 1980)[B] and (Milton 1999)[C]. Volumes are fully distended volumes. Values for humans includes bowel (Tortora *et al.* 2003)[D].

| | ***Ateles*** | | | **human** | |
|---|---|---|---|---|---|
| **GI feature** | **Length[A] (cm)** | **Volume[B] ($cm^3$)** | **Volume calc. as % total GI tract** | **Length[D]** | **Volume as % total GI tract[C]** |
| stomach | 15 | 212 | 35 | N/A | 24 |
| small intestine | 90-120 | 252 | 41 | 5000 | 56 |
| caecum | 22 | 5 | 9 | vestigal | |
| large intestine | 33 | 95 | 16 | 1400-15004 | 20 |

## 2.3 Spider monkey simulated gastric fluid development

Brooks *et al*. (1963) obtained 24 hour collections of gastric fluid content from spider monkeys. The values in Table 2.2 were determined in a gastric aspirate. A variety of pH values were recorded: pH 1.2 from 10am-5pm, rising to pH 2.5 over a 1hr period between 6-7pm, remaining elevated between 8-10pm and then reducing to less than pH2 and remaining at this level between 11pm – 10am. The monkeys were fed during the collection period between 10am – 5pm, a time when a mean pH 1.4 ± 0.3 was recorded. The mean pH recorded during a fasting period was 4.3 ± 1.9. Gastric aspirate contains material from both the *Ateles* saliva and gastric fluid. Therefore, the sum of the values in the formulation of the *ad hoc* simulated saliva and gastric media used in the final model will reflect these values.

Table 2.2 Composition of gastric contents of spider monkey Brooks *et al.* (1963)

| | **Feeding** | **Fasting** |
|---|---|---|
| pH | 1.4 ± 0.3 | 4.3 ± 1.9 |
| Free acid mEq/l | 62 ± 17 | 4 ± 6.6 |
| Total acid mEq/l | 81 ± 20 | 11 ± 9.9 |
| $Na^+$ mEq/l | 56 ± 22 | 97 ± 18 |
| $K^+$ mEq/l | 21 ± 3.0 | 14 ± 3.0 |
| $Cl^-$ mEq/l | 126 ± 23 | 107 ± 12 |

As stated in Section 2.1 a comparison of the 5 models, listed in Oomen et al. (2002) and reviewed by Intawongse *et al*. (2006) showed there was a lack of consistency in methods being used in bioavailability testing. Because none of the existing models reflected the data for *Ateles* (Table 2.2) it was decided to adapt the DIN 19738 method, Table 2.3. This model was initially used to investigate organic and inorganic toxins in soils. It had also been used with modification by The National Institute of Public Health and The Environment (RIVM), The Netherlands, to investigate bioavailability of toxins in foods (Versantvoort et al. 2004, Versantvoort et al. 2005). The RIVM modification involved the use of three digestion fluids modelling mouth and stomach and intestinal fluids. The Danish Environmental Agency also modified this model for their work, taking elements

from both models (Grøn 2005).

The *Ateles ad hoc* model developed in this thesis was based upon the DIN 19738 method simulated media shown in Table 2.3, modified to reflect the data in Table 2.2. The sample/liquid ratio, incubation times and the type of mixing were taken from the 'Swallow-fasted' method (a modified RIVM method) published by Brandon et al. (2006).

Table 2.3 Composition of gastric fluid (GI), DIN 19738

| **Saliva** | | **Gastric Juice** | |
|---|---|---|---|
| **salt** | **mg/100ml** | **salt** | **mg/100ml** |
| NaCl | 50 | NaCl | 290 |
| NaSCN | 15 | KCl | 70 |
| $Na_2SO_4$ | 55 | $KH_2PO_4$ | 27 |
| $NaHCO_3$ | 15 | pepsin* | 100 |
| KCl | 45 | mucin* | 300 |
| $KH_2PO_4$ | 60 | | |
| $CaCl_2.2H_2O$ | 15 | | |
| mucin* | 75 | | |
| α-amylase* | 25 | | |
| urea | 100 | | |
| uric acid | 10 | | |

*amylase, pepsin, mucin = bovine source

The PBET methods usually use a ratio of 1:5 or 1:25 'soil' to fluid ratio. However Oomen *et al*. (2003) reported that even ratios 1:100 – 1:5000 did not produce significantly different extraction profiles. A ratio of 1:16 was chosen to enable the maximum investigation of the limited samples available. Therefore, the volumes chosen were - 10ml simulated saliva + 30ml simulated gastric fluid. The combined volumes of saliva and gastric fluid used were chosen such that the volume occupied approx. 20% of the possible *Ateles* spp. stomach volume to allow for the presence of food.

## 2.3.1 Electrolyte Calculations:

In order to calculate the required amounts of the salts in the media the following calculations were performed, using the following values (Table 2.4):

1 mEq $Na^+$ = 22.99 mg; 1 mEq $K^+$ = 39.098 mg; 1 mEq $Cl^-$ = 35.453 mg

Table 2.4 Calculated results for the range of the major constituent ions, found in the feeding data, Table 2.2, Brooks *et a*l. (1963), required in the final 40ml of combined saliva and gastric fluids.

| **Sodium mEq/l** | **$Na^+$ mg/l** | **$Na^+$ mg/40ml** | **Potassium mEq/l** | **$K^+$ mg/l** | **$K^+$ mg/40ml** | **Chloride mEq/l** | **$Cl^-$ mg/l** | **$Cl^-$ mg/40ml** |
|---|---|---|---|---|---|---|---|---|
| 34 | 781.66 | 31.27 | 18 | 703.76 | 28.15 | 103 | 3651.66 | 146.07 |
| 56 | 1278.44 | 51.14 | 21 | 821.05 | 32.84 | 126 | 4467.08 | 178.68 |
| 78 | 1793.22 | 71.73 | 24 | 938.35 | 37.53 | 149 | 5282.49 | 211.30 |

In order to calculate the ion concentrations in the DIN 19738 media constituents the following proportions used are presented in Table 2.5.

Table 2.5 Percentage of individual ions in each salt, listed in the DIN 19738

| Salt | Molecular wt | $Na^+$ % | $Cl^-$ % | $K^+$ % |
|---|---|---|---|---|
| NaCl | 58.44 | 39.34 | 60.66 | |
| $Na_2SO_4$ | 142.04 | 32.37 | | |
| $NaHCO_3$ | 84.01 | 27.37 | | |
| KCl | 74.55 | | 47.56 | 52.44 |
| $KH_2PO_4$ | 136.09 | | | 28.73 |
| $CaCl_2$ | 110.98 | | 63.89 | |

## 2.3.2 Development of simulated saliva for *Ateles* spp. model

Saliva volume chosen (10ml) is representative of that produced by 2 minutes chewing at a flow rate of 5ml/min in response to a stimulus (Versantvoort *et al*. 2004). The calculations for ions present in 10ml simulated saliva DIN 19738 are presented in Table 2.6

Table 2.6 Calculated sodium, potassium and chloride ions in 10ml, simulated saliva DIN 19738 method

| Saliva | mg/100ml | mg/10ml | $Na^+$ % | $K^+$ % | $Cl^-$ % | $Na^+$mg/10ml | $K^+$ mg/10ml | $Cl^-$ mg/10ml |
|---|---|---|---|---|---|---|---|---|
| NaCl | 50 | 5 | 39.34 | | 60.66 | 1.97 | | 3.03 |
| $Na_2SO_4$ | 55 | 5.5 | 32.37 | | | 1.78 | | |
| $NaHCO_3$ | 15 | 1.5 | 27.37 | | | 0.41 | | |
| KCl | 45 | 4.5 | | 52.44 | 47.56 | | 2.36 | 2.14 |
| $KH_2PO_4$ | 60 | 6 | | 28.73 | | | 1.72 | |
| $CaCl_2$ | 15 | 1.5 | | | 63.89 | | | 0.96 |
| | | | **Total in 10ml saliva** | | | **4.16** | **4.08** | **6.13** |

The initial pH of saliva secretion in humans is pH 7.4 and is produced at a flow rate of 2-10ml/min when responding to stimuli such as to a sour taste or a hard pebble (Versantvoort *et al.* 2004). Saliva, in addition to the electrolytes, contains two major types of protein secretions, a mucous secretion containing mucin for lubrication and other functions and α-amylase enzyme for the digestion of starches.

### 2.3.2.1 Non-electrolyte constituents

Mucins are extracellular large highly glycosylated molecules having 'mucin domains'. Mucin domains are rich in the amino acids threonine, serine and proline. Mucins are an example of salivary Proline-Rich Proteins (PRP), and may be secreted or bound to the mucous membrane (Bansil *et al.* 1995, Bansil. *et al.* 2006). Salivary constituents such as PRP and a second class of peptide, the histantins, vary with species. Both these constituents bind to tannins with histantins binding twice as many tannins as do PRP. Tannins in diet may have detrimental effects as a result of binding to (a) structural proteins and amino acids in the lining of the GI tract; (b) enzymes and food proteins so reducing both the digestion and absorption of proteins and amino acids (Bennek 2002).

Dietary proteins form large complexes with tannins preventing them from being digested and available to the animal. Excretion of such complexes is a significant cause of protein loss in many animals (McArthur *et al.* 1995, Min *et al.* 2003, Mueller-Harvey 2006). Monogastric mammals, e.g. pigs when fed with a diet containing levels of tannins between 0.2 5% dry forage experience depressed growth rates, low protein utilization, damage to the mucosal lining of the digestive tract, alteration in the excretion of certain cations, and increased

excretion of proteins and essential amino acids. Levels above 5% in the pig may be fatal (McLeod in Mueller-Harvey 2006).

There are also species differences in the amount of PRP and histantins secreted. These differences have been linked to dietary type. Grazers primarily consume grasses and consequently have low tannin exposure and have been shown to have very low levels of PRP in their saliva. In contrast browsers, consuming leaf, stem and bark material have a much higher dietary tannin exposure (Robbins *et al.* 1991, Robbins 1994, Bennek 2002) and higher levels of salivary PRP. Amongst the primates, the lowest levels of tannin consumption is found in insect eating tamarins and squirrel monkeys with levels increasing with increasing fruit and leaf consumption (Azen *et al.* 1978). Salivary PRP bind to dietary tannins proportionately greater than to dietary proteins. This preferential binding results in reduced faecal nitrogen losses by preventing protein-tannin complex formation. PRP contain non-specific nitrogen and non-essential amino acids which make them less costly for an animal to exploit rather than using up valuable dietary protein or amino acids (McArthur *et al.* 1995). This is therefore an economical mechanism to deal with potentially deleterious dietary tannin levels.

Tannins are astringent; astringency is the sensation caused by the formation of complexes between tannins and salivary PRP (Reed 1995). *Ateles* are able to discriminate tannic acid solutions when presented at the 0.1mM level (Laska *et al.* 2000). This level of discrimination may allow *Ateles* spp. to avoid higher tannin levels in the selection of food items and additionally utilise plant proteins more efficiently. The high sensitivity of *Ateles* to tannins in foods (Laska *et al*. 2000) was the rationale behind including an oral component to simulate the presence of PRP in saliva.

#### 2.3.2.2 Mucin source

In order to more closely resemble human and primate secretions it was felt necessary to make a modification to the type of mucin used from that used in the DIN Model. Two types of mucin product were commercially available:

mucin from bovine sub-maxillary glands Type 1-S

mucin from porcine stomach Type II

Grazers such as cattle have relatively smaller sub-maxillary glands and have reduced levels of PRP in their saliva compared to browsers and omnivores (Robbins *et al.* 1991). Porcine secreted Mucin type II, is the closest to human secreted mucin (Perez-Vilar *et al.* 1998). It was considered that this product would be more suitable for the *Ateles* model than that of bovine origin used in the original models. No alteration in concentration was made to the values used in the human simulation model.

#### 2.3.2.3 α-amylase source

The source of α-amylase was also changed. α-Amylase enzymes vary from tissue to tissue between species and within a single species (Brayer *et al.* 1995). Differences in amino acid sequences between human salivary and pancreatic amylases have been identified (Brayer *et al.* 1995). As cattle are grazer/ruminants there may be structural differences between bovine enzymes and those of humans and non-human primates.

Table 2.7 Formula used for *Ateles* simulated saliva, modified from DIN 19738

| **Saliva mg/100ml** | |
|---|---|
| NaCl | 50 |
| $Na_2SO_4$ | 55 |
| $NaHCO_3$ | 15 |
| KCl | 45 |
| $KH_2PO_4$ | 60 |
| $CaCl_2.2H_2O$ | 15 |
| Mucin | 75 |
| α-amylase | 20 |
| urea | 100 |
| uric acid | 10 |

However Hwang *et al.* (1997) showed that there was approx. 70% structural homology between human and bacterial α-amylase. Human salivary α-amylase was not widely commercially available thus a bacterial α-amylase was chosen as a substitute for human amylase, in the *Ateles* model, in place of the bovine amylase used in the DIN 17938 model. The enzyme α-amylase acts on internal bonds of long chain carbohydrate polymers. The simulated *Ateles* GI fluid has reduced α-amylase content (Table 2.7). The α-amylase content was reduced to reflect the monkey dietary content of soluble sugars and relative lack of long chain carbohydrates.

## 2.3.3 Development of the Simulated Gastric fluid for the *Ateles* model

The volume of gastric fluid was set at 30ml. The calculations for ions present in 30ml simulated saliva (DIN 19738), are presented in Table 2.8

Table 2.8 Calculated sodium, potassium and chloride ions in 30ml, simulated gastric fluid DIN 19738

| **Gastric fluid** | **mg/100ml** | **mg/30ml** | **$Na^+$ %** | **$K^+$ %** | **$Cl^-$ %** | **$Na^+$ mg/30ml** | **$K^+$ mg/30ml** | **$Cl^-$ mg/30ml** |
|---|---|---|---|---|---|---|---|---|
| NaCl | 290 | 87 | 39.34 | | 60.66 | 34.23 | | 52.77 |
| KCl | 70 | 21 | | 52.44 | 47.56 | | 11.01 | 9.99 |
| $KH_2PO_4$ | 27 | 8.1 | | 28.73 | | | 2.33 | |
| | | | **Total in 30ml Gastric fluid** | | | **34.23** | **13.34** | **62.76** |

The sum of the calculated values found in the combined 40ml of DIN mixed media (10ml saliva and 30ml gastric fluid) are presented in Table 2.9 together with an illustration of the deficits when compared to the ranges found in the *Ateles* data from Brooks (Brooks *et al*. 1963). Calculations were then performed to enable additions to the gastric media formulation in order to address these deficiencies. Several different combinations were attempted and the pH measured, before arriving at the final formula, Table 2.10.

Table 2.9 Comparison of the values in the DIN media with those calculated as present in *Ateles* aspirate (Brooks et al. 1963)

| | **DIN 19738 values mg/ 40ml derived from (10ml Saliva + 30ml Gastric) Table 2.6 and 2.8** | **Range *Ateles* calculated mg/ 40ml gastric aspirate derived from Table 2.2** | **Additional required Saliva +Gastric mg/ 40ml** |
|---|---|---|---|
| $Na^+$ | (4.16+34.27) = 38.30 | 31.27 – 71.73 | Max 33.43 |
| $K^+$ | (4.08+ 13.34) = 17.42 | 28.15 – 37.53 | 10.63 – 20.11 |
| $Cl^-$ | (6.13 + 62.67) = 68.80 | 146.07 – 211.30 | 77.27 – 142.5 |

The final formulae for the salts in gastric solution are presented in Table 2.10. It was necessary to add

conc. HCl acid to provide extra Cl ions and to produce a solution with pH 1.9-2.1.

Table 2.10 Final composition of salts in the *Ateles* ad hoc model gastric media.

| **Gastric fluid** | **mg/100ml** | **mg/30ml** | **$Na^+$ %** | **$K^+$ %** | **$Cl^-$ %** | **$Na^+$ mg/30ml** | **$K^+$ mg/30ml** | **$Cl^-$ mg/30ml** |
|---|---|---|---|---|---|---|---|---|
| NaCl | 500 | 150 | 39.34 | | 60.66 | 59.01 | | 90.99 |
| KCl | 180 | 54 | | 52.44 | 47.56 | | 28.32 | 25.68 |
| $KH_2PO_4$ | 27 | 8.1 | | 28.73 | | | 2.33 | |
| | | | **Total in 30ml Gastric fluid** | | | **59.01** | **30.65** | **116.67** |

Conc. HCl acid 36% w/v was used, this equates to a concentration of 55mg HCl/150µl. Addition of 150µl contributed 52.50 mg Cl ions to the final 40ml of combined media, and achieved the desired pH range (Table 2.11). When a higher volume was attempted, the pH fell below 1.7 and precipitation was observed.

Table 2.11 Value for the combined *Ateles* simulated saliva (10ml) and gastric fluid (30ml)

| **ad hoc model *Ateles* media values** | **mg/ 10ml saliva media** | **mg/ 30ml gastric media** | **mg/40ml combined *Ateles* media** |
|---|---|---|---|
| $Na^+$ | 4.16 | 59.01 | 63 .17 |
| $K^+$ | 4.08 | 30.65 | 34.73 |
| $Cl^-$ | 6.13 | 116.67 | 122.80 |
| $Cl^-$ from 150µl Conc HCl | | 52.50 | |
| **Total $Cl^-$ions** | | | **(52.5 + 122.80) =175.20** |

### 2.3.3.1 Pepsin source

The Pepsinogen A gene (PAG) which is responsible for controlling secretion of pepsin is found in humans, chimpanzees and pigs. In these species it is activated by the same tissue specific mRNA, found only in gastric mucosa (Sogawa *et al.* 1983). This was a consideration in the select ion of pepsin from porcine source as it is genetically orthologous to human and more readily available, (Hughes *et al.* 2003) rather than the bovine pepsin used in the original models. There was no further necessity to adjust the pH as the DIN 19738 method has a pH 2, which is consistent with the pH characteristics identified in Brooks *et al.* (1963). The final solution, including the mucin and pepsin was prepared (Table 2.12) stored in a refrigerator at $4^0$C and examined after 24 and 48 hours for signs of precipitation. Precipitation was seen in 3/5 samples of gastric media between 24-48 hours. Freshly prepared media was therefore used for each set of digests.

Table 2.12 Formula used for *Ateles* simulated gastric media modified from, from DIN 1973.

| **Gastric fluid *Ateles ad hoc* model mg/ 100ml** | |
|---|---|
| NaCl | 500 |
| $KH_2PO_4$ | 27 |
| KCl | 180 |
| mucin | 240 |
| pepsin | 80 |

## 2.3.4 Remaining Modifications

In addition to choosing different origins for the amylase, mucin and pepsin the following items in the DIN model were changed:

- It was necessary to reduce the digestion times, to reflect the more rapid passage of food through the

*Ateles* gut. The DIN 19738 model uses a digestion time of 2 hours for the stomach and 6 hours for the intestine whilst the RIVM model used 2 hours for both. The modification to reflect the *Ateles* transit times were mixing with saliva fluid of 3 mins followed by 60 mins after addition of the gastric media.

- NaSCN Sodium thiocyanate - is secreted as an anti-oxidant in saliva, was not added to the *Ateles* fluids, due to COSHH regulations.
- mucin and pepsin in gastric media - levels were reduced by ~ 20%, to reflect the reduced time available for secretion in *Ateles* associated with a reduced transit time and differences in volume/scale of the stomach and intestine.

## 2.3.5 Model Schematic

The schematic diagram of the ad hoc *Ateles* model is shown in Figure 2.2, which is based on the modified DIN 17938 /Swallow-fasted model of Brandon e*t al. (2006)*.

### 2.3.5.1 Sample Size

Upon drying both the *Viscum album* (used in method development) and *Phoradendron*, (Section 4.2), showed a loss of approx. 55-65%, thus the 2.5g of dried leaf sample would represent ~5g wet leaf mass. This would equate to > 20 leaves depending upon the species of *Phoradendron*. In order to maximise the number of possible analyses the above schematic was reduced to use 500mg leaf and/or 200mg geophagy sample, 2ml saliva and 6ml gastric media. This permitted replicate analyses to be performed. 15ml polypropylene centrifuge tubes were used for the digestions; this reduced the residual volume of air in the tubes whist permitting adequate mixing.

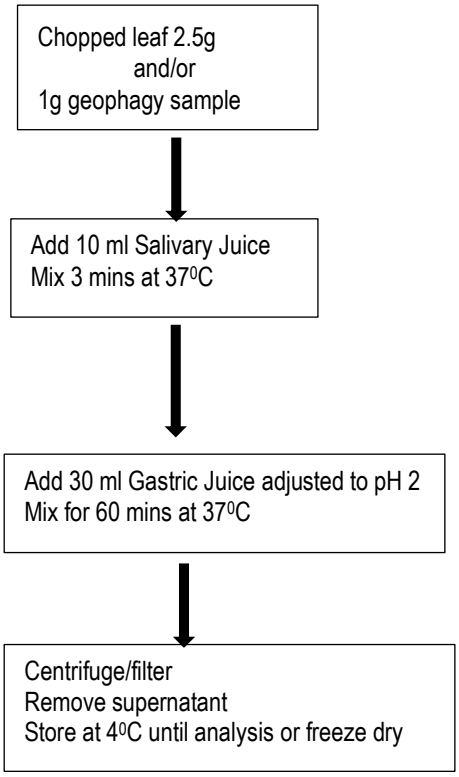



Figure 2.2 Schematic showing the parameters of the *ad hoc Ateles* Model.

# Chapter 3 Analysis of Geophagic Samples

## 3.1 Introduction

This chapter relates to Specific Aim 1(Section 1.11) to characterise the geophagic material and relate the analytical results to the probable functions hypothesised for geophagy (Section 1.9). Briefly the hypotheses are classified: 1) Adaptive Beneficial e.g. protection from toxins such as plant secondary metabolites or bacterial toxins and pathogenic organisms; providing mineral or micronutrients; alleviating symptoms such as diarrhoea or excess acid or as 2) A Non-adaptive aberrant behaviour. The latter is unlikely to be addressed by physico-chemical analyses.

Table 3.1 Examples of post 1999 primate/geophagy publications.

| **Species** | | **Reference** | **Site** |
|---|---|---|---|
| chacma baboons | *Papio cynocephalus ursinus* | Pebsworth *et al.* (2012, 2013) | South Africa |
| bonobo | *Pan paniscus* | Beaune *et al.* (2013) | D. R. Congo |
| chimpanzee | *Pan troglodytes schweinfurthii* | Aufreiter *et al.* (2001), Ketch *et al.*(2001) | Tanzania and Gombe |
| chimpanzee | *Pan troglodytes schweinfurthii* | Mahaney *et al.* (2005) | Uganda |
| red howler monkeys | *Alouatta seniculus* | Blake *et al.* (2010) | E. Ecuador |
| red handed howler monkey | *Alouatta belzebul* | de Souza *et al.* (2002) | E. Brazil |
| Phayre's leaf monkeys | *Trachypithecus phayrei* | Pages *et al.* (2005) | Thailand |
| golden bamboo lemur | *Hapalemur aureus* | Jeannoda *et al.* (2003) | Madagascar |
| bonnet macaques | *Macaca radiata* | Voros *et al.* (2001) | India |
| Japanese macaques | *Macaca fuscata* | Wakibara *et al.* (2001) | Japan |
| orangutans | *Pongo pygmaeus.* | Matsubayashi *et al.* (2007, 2011) | Sabah, Borneo |
| bearded Saki monkey | *Chiropotes satanas* | Veiga *et al.* (2007) | S. E. Brazil |
| bald-faced Saki monkeys | *Pithecia irrorata* | Adams *et al.* (2011) | Peru |
| Milne-Edwards' sifaka | *Propithecus edwardsi* | Arrigo-Nelson *et al.* (2010) | Madagascar |
| white-bellied Spider monkeys | *Ateles belzebuth* | Blake *et al.* (2010) | E. Ecuador |
| brown spider monkey | *Ateles hybridus* | Link *et al.* (2011a) | Columbia |

Geophagy in animals has increasingly been reported, resulting in many more species being identified as exhibiting this behaviour e.g. with the first instance of a geophagy in a marsupial, the eastern grey kangaroo (*Macropus giganteus)* being reported (Best *et al.* 2013). The potential importance of geophagy was highlighted in a doctoral thesis (Montenegro 2004) which suggested that salt/mineral licks should be classed as a Keystone Resource for both humans and animals. A keystone resource is one "*whose impact on its community or ecosystem is large and disproportionately large relative to its abundance*" (Power 1996 in Watson 2001). There have also been many publications relating to human geophagy attempting to determine the function of geophagy e.g. (Henry *et al.* 2003, Abrahams *et al.* 2006, Kawai *et al.* 2009, Young *et al.* 2010, Young *et al.* 2011, Abrahams *et al.* 2013). The publications, prior to 1999, specifically relating to geophagy in primates were collated and reviewed (Krishnamani *et al.* 2000). Since then there have been further publications, e.g. those in Table 3.1. Ferrari *et al.* (2008) reviewed those specific to New World primates.

## 3.2 Santa Rosa Primary Geology and Volcanic history

Figure 3.1 shows the underlying morphology of the Santa Elena peninsula and the tectonic features of the area. The Santa Elena Nappe is an overthrust of allochthonous material composed of a) ultramafic complex derived from mid ocean ridge basalts and b) mafic material which has a signature suggestive of island arc origin with low-grade metamorphism and hydrothermal alteration. (Glossary of Geological terminology Appendix 1.2).

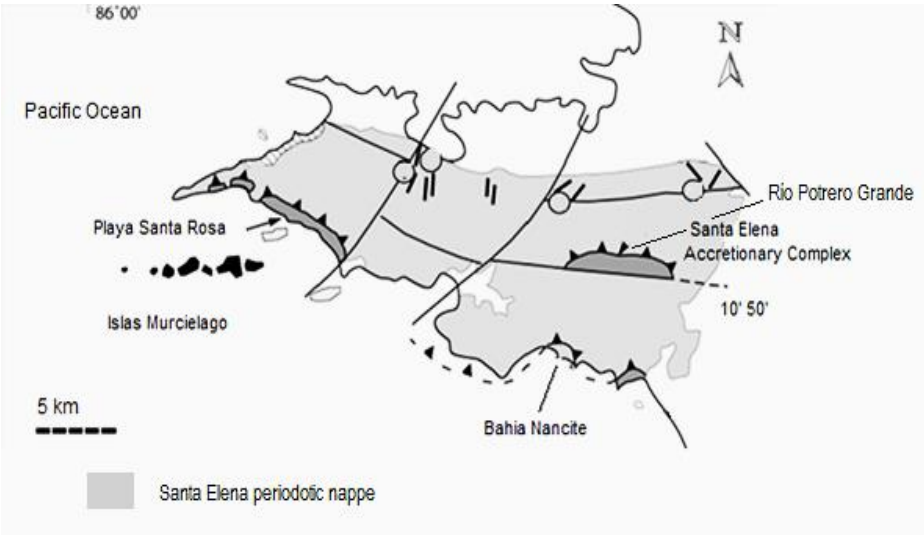



Figure 3.1 Geological map of the Santa Elena Peninsula modified from Gazel *et al*. (2006)

The Santa Elena Accretionary Complex is an autochthonous basaltic sedimentary suite, resting immediately below the overthrust. This includes pelagic and volcanoclastic sediments, tuffs and magmatic rocks. The known geophagy sample sites are close to the E-W fault adjacent to the Santa Elena Accretionary Complex, (Figure 3.1). This is exposed within the tectonic-erosional window at Río Potrero Grande. This unit includes pillow lavas and radiolarian cherts i.e. sedimentary rocks with high silica content (Gazel *et al.* 2006).

The Santa Rosa plateau occupies an area of approximately 1500 $Km^2$ between the Pacific coast to the West and the Cordillera de Guanacaste which includes the stratovulcanic complex of Rincón de la Vieja to the east ($10^0$49'48"N $85^0$ 19'26"W). The vulcanology of the area (Kempter 1997) contributes to the secondary geology of the research area as the sedimentary rocks are covered by the ignimbrites of Santa Rosa plateau (Chiesa *et al.* 1987, Chiesa *et al.* 1992, Vogel *et al.* 2004).

Large volumes of these silicic ignimbrites, with an estimated volume of 130 cu km, are found in central and northern Costa Rica, extending from the border with Nicaragua to the city of Cañas (Mora 1988). The geological map of Guanacaste (Figure 3.2) shows the extent of the ignimbrites (Orange area) which contains the study area. These ignimbrites originate from large explosions of ancient volcanic sources, probably located below the current volcanic cordillera de Guanacaste. Some of these flows extended 40-50 km from the point of eruption. Between the levels of ignimbrites are small continental sedimentary deposits called epiclastitic units. The Santa Rosa plateau thus consists of a sequence of several deposits of pyroclastic flows, and ignimbrites, deposits of pumice and pyroclastic material, interspersed with fluvio-lacustrine sediments and some andesitic lava (Chiesa 1991).

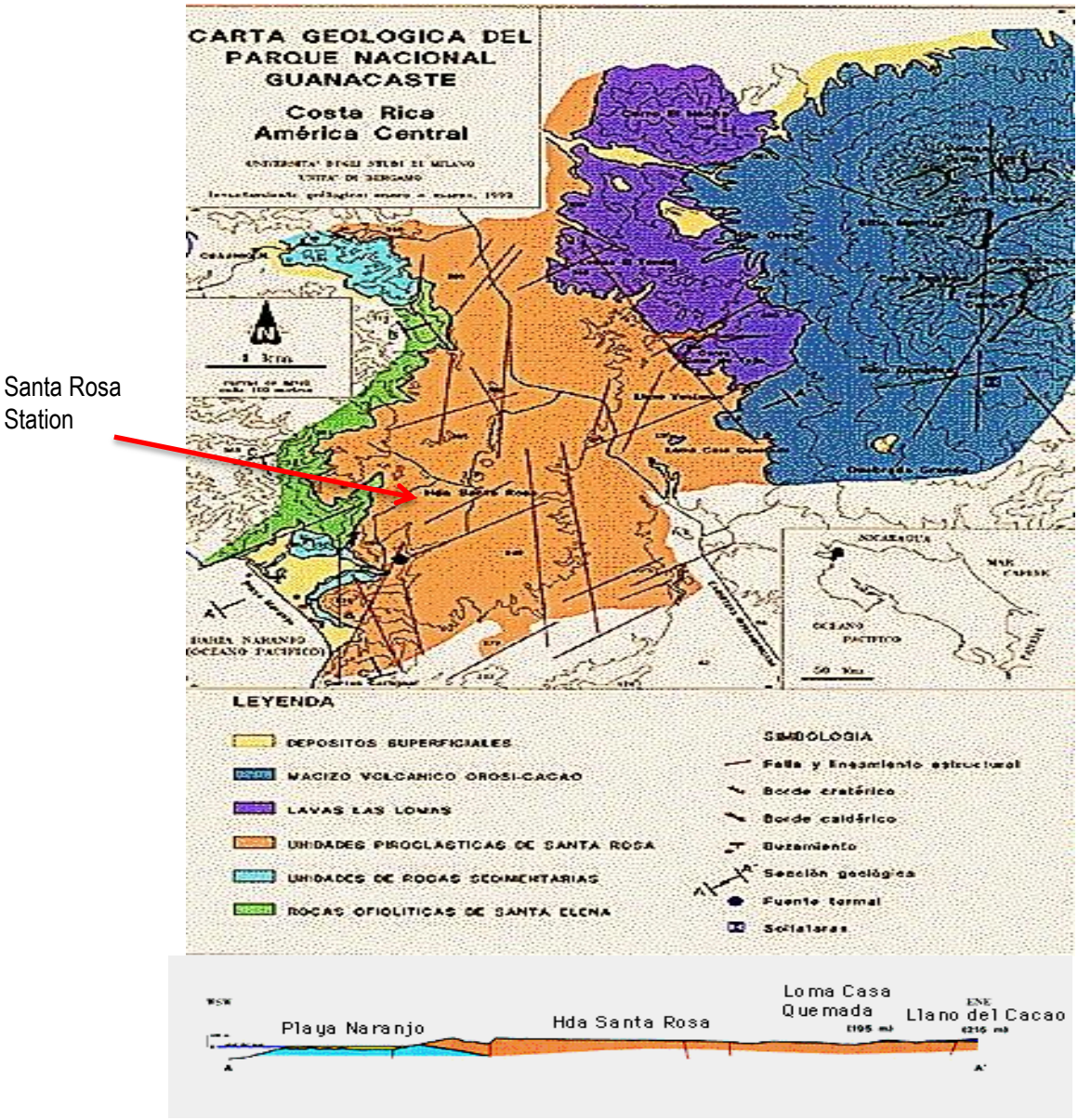



Figure 3.2 Geological map showing the extent of the ignimbrites with reference to the study area and a geological profile, modified from (Chiesa *et al.* 1987).

## 3.3 Methods

Mahaney *et al.* (2003) provide a framework for both the collection of samples, identification of potential control sites and the range of analyses, attempting to standardise data collected and so permitting improved comparisons with other publications.

### 3.3.1 Geophagy Sample Area Description

The known *Ateles* geophagy sites were situated along the edge of the mesa (Figure 3.3). The top of the mesa is heavily wooded with a mixture of mature trees and understorey vegetation (Figures 3.3-3.4). The top is also relatively flat; there is no gradual slope away from the edge which resembles the edge of an escarpment. In many places, trees overhang the vertical faces, and there are fallen trees on the sloping areas below. On the top, there is very little visible depth of soil and tree roots can be seen where there have been tree/rock falls. The sloping area was wooded mostly with small trees near the base of the vertical faces. There were steep sided ridges, which extended westward and down into the bottom of the steep sided ravine/valleys (Figure 3.4). In the valleys between the ridges, there were tall trees. Scattered across the area also were


Figure 3.3 Highlighting the edge of the 'mesa' and the heavily wooded area below, looking towards Rincón de la Vieja (Author's own photograph).

boulders (1.5 - 2.0m horizontal and 1.5m vertical). These were mostly dark brown and heavily covered with green algae, moss and fern growths and other small plants. When the boulders were hit with a small geology hammer they sounded dull and wooden, like hitting wet material and material broke away easily with the hand, exposing fine fibrous roots.

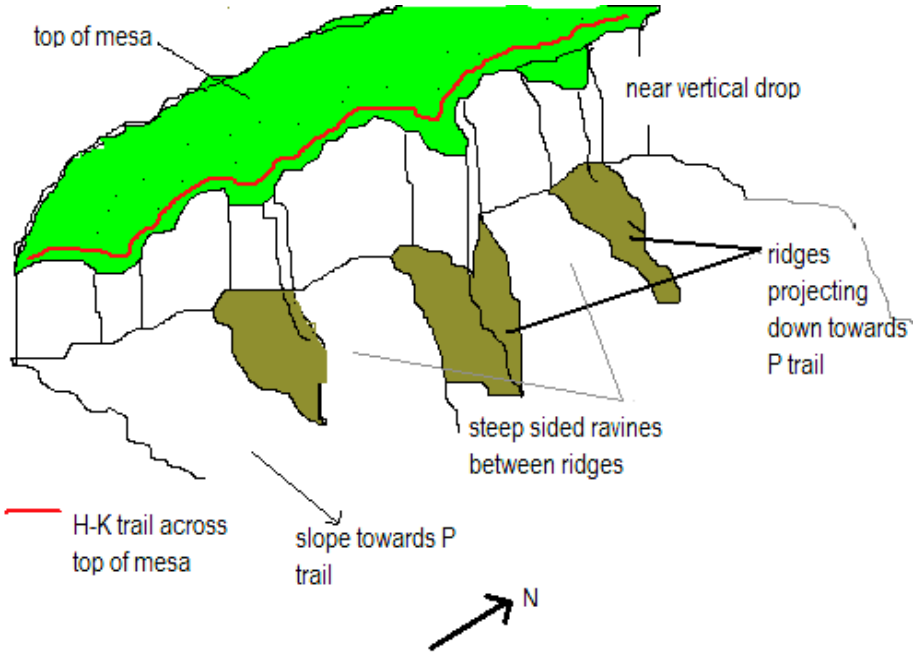



Figure 3.4 A sketch diagram showing the general topography of the area encompassing the sample sites

The vertical areas had some shelves and overhangs with many deep vertical and horizontal fissures. There were distinct colour changes associated with these deep fissures. The colour of the face varied between bright light grey, pale cream, dark red and green, almost black and areas with significant surface algal growth. The sloping area was very steep, the soil material loose and crumbly, making climbing difficult. When wet the surface was extremely slippery. The geophagy sites were on the steep vertical faces, adjacent to the top of the mesa, below H-K trails (Figures 3.5-3.6). GPS and elevation data are presented in Appendix 1.4, Table 1.2. An

area of home range between Trail B and the stream, Quebrada San Emilio, (Figure 3.5) included the three previously identified *Ateles* geophagy sites. The known sites were close to the top of the mesa, were overlooked by trees regularly used for feeding or resting by the monkeys and had several possible clear and easy access routes to the geophagy sites for the monkeys. The area along the edge of the mesa (trails K and H) was surveyed for additional sites and suitable potential control sites.

Samples were taken from a total of 10 sites, these comprised: three confirmed *Ateles* eating sites; a single site identified as being used by *Cebus* monkeys, a site suspected as being used by *Cebus* monkeys, 5 further control sites identified as potential *Ateles* geophagy sites.

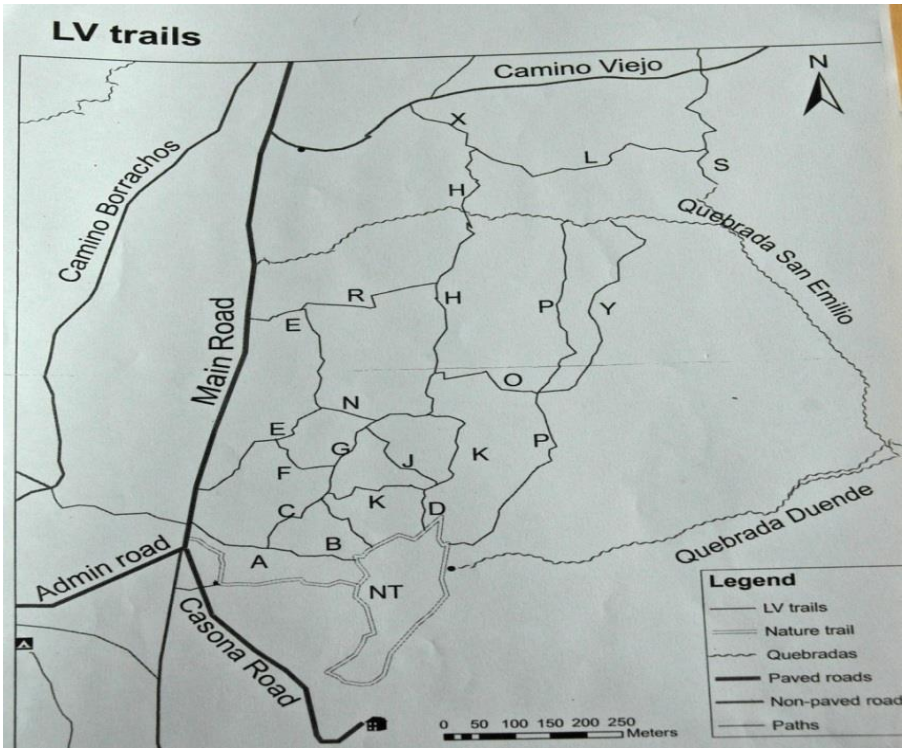



Figure 3.5 Map showing the major marked trails used by primate researchers at Santa Rosa.

Site 2

Figure 3.6 View looking up towards Site 2 (Author's photograph).

The geophagy sites used by *Ateles* were in situations that would be difficult to access by the only potential predators large enough to be a threat to the large bodied *Ateles*. Typically these predators are: jaguar (*Panthera onca*), puma (*Felis concolor*), and ocelot (*Leopardus pardalis*) Link *et al.* (2011b). Predation by felid

had been reported at Santa Rosa (Santorelli *et al.* 2011). The known *Ateles* sites were not easily accessed from either above or below and had suitable trees for observation and quick access and retreat. (Figure 3.6, Site 2, an *Ateles* geophagy site). Control sites were selected to meet these criteria and be within the home range and adjacent to a source of mistletoe. Sites were also chosen to meet criteria suggested by Mahaney *et al.* (2003), in that sites should be close to current geophagy sites and offer possibilities to the monkeys.

### 3.3.2 Site Collection Methods

The conditions attached to the export permits limited combined total geophagy sample material to 3Kg, limiting material available for testing. There were no analytical facilities on site. Within each site area, sections of rock-face surface with visible differences in colour or texture (Figures 3.7-3.8) were identified in accordance with Mahaney *et al.* (2003).

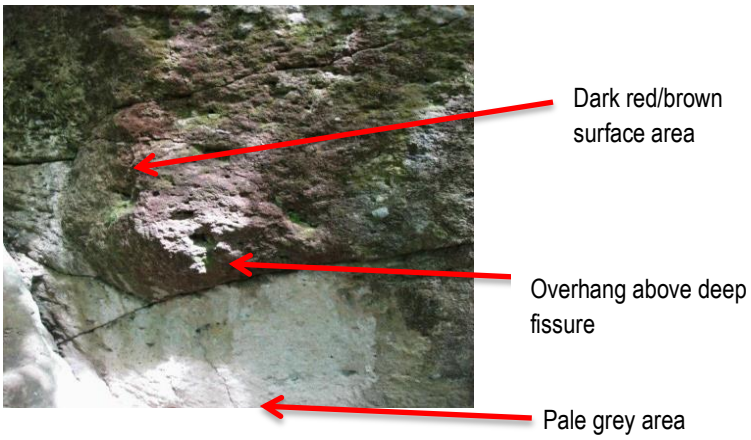



Figure 3.7 Photograph illustrating variation within Site 4

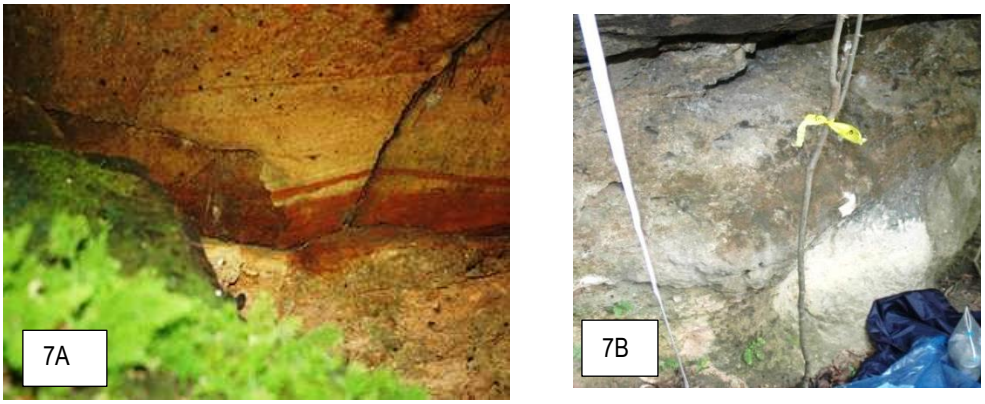



Figure 3.8 Site 7A showing the deep orange/brown coloured bands; adjacent Site 7B, (less than 4 ft. distance horizontally) creamy/grey colour.

Site 10, a confirmed *Ateles* geophagy site, required deployment of climbing equipment and specialist rope-work to be able to access the site, and meet Health and Safety requirements. The sample was collected by Elvin Murillo Chacon and Norberto Asensio, on behalf of the author. Site 4 is presented as an example of sampling procedures (Figures 3.9-3.10). Sampling points were chosen to be as representative as possible of the visible differences within the individual sites (Figure 3.10) as suggested by Wilson (2003).

Multiple samples were taken in order to provide as wide a range of material for analysis, taking into consideration the variety of potential geological sources. The dry surface was brushed, with a stiff bristle paintbrush, to remove loose debris and any obvious surface contamination e.g. algal growth. Table 3.2 is an

example of sample collection record. The remaining Site Sample records are presented in Appendix 1.4, Table 1.3.

Table 3.2 Example of site Sample Record – Site 4

| No. | (g) | description | comments |
|---|---|---|---|
| 19 | 38 | friable | Material sampled 3-4 cm depth |
| 20 | 12 | friable | Material sampled 5-7 cm depth (position as 19) |
| 21 | 46 | friable | Material sampled 1-3 cm depth |
| 22 | 30 | friable | Material sampled 5-8 cm depth (position as 21) |
| 23 | 24 | hard fragments | 3 hard isolated fragments |
| 24 | 28 | friable | Material 1-2 cm depth adjacent to scrape marks |
| 25 | 76 | friable | Material from back wall 1-3 cm very pale grey colour |
| 26 | 102 | friable | Material from overhang 1-3 cm darker – brown colour |
| 27 | 34 | friable | Material from deeper 5-8 cm contained solid fragments (position as sample 26) |
| 28 | 20 | pumice? | Solid fragment appearance similar to pumice |
| 29 | 18 | pumice? | Solid fragment appearance similar to pumice |
| 30 | 14 | fragment | 3 hard isolated fragments |
| 31 | 42 | friable | Material 1-2 cm depth adjacent to scrape marks |
| 32 | 108 | hard fragment | Dense pebble like material adjacent to scrape mark |

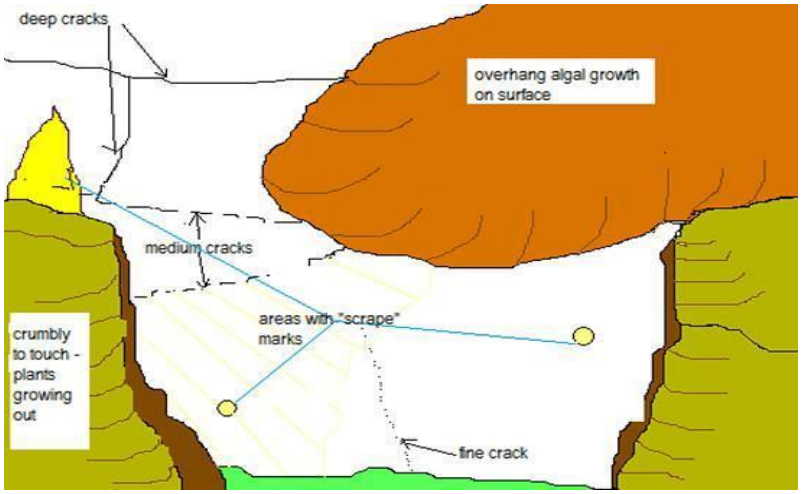



Figure 3.9 Site 4 Sketch to illustrate the varieties of visually different zones

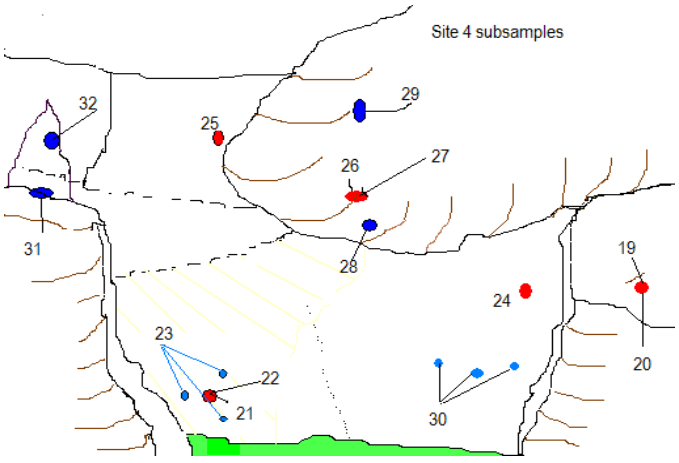



Figure 3.10 Areas sampled within Site 4

Samples were weighed before drying and again before placing in Ziploc bags. Each sample and site was then processed independently, to prevent any cross-contamination. Example images (Figure 3.11) of Sample 19 show a coarse powdery material, containing hard fragments, produced following gentle hand pressure. Sample 27, was friable material taken from a depth of 5-8 cm and contained hard angular material. Sample 30

fragments of rough surfaced coarse open texture, light weight material similar in texture to pumice stone. Sample 32 was a relatively large heavy pebble.

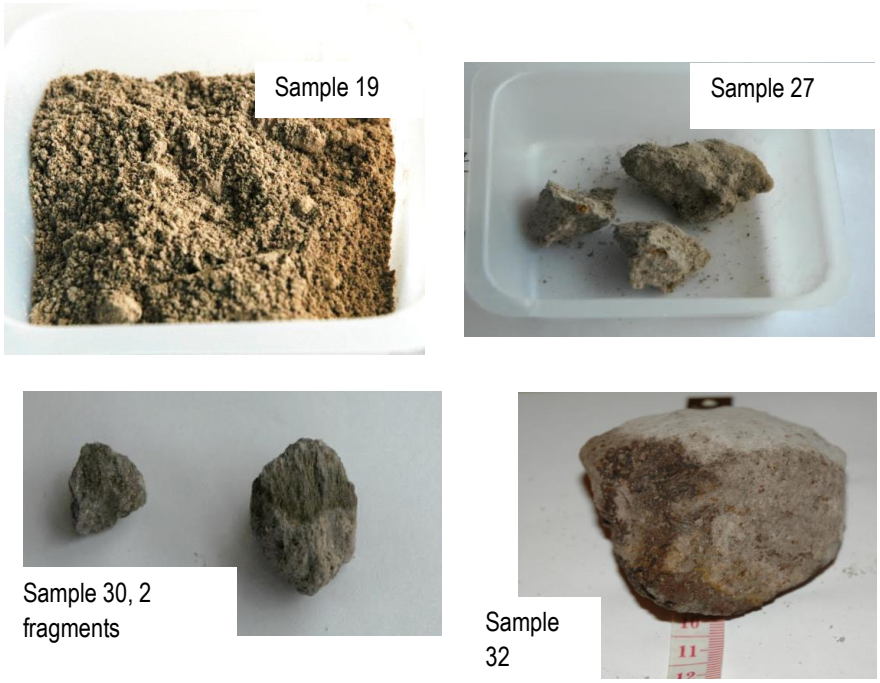



Figure 3.11 Author's own images of samples from Site 4, during collection.

### 3.3.3 Sample Drying method development

Sampling and storage procedures were developed on site taking into consideration the limited facilities available. Material was collected from potential sites. Samples were then taken where possible from an initial depth between 2-5cm and then a deeper depth 5-10cm from the surface. This avoids material which would have been exposed to rain and so may not be truly representative of the underlying material. A rigid 'plastic' rod, marked at cm intervals, was hammered into the surface to the required depth then sample material excavated using a rigid long handled plastic palette knife. Samples were collected in clean screw topped glass jars and labelled, and taken back to the onsite laboratory.

Samples to be used in method development were divided into two, placed in polypropylene weighing boats and weighed. Samples were then either placed on the work bench and covered with aluminium foil or placed in a snap-lock polypropylene storage box together with a jar containing Self-indicating silica gel granules (GeeJay Chemicals Ltd). This silica gel contains a moisture sensitive indicator that changes colour orange to green as moisture is adsorbed giving a visual indication of the activity level of granules. The samples were examined daily and reweighed after 2-3 days. During this period the relative humidity was > 80%. The foil covered samples failed to dry suitably in these conditions and became contaminated by insects. The samples dried in the silica gel/box conditions showed a small initial mass loss (<2%) with the silica gel taking on a slight green colouration and no insect contamination. After 7 days, there was no further change in mass. Figure 3.12 illustrates the colour change on drying.

(Left)- Example Site 8 after 7 days drying (Right) Example Site 8 undried sample

Figure 3.12 Illustrating the effect of drying with silica gel, after 5-7 days

The dried samples were transferred to Ziploc bags, double bagged. The bags were placed in snap-lock polypropylene boxes, containing silica gel, for long-term storage. The silica gel drying and box storage method was adopted. Situations of sampled sites are presented in Appendix 1.4, Table 1.3.

The analytical techniques used in this investigation and their relationship to the potential functional hypotheses are presented in Table 3.3.

Table 3.3 Summary of the analytical techniques used, rational for analysis and relationship to potential functional hypotheses.

| | Rational for analysis and relationship to potential functional hypothesis (Chapter 1 Section 1.9) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Technique- characteristic determined** | **Sample Characterisation** | **Comparison with published data** | **Anti-diarrhoeal** | **Antacid/acid neutralisation/gastro protection** | **Nutrition/ physiological imbalance** | **Detoxification** | **Anti-bacterial /anti-infective properties** |
| Munsell colour characteristic | ✓ | ✓ | | | | | |
| Measurement of water content | ✓ | | | | | | |
| Loss on Ignition/ presence of carbonates/organic material | ✓ | | | ✓ | | | |
| XRD –presence of clay or other minerals | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ |
| IR – presence of clay minerals/organic matter/water | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ |
| XRF– detectable elements present | ✓ | ✓ | | ✓ | ✓ | | |
| ICP– of elements released into gastric extract<br>Effects of geophagy material on potentially available Fe | | ✓ | ✓ | | ✓ | | ✓ |
| Laser diffraction particle size in variable pH-media conditions/presence of clay sized particles | ✓ | ✓ | ✓ | | | ✓ | |
| Sample pH in water and KCl solution | ✓ | | | ✓ | | | |
| UV– analysis of gastric extract following exposure to example PSM in gastric conditions | | ✓ | | | | ✓ | |
| Microbiological screening of gastric digest | | | | | | | ✓ |

## 3.4 General Sample Preparation – Analytical methods

Several of the analytical techniques required dry sample material of less than 2mm size. Approx. one third of a sample from each site was gently processed in an agate mortar using a rubber pestle, to prevent cross contamination. The resulting material was sieved using a 2mm mesh stainless steel sieve. The sieved material was then Colour characterised and used to determine the Water content and Loss on Ignition (LOI). The remaining material was stored in sealed containers, containing silica gel, for the remaining analyses.

Samples 33-36 from Site 5 (a potential *Cebus* monkey site) were discarded following examination in the laboratory at LJMU, as there were obvious signs of white thread-like material (probably fungal hyphae) contaminating the samples and condensation on the inner surface of the bags. Section 3.4.12, Table 3.16 provides a summary of samples collected and analyses undertaken.

### 3.4.1 Munsell© Colour Characterisation

#### Method

Using the standard method from Rowell (1994), previously sieved, (<2mm) air dried material was placed between clean glass slides to produce a smooth uniform surface. The top slide was removed and the sample colours determined using the Munsell® Soil Colour Charts, 1998 Revised Edition. Colours were determined between 11.00-14.00 hours in bright daylight conditions.

The value obtained from the charts e.g. 7.5YR 5/3 has three components, hue – colour specific (e.g. 7.5YR), value – light or darkness (e.g. 5) and chroma - colour intensity (e.g.3). Chart for Hue 7.5YR can be found (Appendix 1.4, Figure 1). A typical colour descriptor would take the form 7.5Y 5/3, colour 'brown'. The results are presented in Section 3.5.1.

### 3.4.2 Determination of Water Content at $105^0$C

Soil materials contain non-mineral related water, the amount depending upon the presence of organic material and the preceding weather conditions. Even after air-drying the material will have residual water, the amount dependent upon the characteristics of the material and the humidity of the room used for air-drying. Moisture content of collected air-dried soil samples may change during storage (fluctuations in air moisture, temperature, oxidation of organic matter, loss of volatile constituents). The storage conditions adopted were attempts to mitigate these changes.

Water content is determined after drying in an oven at $105^0$C. The methods used were those routinely employed by soil scientists (Rowell 1994, Pansu *et al.* 2007). The temperature maintained for a defined period of time, is sufficiently high to eliminate free forms of water and sufficiently low not to cause a significant loss of organic matter and unstable salts by volatilization (Pansu *et al.* 2007).

This analysis was conducted approx. 10 weeks after collection, following arrival of samples at the laboratory in Liverpool following the methods in Pansu *et al*. (2007 pages 1-13). All samples were analysed as a single batch.

Method

10.0±0.2g previously stored, dried, sieved < 2mm fraction of each sample was weighed into a pre-weighed porcelain crucible. The samples were then placed in a preheated oven at 105$^0$C overnight (16 hours). After removal samples were allowed to cool in a desiccator for 2 hours before weighing. All the determinations were made in a single session, to standardise conditions. A single determination was made on each sample, with multiple samples tested from each site, with the exception of Site 10 where only a single sample was obtained by the climbing team. Results are presented in Section 3.5.2.

### 3.4.3 Determination of Loss on Ignition (LOI)

The loss on ignition value is used as an estimate of the content of non-volatile organic matter in soil samples e.g. microorganisms, roots/organic waste products. Organic matter begins ignition at ~200$^0$C and is completely depleted at about 550$^0$C.

LOI may also contain a contribution from water linked to the mineral crystal lattice plus a little residual non-structural adsorbed water (Pansu *et al.* 2007). Additionally sulphide minerals, carbonates and metallic oxy-hydroxides can modify LOI values as well, via oxidation or dehydration and this may be significant where there is a high clay mineral content (Dean 1974, Santisteban *et al.* 2004).

Method

Organic matter content was determined by measuring the Loss on Ignition, (LOI), at 500$^0$C ±25$^0$C, for 2 hours in accordance with the European Standard Method EN 15934. Following reweighing of desiccator-stored samples used for the determination of water the oven dried samples in the porcelain crucibles were placed in a, previously heated muffle furnace and kept at 550$^0$C ± 10$^0$C for 2 hours. After 2 hours at 550$^0$C the furnace door was released and whilst still hot the crucibles were placed in a desiccator containing fresh silica gel and allowed to cool before weighing. Results are presented in Section 3.5.3.

### 3.4.4 X-Ray Diffraction (XRD)

Each crystalline solid has its unique characteristic X-ray powder diffraction pattern that may be used as a fingerprint for its identification, seen both when analysed as a pure material and as part of a mixture. X-ray powder diffraction is used for the fingerprint characterization of crystalline materials and the determination of their crystal structure. Approx. 95% of solid materials are described as crystalline. When X-rays interact with crystalline material, an X-Ray Diffraction pattern is generated. XRD is commonly used for the qualitative and semi-quantitative determination of clay minerals (Kodama *et al.* 1989).

The X-ray radiation most commonly used is that emitted by a copper source, whose characteristic wavelength of the radiation is =1.5418Å. When the incident beam strikes a powder sample, diffraction occurs (Figure 3.13) in every possible orientation of 2theta (2θ).

Using the angles and intensities of the diffracted beams a three dimensional structure may be constructed and this subsequently used to identify materials. In order for there to be interference between the reflected waves, the path difference must be an integral number of wavelengths: nλ= 2x. The path difference between two incident waves: 2λ= 2d sinθ. This leads to the derivation of the Bragg equation: nλ = 2d sinθ;

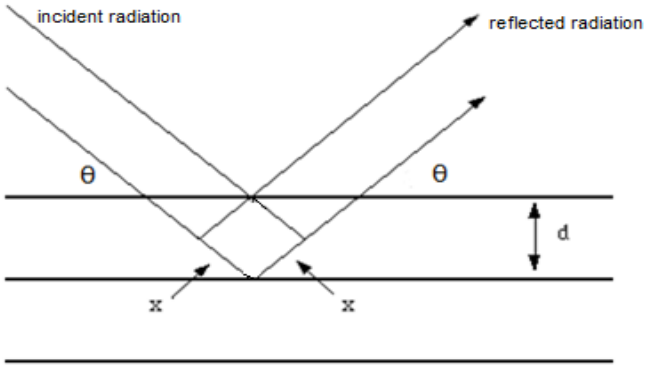



Figure 3.13 Reflection of x-rays from two planes of atoms in a solid, from (Wilson 1987).

An example calculation, substituting the following: n=1, λ = 1.315Å, θ = 22.25 enables a value for d to be calculated:

2x1.315 = 2d sin(22.25); d = 1.541Å

The diffracted beam is detected by using a moveable detector such as a Geiger counter, which is connected to a chart recorder. In normal use, the counter is set to scan over a range of 2θ values at a constant angular velocity. Routinely, a 2θ range of 3 to 70 degrees is sufficient to cover the most useful part of the powder diffraction pattern for characterisations.

## Determination of an Unknown

The d-spacing of each peak is obtained by solution of the Bragg equation for the appropriate value of λ. Once all d-spacings have been determined, automated search/match routines compare the ds of the unknown to those of known materials. Because each mineral has a unique set of d-spacings, matching these d-spacings provides an identification of the unknown sample. A systematic procedure is used by ordering the d-spacings in terms of their intensity beginning with the most intense peak. The relative intensity is recorded as the ratio of the peak intensity from pure sample to that of the most intense observed peak (*relative intensity* = $I/I_0$) and is compared to values from databases e.g. The International Centre for Diffraction Data® (ICDD®) to identify matches.

## Method

Two samples of Clay Certified Reference Materials (CRM) the kaolinite KGa-2 and the montmorillonite SWy-2 were obtained from The Clay Minerals Society, The Source Clays Repository, P.O.Box, 4416, Boulder, Colorado, USA.

## Sample Preparation

Samples were prepared according the Instrument Standard Operating Procedures (SOP) for the Rigaku equipment, under the instruction of Dr N Dempster, Research Technical Officer, LJMU.

Preferred orientation, non-random distribution of the crystallites is usually the most important single cause of intensity variations in a diffraction pattern. Random orientation ensures that incident X-rays have an equal chance of diffracting off any given crystal lattice face of the minerals in the sample. Although some

orientation is inevitable (platy minerals e.g. clays tend toward some preferred orientation), the United States Geological Survey (USGS) method is considered sufficient for most applications.

Samples ~ 1g were dried at $60^0$C before grinding in the presence of ethanol with an agate mortar and pestle. Ground samples were then sieved using a Standard Sieve, Stainless Steel Mesh, 0.063 mm sieve. Sieving is used to achieve even distribution. Sieved material was then tapped into the spinner holder, and a glass slide applied to produce a flat surface. The sample is irradiated with x-rays and the intensity of the reflected radiation is recorded using a goniometer. This reflection data is then analysed for the reflection angle to calculate the inter-atomic spacing (d value in Angstrom units - $10^{-8}$ cm). Preliminary diffraction analysis was performed using a Rigaku Miniflex instrument (Table 3.4).

## Instrumentation

Table 3.4 Equipment and Conditions for XRD analyses.

| **Rigaku Mini-Flex** | | **Phillips PW 1730** |
|---|---|---|
| Goniometer | Miniflex goniometer | |
| Attachment | Top filled aluminium holders (spinning mode). | Top filled aluminium holders (spinning mode). |
| Scanning Mode | 2theta/theta | 2theta/theta |
| Scanning Type | Continuous Scanning | Continuous Scanning |
| X-Ray | Cu tube 30kV/15mA | Cu tube 50kV/40mA |
| Divergence slit | Variable | |
| Scattering slit | 4.2deg | |
| Receiving slit | 0.3mm. | |
| Start | 30 2theta | 1.50 2theta |
| Stop | 600 2theta | 700 2theta |
| Step | 0.01s | 0.01s |

A total of 39 samples were analysed (Table 3.16) using a Rigaku Miniflex and from these 15 (Table 3.5) were selected for more detailed analysis and peak matching at Rockwood Industries, using a Philips PW1730 XRD instrument. Sample preparation and analyses at Rockwood Industries, were performed by Ms D. McCarthy, in accordance with the Rockwood SOP. Samples were selected for analysis at the Rockwood facilities to provide information on the known *Ateles* and *Cebus* eating geophagy material and a selection of control samples. The number of samples analysed was limited by access to the Rockwood facilities, kindly made available free of charge.

Rockwood Industries (Widnes, Cheshire), is a subsidiary of Rockwood Specialties, Inc. and Rockwood Clay Additives GmbH and Southern Clay Products, Inc. Rockwood produce and market an extensive range of additives using natural and synthetic clays. Rockwood Additives regularly analyse unknown clay minerals of this type and produce their own reference standards.

The diffractograms obtained at Rockwood Industries were compared with the internationally recognised International Centre for Diffraction Data-JCPDS (Joint Committee on Powder Diffraction Standards) database, using the DIFFRACTPLUS BASIC 5 (Brukker) software, to determine the probable constituent minerals present. This software uses the Rietveld Method (Rietveld 1969) which takes into account the instrumental and sample produced variables to produce a match. This allows the use of multiple peak matching, increasing the ability to match samples in complex matrices. Results are presented in Section 3.5.4.

Table 3.5 Samples selected for analyses at Rockwood Industries.

| Site Number | Sample Number | Site Function |
|---|---|---|
| 1 | 1 | control |
| 1 | 9 | control |
| 1 | 11 | control |
| 2 | 13 | *Ateles* eating site |
| 2 | 14 | *Ateles* eating site |
| 2 | 15 | *Ateles* eating site |
| 6 | 40 | control |
| 6 | 46 | control |
| 7A | 56 | control |
| 7A | 60 | control |
| 7B | 62 | control |
| 8 | 70 | *Cebus* eating site |
| 9 | 75 | *Ateles* eating sit |
| 9 | 77 | *Ateles* eating sit |
| 10 | 100 | *Ateles* eating sit |

### 3.4.5 Infrared Analysis (IR)

Infrared (IR) spectroscopy has been used for decades to investigate the structure and chemistry of clay minerals. The infrared spectrum of clay minerals is sensitive to chemical composition and isomorphous substitution providing information on chemical composition, surface properties and on mineral identification (Russell 1987). The absorption of infrared radiation is influenced, to different degrees, by the degree of crystalline order and the size and shape of the mineral particles and the effects of particle orientation.

Attenuated Total Reflectance (ATR) Infrared analysis combined with high sensitivity Fourier-transform Infrared (FTIR) is considered a simple and routine technique for real world samples including clay analysis ((Rintoul *et al.* 1998). ATR is a rapid, non-destructive technique, the method making it possible to obtain spectra with little or no sample preparation, in particular making the preparation of pressed KBr pellets unnecessary (Madejova *et al.* 2001).

#### Method

Kaolinites have a characteristic diagnostic absorption band at 3700cm$^{-1}$ due to the inner hydroxyl group. This band has been used to quantify kaolinites (Kodama *et al.* 1963). This absorption band was found to become featureless with grinding or weathering (Kodama *et al.* 1989). Methods were trialled using the Kaolinite CRM.

#### Sample preparation

Samples of previously dried <2mm material, which had been stored in a desiccator with silica gel were gently ground in an agate mortar, for approx. 3 min., to reduce the particle size. In order to minimize damage isopropyl alcohol was used as a lubricant. Isopropyl alcohol was selected for the lubricant/moistening agent, as it is inert, and volatile. This technique has minimal effect on mineral structure and following evaporation produces particles of a suitable size (Russell 1987, Madejova *et al.* 2001).

Instrumentation

The spectra were obtained using a Perkin Elmer BX, FTIR spectrophotometer and its associated software package Spectrum for Windows© PerkinElmer Inc. ATR spectra were obtained using the MIRacle, single reflection horizontal ATR accessory from Pike Technologies (Madison WI, USA). The sampling plate features a small round ZnSe crystal, which allows analysis of small samples in the spectral range of 20,000 to 650 $cm^{-1}$. The sample is placed on the ZnSe crystal in a manner to ensure random orientation, and pressed with a micrometer-controlled compression clamp. Good contact between the sample and the crystal is necessary to obtain high-quality spectra (Madejova *et al.* 2001). All samples were analysed at room temperature, 20 scans were performed using a resolution of 4$cm^{-1}$, interval of 2$cm^{-1}$ and % Transmission (%T) as the unit of measurement and a range of 4000-600 $cm^{-1}$.

Use of the MIRacle, ATR accessory, from Pike Technologies (Madison WI, USA) allowed direct comparison with the values in Madejova *et al.* (2001). Specific regions of the spectra were examined to determine the presence of clay minerals together with organic material and structural water. Results are presented in Section 3.5.5.

### 3.4.6 X-Ray Fluorescence (XRF)

When materials are excited with high-energy, short wavelength radiation (e.g. X-rays), they become ionized. If the energy of the radiation is sufficient to dislodge a tightly held inner electron, the atom becomes unstable and an outer electron replaces the promoted inner electron. When this happens, energy is released due to the decreased binding energy of the inner electron orbital compared with an outer one. The emitted radiation is of lower energy than the primary incident X-rays and is termed fluorescent radiation. Because the energy of the emitted photon is characteristic of a transition between specific electron orbitals in a particular element, the resulting fluorescent radiation can be used to detect the abundances of elements that are present in the sample. Results are commonly reported by instrument software as oxides.

These properties are used in the analysis of major and trace elements in geological materials. The intensity of the energy measured is proportional to the abundance of the element in the sample. A set of calibrations are performed using Certified Reference Materials (CRM) or by preparing specific samples with known ratios of components. The unknown sample values are measured and the amounts present determined from the calibration data. X-Ray fluorescence is particularly well suited for investigations that involve bulk chemical analyses of major elements (e.g. Si, Ti, Al, Fe, Mn, Mg, Ca, Na, K, P) in rock and sediment. It is also utilised in the analysis of trace elements (in abundances >1 ppm; Ba, Ce, Co, Cr, Cu, Ga, La, Nb, Ni, Rb, Sc, Sr, Rh, U, V, Y, Zr, Zn) in rock and sediment. Detection limits for trace elements are typically of the order of a few parts per million.

Preliminary data were obtained using a Shimadzu EDX-720, however it was not possible to detect light elements e.g. (Na) at levels permitting quantification and high levels of iron (Fe) caused interference due to secondary excitation effects. A further major difficulty was the lack of a set of suitable Certified Reference Material that closely matched the origin and matrix of the unknown samples, necessary to produce a suitable set of calibration plots. Limited XRF analysis was undertaken at the Rockwood Industries facilities at Widnes, on the sample set analysed by XRD at Rockwood (Table 3.5).

## Method

Reference standards were prepared in accordance with the Rockwood Industries XRF, SOP (Table 3.6). The preparation of samples and standards was undertaken following the Rockwood Industries SOP, Ms. D McCarthy Senior Analytical Technician.

## Sample preparation

The samples were milled for 15 min. to produce a homogeneous finely powdered sample, using a Retsch model MM2000, ball mill, equipped with zirconium grinding jars. The milled sample was dried in an oven at $105^0C \pm 5^0C$ for minimum of 4 hours (usually overnight) and then allowed to cool in a desiccator. Approx. 1.5000g (±0.0005g) of sample was placed in a platinum-gold crucible together with 7.5g (±0.0005g) of Spectroflux 100B and 0.1g (±0.01g) of ammonium iodide. The contents of the crucible were mixed and transferred to PANalytical Perl'X 3 equipment. This is a fully automated bead preparation system and is used to fuse beads for XRF analysis. Fused beads were stored in plastic containers with foam inserts in a desiccator, as $Li_2B_4O_7$ (contained in the Spectroflux) is strongly hygroscopic.

Table 3.6 % Composition of prepared XRF standards, using Puratronic®, J Matthey reagents (number *)

| | **$SiO_2$ 010856*** | **$Al_2O_3$. 902001** | **$Fe_2O_3$ 10716*** | **$TiO_2$ 010897*** | **MgO 951001*** | **$CaCO_3$ 010679*** | **$Na_2CO_3$ 010861*** | **$K_2CO_3$ 010838*** |
|---|---|---|---|---|---|---|---|---|
| Std 1 | 100 | | | | | | | |
| Std 2 | | 100 | | | | | | |
| Std 3 | | 30 | | 2 | 20 | 30 | 8 | 10 |
| Std 4 | 10 | 25 | 12 | 3 | 36 | 4 | 2 | 8 |
| Std 5 | 25 | 20 | | 5 | 40 | | 4 | 6 |
| Std 6 | 40 | 15 | 30 | | 15 | | | |
| Std 7 | 45 | 10 | | | 8 | 25 | 12 | |
| Std 8 | 50 | 5 | 13 | 2 | 6 | 20 | | 4 |
| Std 9 | 55 | | 5 | 1 | 4 | 15 | 18 | 2 |
| Std 10 | 60 | 8 | 15 | 4 | 2 | 5 | 6 | |
| Std 11 | 65 | | 10 | | 25 | | | |
| Std 12 | 70 | | 20 | | | | 10 | |

## Equipment and Conditions:

Phillips PW2400 Spectrometer, Instrument parameters used for analysis, Table 3.7

Table 3.7 PW2400 Instrumental Analysis conditions

| | **Al** | **Fe** | **Ca** | **K** | **Si** | **Ti** | **Mg** | **Na** |
|---|---|---|---|---|---|---|---|---|
| X-Ray Tube | Rh | Rh | Rh | Rh | Rh | Rh | Rh | Rh |
| X-Ray Power KV/MA | 30/100 | 60/50 | 30/100 | 30/100 | 30/100 | 40/75 | 30/100 | 30/100 |
| X-Ray Path | Vac | Vac | Vac | Vac | Vac | Vac | Vac | Vac |
| Collimator (µm) | 700 | 300 | 300 | 300 | 700 | 300 | 700 | 700 |
| Collimator mask (mm) | 37 | 37 | 37 | 37 | 37 | 37 | 37 | 37 |
| Detector | Flow | Flow | Flow | Flow | Flow | Flow | Flow | Flow |
| Count Time (secs) | 14 | 4 | 4 | 4 | 12 | 4 | 12 | 12 |
| Spinner | On | On | On | On | On | On | On | On |
| Filter | None | Al | None | None | None | None | None | None |

Two samples were prepared for each CRM and site sample; five determinations were obtained for each. The data were pooled and the mean % values reported. Results are presented in Section 3.5.6.

### 3.4.7 Determination of sample pH

The standard method for measurement of pH (Rowell 1994) uses 10g of sample material and 25 ml freshly deionised water. This method was modified due to the sample size constraints imposed by the export licenses.

#### Method

Samples of 5.0±0.1g, air-dried, <2mm material were placed in screw capped polypropylene sample tubes. 12.5ml of deionised 18MΩ water was added to the samples that were mixed on a Grant Multifunction Rotating Mixer for 15 mins. Samples were removed from the shaker, stirred and pH measured within 30 seconds. The pH was determined using a PHM85 precision pH meter.

Differences in pH occur when the electrode is placed in a soil-slurry compared to the supernatant after the soil has settled. The differences are more pronounced with soil pH in water compared to electrolyte solutions. The procedure was repeated substituting 0.1M KCl solution, to determine the salt pH. Use of electrolyte solutions avoids variable soil-water pH due to differences in background salt levels in different soils and improves electrical conductivity in the electrical circuit for pH measurement (Miller *et al.* 2010).

The comparison of pH (KCl) and pH ($H_2O$) provides an assessment of the nature of the net charge on a colloidal system: The formula used is:

$$pH(KCl) - pH(H_2O) = \Delta ph$$

Results are presented in Section 3.5.7.

### 3.4.8 Laser Diffraction Particle Size Analysis

Traditional particle size methods such as sedimentation and pipette were not suitable for the very limited amount of each sample available; it was therefore decided that Laser Diffraction (LD) was the most appropriate technique.

LD has the following advantages: it provides a wide range of size classes including many data points <2 µm, it is a rapid technique, independent of the particle density because it provides a volume-based distribution. Pieri *et al* (2006) compared particle sizes obtained by three techniques, sedimentation, LD and Scanning Electron Microscopy. For all the soil samples Pieri *et al* (2006) measured, the LD method yielded a smaller clay fraction than the pipette method and a larger silt fraction. Pieri *et al* (2006) concluded that the LD method is a valid method for particle size differential analysis, even though it provides data that are not fully comparable with the classical sedimentation methods.

A Coulter LS 13 320 LD Particle Size Analyser was used for analysis. The associated software utilises two measurement techniques, Laser Diffraction Scattering with Mie deconvolution and Polarised Intensity Differential Scattering (PIDS).

Elastic light scattering (ELS) is the main method for the characterization of particles of sizes ranging from microns to mm. In ELS the scattered light has the same frequency as the incident light, and the intensity of scattered light is a function of the particle's optical properties and dimensions (Table 3.8).

Table 3.8 Factors effecting light scattering from particles and particulate suspensions

| | **variables** |
|---|---|
| scattered light intensity of a particle | particle dimension, particle refractive index, medium refractive index, light wavelength, polarization, and scattering angle. |
| scattered light intensity from a particulate suspension | particle concentration and particle-particle interaction. |

Sample concentration is adjusted such that the sample will scatter enough intensity to enable the measurement to be completed with a desired signal to noise ratio, but not to scatter so much as to saturate the detecting system.

If the sample is uniform, the scattered intensity is a function of scattering angle, particle shape, and particle size. A major assumption in a light scattering measurement is that the refractive index and density of particles in the sample are uniform, which is unlikely for geological samples. This can be mitigated by the choice of appropriate Optical Model parameters (Table 3.9). A composite scattering pattern is measured by detectors placed at angles up to ~ 35 degrees from the optical axis. The size distribution of this composite scattering pattern is deconvoluted and the relative amplitude of each pattern is used to measure the relative volume of spherical particles of that size.

Deconvolution based on Mie theory of light scattering is based on assumption that the particles are spherical.

### Mie Theory d < λ

Mie theory describes the interaction of light with a particle of arbitrary size (sizes < incident light wavelength) as a function of angle, given that the wavelength and polarization of the light are known and that the particle is smooth, spherical, homogeneous, and of known refractive index. It can be used for particles < 20μm.

### Polarised Intensity Differential Scattering (PIDS) - d << λ

Combining the polarization effects of light scattering with the wavelength dependence at high angles, extends the lower size limit to as low as 40nm, almost reaching the theoretical limit. The PIDS assembly provides size information for particles in the 0.04μm to 0.4μm range. It also enhances the resolution of the particle size distributions up to 0.8μm.

### Application of Laser Diffraction (LD) analysis to Non-Spherical Particles.

The particles in the sand and silt fractions are more likely to resemble a sphere, while in the clay fraction particles are often flat disc-shaped depending on the type of clay minerals characterizing the clay-size fraction. In sedimentation, a non-spherical particle tends to settle with the maximum cross-sectional dimension perpendicular to the direction of motion. This assumption results in decreasing the equivalent diameter (longer settling times) with overestimation of the clay fraction. In LD techniques, a non-spherical particle reflects a

cross-sectional area which is larger than a theoretical sphere of the same volume. This effect results in increasing the equivalent diameter, with underestimation of the clay fraction, because a particle is assigned to a larger size section of the distribution (Pieri *et al.* 2006).

#### 3.4.8.1 Sample Preparation, primary analysis

The sample preparation method of Eshel *et al.* (2004) and Smart (2002) were used. Approx. 200mg of < 2mm sieved material, was dispersed in 10ml of a sodium hexametaphosphate solution 0.5%w/v. Sodium hexametaphosphate is present to maintain particles in suspension and retard settling or flocculation. Two replicates of each sample were prepared. The samples were placed on a Grant Multifunction Rotating Mixer, rotated at room temperature for 24 hours. Samples were then introduced to the LS 13320 and data recorded for 10 consecutive runs of 1 min.

### Instrument and Analysis Conditions

The Mie optical model (Table 3.9) was used as it is considered as providing a better estimate of size distributions in clay materials (deBoer *et al.* 1987, Pieri *et al.* 2006). Table 3.10 contains instrument settings.

Table 3.9 Optical Model Parameters, particle size

| **Optical Parameters** | |
|---|---|
| Refractive index water | 1.33 |
| Refractive index geophagy material* | 1.53-1.56 |
| Imaginary Refractive Index content** | 1 |

*Refractive index Soils 1.53 ((Pieri *et al.* 2006) and 1.56 (Buurman *et al.* 1997) were used for size analysis. **Imaginary content for Refractive index of 1.0 was chosen as this was suitable for lightly pigmented metal oxides or highly coloured materials.

Table 3.10 Instrumental conditions, particle size

| **Parameter** | **Conditions** |
|---|---|
| Sample loading | Standard obscuration > 10% |
| Sonication prior to initial analysis run | 10 seconds Level 3 |
| Run time | 60 seconds |
| Pump speed | 50% |

Data corresponding to the particle size fractions, of the Udden Wentworth classification (Table 3.11) were obtained.

Table 3.11 Udden-Wentworth grade scales, particle size

| **particle size µm** | **Descriptive terminology** | |
|---|---|---|
| <2 | clay | Clay |
| 2-4 | very fine silt | Silt |
| 4 to 31 | fine , medium and coarse silts | |
| 31 to 62.5 | coarse silt | |
| 62.5 to 250 | very fine and fine sand | Sand |
| 250 to 500 | medium sand | |
| 500 to 2000 | coarse and very coarse sand | |

The scale devised (1898) by the American sedimentary petrologist J.A. Udden was adapted (1922) by C.K. Wentworth, who expanded the definitions of the various grades to conform to actual usage by researchers (Rowell 1994). Data for each site was pooled for analysis. Results are presented in Section 3.5.8.

#### 3.4.8.2 Effect of pH and Gastric media on Particle Size

Mucin as a macromolecule (present in the gastric media) may exert an influence on the particle size distribution. Commercially produced mucin contains a range of different molecular weights (Bansil *et al.* 1995). Epithelial mucins are responsible for the viscoelastic and gel-forming properties of mucus, which forms the protective layer covering epithelial organs. This layer protects the stomach lining from auto-digestion by the HCl acid in the lumen. Porcine gastric mucin at concentration > 10mg/ml in solutions of low ionic strength showed a 100 fold increase in viscosity when the pH is reduced from 7-2 (Bansil *et al.*, 1995). The increase in viscosity was due to the formation of very large aggregates, ~10,000µm, at pH2 (Bhaskar *et al.* 1991), who also reported a similar change occurring in a simulated gastric fluid Bhaskar *et al.* (1991) had prepared. However, the pH induced mucin aggregation was prevented by increasing ionic strength.

The mixture of simulated saliva and gastric fluid (10:30) contained 30mg/ml mucin. The *Ateles* simulated media differs from the DIN 179738 (DIN. 2004) human replacement media in its concentrations of NaCl and KCl. The calculated ionic strength due to these salts at the DIN fluid concentrations is 0.0368 and that in the *Ateles* media is 0.0621, (calculations performed using the Ionic strength calculator, www.lenntech.com). This increased ionic strength in the *Ateles* media may influence the degree of aggregation.

##### Sample Preparation and Method

The effect of both pH and simulated gastric media on particle size distribution of the CRMs kaolinite KGa-2 and montmorillonite SWy-2, and site samples was investigated. 100mg samples were dispersed in 5ml of the following conditions in Table 3.12.

Table 3.12 Test solutions used in investigation of pH and Gastric media on Particle Size

| **solutions** | **pH** |
|---|---|
| 18 MΩ deionised water/ sodium hexametaphosphate solution 0.5%w/v. | 6.7 |
| 18 MΩ deionised water/ sodium hexametaphosphate solution 0.5%w/v./ pH adjusted | 2 |
| Combined (10:30) simulated gastric media | 2 |

The solutions were pH adjusted using High Purity HCl acid (Hydrochloric acid 37 w/w % in $H_2O$, 99.999% trace metals basis; Sigma-Aldrich) and measured using a PHM85 precision pH meter. Samples were mixed on a Grant Multifunction Rotating Mixer, placed in an incubator at $37^0$C for 60 mins. Two replicates of each sample were prepared. Samples were then measured as previously stated. Results are presented in Section 3.5.8

### 3.4.9 Adsorption of Plant Secondary Metabolites

Clay minerals and clay sized particles have a significant environmental role, behaving as a natural adsorbent of potentially toxic pollutants e.g. heavy metals and organic compounds such as pesticides, (Bergaya *et al.* 2006a, Bergaya *et al.* 2006b, Churchman *et al.* 2006, Nir *et al.* 2006). Detoxification of PSM by this mechanism has been one of the major hypotheses for the functional use of geophagy in both humans

(Johns 1986, Young *et al.* 2011), birds (Gilardi *et al.* 1999, Brightsmith *et al.* 2008) and bats (Voigt *et al.* 2008) detailed in Section 1.9.1.

#### 3.4.9.1 Potential type of PSM exposure from diet

Predominantly PSM belong to either the polyphenols or alkaloids classes. The presence of both together may render them inactive as polyphenols and alkaloids interact to form insoluble complexes (Hagerman *et al.* 1981, Janzen *et al.* 1984) Janzen also noted that in tropical and neo-tropical vegetation that alkaloid content decreases with increased concentration of condensed and hydrolysable tannins (Janzen *et al*., 1984). Alkaloids can reach levels of 1% dry weight in tissues related to reproduction e.g. flowers and seeds whereas flavonoids are commonly found in leaves, stems and fruits (Wink 2010). Plant phenolics increase with exposure to UV-B radiation (Sullivan *et al*. 1992). Altitude and latitude also influence levels (Jansen *et al.* 1998, Frohnmeyer *et al.* 2003).

There has been much speculation in the literature about the presence of PSM in fruits. Fruit pulp is seen as a plant mechanism to aid with dispersal and so it would be counterproductive to have high values of deterrent compounds present (Schaefer *et al.* 2003, Cazetta *et al.* 2008). Smaller amounts remaining from protection of immature fruits may however persist (Mack 1990). Unripe fruits have higher levels of phenolic acids (tannins) in their outer cell layers (Crozier *et al.* 2008). Fig trees in particular have a high diversity of consumer species (Kalko *et al.* 1996) and it was argued by Janzen (1979) that this was due to an absence of toxic secondary compounds. Figs constitute a significant proportion of *Ateles* food items; this suggests that there is little toxic material present or that *Ateles* have a mechanism for dealing with its presence or any PSM compounds present may be beneficial. Janzen *et al.* (1984) detected high levels of condensed polyphenols in the leaves of the strangler fig varieties found at Santa Rosa; this would suggest that condensed polyphenols and not alkaloids would be potentially present in fig fruits. Alkaloids are reported as being absent in fig fruits (Jordano 1983).

#### 3.4.9.2 Potential type of PSM exposure from mistletoe

A literature review of the PSM classes likely to be present in several species of Viscaceae was conducted. The review indicated the main types of potentially toxic compounds likely to be present (Sections 1.8.4-1.8.6). Adsorption studies were limited to compounds with aqueous solubility and were not attempted for the potential mistletoe constituent peptides and terpenes.

There is much literature relating to the adsorption of amino acids, peptides and polyamino acids onto clay minerals (Hedges *et al.* 1987, Ding *et al.* 2002, Ikhsan *et al.* 2004). Hedges *et al.* (1987) found that kaolinite adsorbed (acidic) anionic amino acids 22-34% uptake whilst adsorbing < 6% polar, neutral amino acids. Kaolinite also adsorbed basic cationic amino acids (~80% removal). In contrast 10-15% of the neutral amino acids were taken up by montmorillonite abut very little of the acidic (anionic) amino acids were adsorbed on to montmorillonites. Both the viscotoxins and thionins are cationic species and can be extracted in acidic aqueous conditions, and so would be expected to be extracted and so present in simulated gastric media. The toxins potentially may be adsorbed. It was decided that adsorption studies of the peptide types, viscotoxins and lectins, present in mistletoes would not be undertaken due to the highly toxic nature of the suitable reference material, and their highly controlled status.

Terpenes are a large family of PSM and are known to be the primary constituents of essential oils (Harborne 1998b). Terpenes generally exhibit poor aqueous solubility and so will not be considered for adsorption studies (Breitmaier 2006). It is unlikely that terpenes would be extracted in the short potential extraction exposure to gastric media solutions.

The specific model compounds used for the study were the alkaloids ephedrine HCl, quinine HCl and berberine; the tannins gallic and tannic acid, and naringin as a flavonoid. Model compounds were analytical grade obtained from Sigma Aldrich. Whilst these specific individual compounds were not identified as present in diet or mistletoes they represent classes of compounds potentially to be present in aqueous extracts. Model compounds were also selected on the advice from Rockwood Industries. The compounds represent a variety of different shapes and sizes and complexity of molecules, factors which would influence the ability of a clay mineral to function as an adsorbent. The compounds chosen also represented both potentially toxic and beneficial classes of PSM and compounds used by other authors, permitting potential comparisons. Following identification of chlorogenic acid, a phenylpropanoid, in *Phoradendron quadrangulare* extracts chlorogenic acid was included in the study.

These compounds were analysed using UV spectroscopy. The application of standardized UV (or UV–Vis) spectroscopy has been used for many years (and remains so) in analyses of plant phytochemicals e.g (Perry *et al.* 2001, Andersen *et al.* 2010, Stalmach *et al.* 2012, Takemoto *et al.* 2012, Khoddami *et al.* 2013). Polyphenol compounds have two characteristic UV absorption bands with $\lambda_{max}$ in the 240-285 and 300-550 nm range. The various flavonoid classes can be recognized by their UV spectra (Andersen *et al.* 2010). The number of compounds tested was also limited by the quantity of geophagy sample available.

#### 3.4.9.3 General Method Development

The method of Brightsmith *et al.* (2008) was used as the starting point for the adsorption development work.

Ranges of different concentrations of quinine hydrochloride dihydrate (Sigma Aldrich) were prepared in fresh deionised water pH adjusted to ~2.00. The solutions were mixed for 50mins on a Grant Multifunction Rotating Mixer placed within an incubator equilibrated to $37^0$C. Samples were allowed to cool to room temperature before measurement of the $\lambda_{max}$ under these pH conditions, using a Perkin Elmer Lambda 25 UV/Visible Spectrophotometer. Using this $\lambda_{max}$ wavelength calibration data were obtained. The aqueous pH2 calibration data was used to determine a suitable concentration range to be used for obtaining the calibration data in combined gastric media, pH~2.00 (Figure 3.14).

##### Method

Freshly prepared simulated saliva and gastric media were combined in the proportions 10:30, in 15ml polypropylene centrifuge tubes and the appropriate volume of quinine stock solution added to provide solutions of the same concentration range of the pH2 aqueous calibration plot. The solutions were mixed for 60mins on a Grant Multifunction Rotating Mixer placed within an incubator equilibrated to $37^0$C. Samples were allowed to

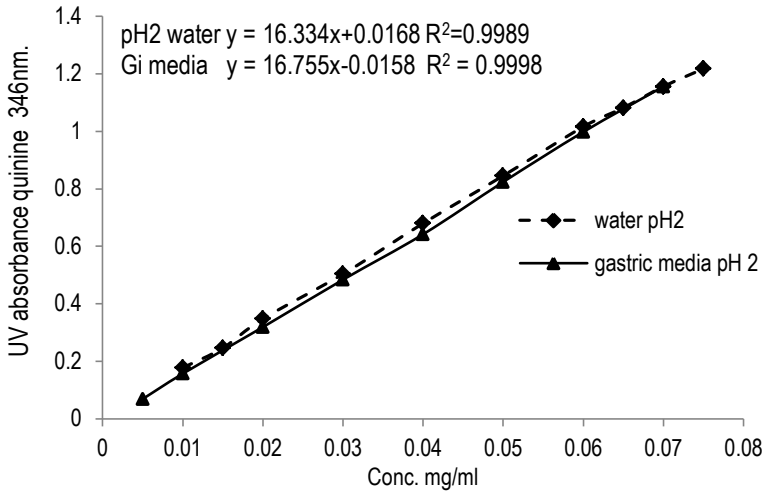



Figure 3.14 Calibration data for quinine HCl in both water pH2 and Gastric media pH2, centrifuged for 45 mins.

cool to room temperature before measurement of the $\lambda_{max}$. The solutions in simulated media were cloudy and not suitable for UV analysis. Samples were withdrawn and placed in micro centrifuge tubes and centrifuged for 15, 30, 45 and 60 mins at 12,500 rpm. The $\lambda_{max}$ and absorbance of the supernatant was determined, using a Perkin Elmer Lambda 25 UV/Visible Spectrophotometer. Solutions centrifuged at 45 and 60 mins were clear and provided calibration data similar to that of the aqueous pH2 solutions. Future work used a centrifuge time of 45 mins, for the remaining adsorption work.

Preliminary adsorption work used four samples of approx. 100mg of Sample 41 for the initial adsorption determinations in pH2 water using two concentrations of quinine HCl solution. Sample 41 was chosen as this was one of the largest samples available so was suitable for this sacrificial work (Table 3.13).

Table 3.13 Adsorption data for Sample 41, method development.

| Sample mg | initial quinine conc. mg/ml | total quinine mg | UV Absorbance | quinine remaining mg/ml | total quinine adsorbed mg/mg sample |
|---|---|---|---|---|---|
| 99.92 | 0.075 | 1.875 | 0.9022 | 0.0564 | 0.0047 |
| 93.50 | 0.075 | 1.875 | 0.9054 | 0.0566 | 0.0049 |
| 97.53 | 0.060 | 1.500 | 0.7100 | 0.0442 | 0.0040 |
| 96.98 | 0.060 | 1.500 | 0.7096 | 0.0442 | 0.0041 |
| 99.92 | 0.075 | 1.875 | 0.9022 | 0.0564 | 0.0047 |

As a result of the development work it was decided to use a concentration of 0.06mg/ml for future quinine/rock adsorption work. This development process was then applied to the remaining model compounds.

### 3.4.9.4 Method Development - tannic acid and gallic acid.

The presence of mucin in the simulated media presented a potential problem for the application of the quinine HCl method to tannins. Polymeric porcine mucin comprises 16% protein per mg of glycoprotein and is rich in serine, threonine and proline equivalent to 43% of total amino acids (Fogg *et al.* 1996). Tannins bind to salivary proline rich peptides.

Calibrations were obtained using pH2 aqueous, simulated media electrolyte solution pH2 and simulated media (proportions 10:30) pH2 without mucin and with mucin. The calibration plots (Figure 3.15) for media including the mucin is suggestive of an initial adsorption with little change in absorbance at the three lowest concentrations (0.01-0.018mg/ml) followed by a steep rise between (0.022-0.026mg/ml).

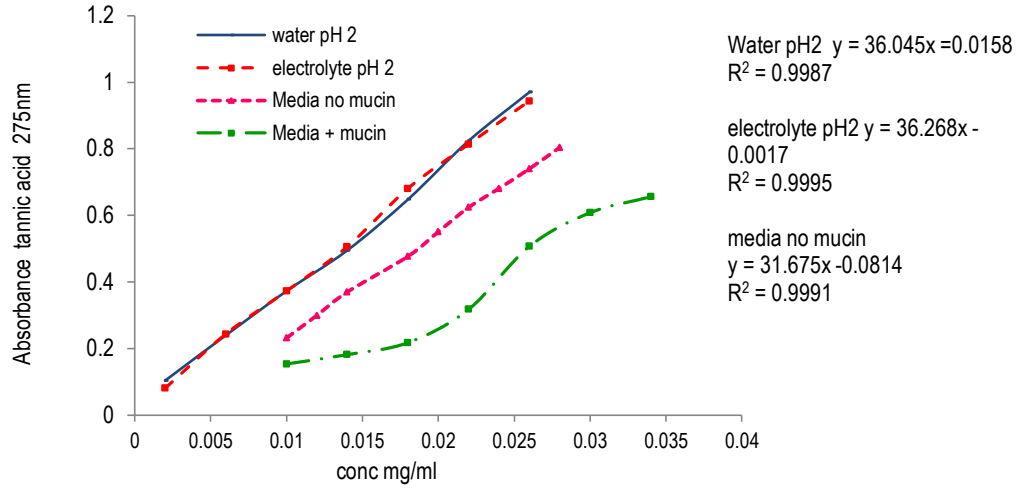



Figure 3.15 Comparison of effect of calibration media on tannic acid UV Absorbance

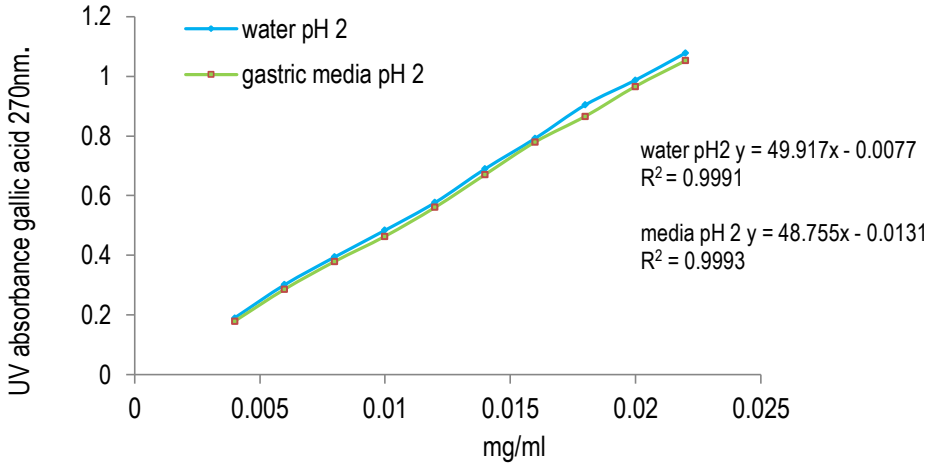



Figure 3.16 Comparison of Calibration plots for gallic acid.

This may be due to saturation of the available binding sites with tannic acid. As can be seen there is no major difference between water pH2 and the pH2 electrolyte mixture (without any mucin or enzymes) used in the GI media. It was decided to investigate the adsorption of tannic acid using the pH2 media without the mucin present. A similar problem was not experienced with gallic acid (Figure 3.16).

## Method Adsorption studies

Concentrated solutions of the PSM model compounds were prepared in pH2 adjusted deionised water, such that 200µl added to 7.8ml media gave a UV absorbance near the upper end of the calibration plots. Fresh simulated media solutions were prepared using pH adjusted deionised water. Media was then mixed in the ratio 10:30 saliva/gastric media. 7.8ml aliquots were placed in 15ml conical polypropylene centrifuge tubes and 200mg±10mg of the geophagic samples or 10-25mg SWy-2 or 50mg±5mg KGa-2 were added to the tubes. The geophagic sample mass to media ratio would equate to 1g geophagic material in 40ml stomach contents (1g occupied approx. 1ml). Lower amounts for CRM were used following results of adsorption development work.

Tubes were then mixed on a Grant Multifunction Rotating Mixer for 60 mins, in an incubator at 37$^0$C. After 10 mins 200 µl of PSM test solution was added to incubated tubes and the mixtures incubated for a

further 50 mins. A tube of media alone was prepared as a blank for the analysis. A further tube of media and test compound was prepared to use as a control standard.

Following incubation the sample tubes were placed in a refrigerator for 10 mins to cool and allow the clay materials to sediment out, aliquots were then removed and placed in micro centrifuge tubes and centrifuged for 45 mins at 12,500 rpm. Samples were then analysed using a Perkin Elmer Lambda 25 UV/Visible Spectrophotometer, the residual concentrations of model compounds in the tubes calculated and the adsorption of the model PSM (mg/g clay/geophagic sample) calculated and plotted in Excel© 2010. Results are presented in Section 3.5.9.

### 3.4.10 ICP-MS Determination of mineral released

High concentrations of protein materials and inorganic salts may cause deposition in the instrument and may cause the nebuliser and the aperture in the sampling cone to become clogged (Vandecasteele *et al.* 1993). This may cause variable flow rates and reproducibility issues. Therefore, it was necessary, before any investigation of the geophagic samples was undertaken, to investigate the impact of the gastric media solutions on the analytical technique.

An ICP-MS instrument uses a plasma as the ionisation source (ICP) and a mass spectrometer (MS) analyser to detect the ions produced. It can simultaneously measure most elements in the periodic table and determine analyte concentration down to the sub nanogram/litre (ng/l) or part-per trillion (ppt) levels. It can perform qualitative, semi-quantitative, and quantitative analysis. Sample preparation and use of the instrument were undertaken under the supervision of Dr. P. Riby, LJMU.

Polyatomic interferences in ICP-MS are caused when ions generated from the plasma, the sample, or a combination of the two carry a mass-to-charge ratio that is identical to that of the analyte ion e.g. $Fe^{56}$ has interference due to combination of Argon from the plasma and oxygen $^{56}ArO^{+}$. The isotope with the highest abundance is usually the form measured however in this case it is compromised by the interference. This leads to incorrect ion counts being recorded. It is therefore necessary to choose to measure a different isotope e.g. $Fe^{54}$ and avoid the high background due to interference by 56 mass unit ions.

Non-spectral interference is due to a change in signal intensity that is not related to the quadrupole mass analyser, and may be enhancement or suppression and results in over/under detection (Hsiung *et al.* 1997). These are due to the components within the matrix. Olivares *et al.* (1986) showed that matrix effects, due to easily ionised elements, was in the order Na >Mg >I >Br, and suggested that biological specimens containing relatively large concentrations of easily ionized matrix elements, e.g. Na, K, Ca, and Mg makes analysis of these samples difficult. The *Ateles* gastric media has high concentrations of Na and K.

These limitations are taken into account with the use of external calibration and internal standard addition. Any internal standard should undergo identical matrix suppression or enhancement as that of the analyte elements. Internal standards are most appropriate for detection of altered delivery rates such as blocked orifices (Finley-Jones *et al.* 2008).

#### Instrument:

A Thermo X SERIES 2; (Thermo Scientific, MA), Inductively Coupled Plasma Mass Spectrometer (ICP-MS), with associated control and analysis software.

## Internal Standard - Rhodium, $Rh^{103}$ recovery

Rhodium (Rh) is added as an internal standard and used to calculate concentrations and correct for a decrease in signal during analysis. It was therefore necessary to determine if there was any signal reduction due to the media components.

## Method

Prior to use all tubes and glassware equipment was soaked in 2% nitric acid in deionised water for 24hours, removed from the acid wash and rinsed three times with 18 MΩ high purity deionised water, and placed upside down to drain. Once dry all tubes were capped, before use.

Blank solution of 18MΩ deionised water and 5 samples of simulated media mix (10:30) were placed in clean conical polypropylene centrifuge tubes. Samples were mixed on a Grant Multifunction Rotating Mixer for 60 mins, in an incubator at $37^0$C. Following incubation, the sample tubes were centrifuged for 45 mins at 13,000 rpm, to attempt precipitate the protein, before ICP-MS analysis. The centrifuged gastric media solutions were filtered through nylon 0.45µm syringe filter. Filtered media was then placed in clean polypropylene centrifuge tubes, with nitric acid (nitric acid, 70% w/w, purified by redistillation; ≥99.999% trace metals basis; Sigma Aldrich) and Rhodium standard $Rh^{103}$ solution (PlasmaCal, 1000ppm, SCPScience) 10ppm to provide 1:5, 1:10; 1:20, and 1:100 dilutions (Table 3.14).

Five determinations were obtained for each dilution, a including the blank. The Rh recovery was determined for each of these dilutions.

Table 3.14 Schedule for preparation of diluted, ICP samples

| | **Dilutions** | | | | | |
|---|---|---|---|---|---|---|
| | **1 in 10** | **1 in 20** | **1in 25** | **1 in 40** | **1 in 50** | **Blank** |
| Gastric media(ml) | 2 | 1 | 0.8 | 0.5 | 1 | 0 |
| Nitric acid (ml) | 0.4 | 0.4 | 0.4 | 0.4 | 0.5 | 0.5 |
| $Rh^{103}$ Standard (µl) | 20 | 20 | 20 | 20 | 50 | 50 |
| Deionised water to (ml) | 20 | 20 | 20 | 20 | 50 | 50 |

## Results of Rh Recovery for Gastric Media

Initial $Rh^{103}$ recovery for the 1:100 and 1: 5 dilutions were 88% and 76%, with RSD values ≥3% The values for the 1:100 dilution at the 5th determination had dropped to 82% with %RSD >5%. There were visible signs of deposition on the cone surfaces at the 1:100 dilution. The low $Rh^{103}$ recovery and its deterioration over the time of the analysis may be due to the presence of the protein materials and inorganic salts in the gastric media having several effects on the equipment. Taking into consideration these issues and the small amount of samples available it was decided that the geophagic digests would be undertaken using deionised water, adjusted to pH2 using HCl (Hydrochloric acid, 37 w/w %, 99.999% trace metals basis; Sigma-Aldrich). The presence of mucin in the gastric media would have created a further problem as gastrointestinal mucin has an affinity for metal ions in the following pattern: $Fe^{3+}$> $Al^{3+}$ $Cr^{3+}$> $Pb^{2+}$ > $Zn^{2+}$ > $Co^{2+}$ > $Ca^{2+}$ > $Na^{+}$, $Cs^{+}$ (i.e. $M^{3+}$ >$M^{2+}$ >$M^{+}$ (Powell *et al.* 1999). This may have reduced levels to below the Limit of quantification (LOQ).

### Sample preparation- Calibration Standards

A multi-element, 10ppm stock solution was prepared from commercial single-element 1000ppm calibration solutions (PlasmaCAL Single Element Calibration Standards, SPC Science) in ultrahigh purity 18MΩ deionised water containing 1% nitric acid (nitric acid, 70% w/w, purified by redistillation; ≥99.999% trace metals basis; Sigma Aldrich). Calibration dilutions of the multi element stock solution at 10, 50, 100, 150, 200, 250, 300, 350 and 400ppb were prepared containing 100 µl of 10ppm $Rh^{103}$ stock solution (Rh103 1000 ppm PlasmaCAL) and 1ml nitric acid ($HNO_3$, 70% w/w, purified by redistillation; ≥99.999% trace metals basis; Sigma Aldrich) in high purity 18MΩ deionised water. The elements selected for determination were chosen following a preliminary full element survey scan. These were elements of potential biological activity which were also present at levels which would permit quantification. The elements were : B, Na, Al, P, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Co, Zn, Se, Mo and Ba.

### Sample Preparation

500mg ± 10% geophagic samples were placed in polypropylene tubes together with 8 ml of high purity 18MΩ deionised water adjusted to pH2 with HCl (Hydrochloric acid 37 % w/w, 99.999% trace metals basis; Sigma-Aldrich). The samples were mixed on a Grant Multifunction Rotating Mixer for 60 mins, in an incubator at $37^0$C. Following incubation, the sample tubes were centrifuged for 45 mins at 13,000 rpm, to remove particulates before analysis. The supernatant was filtered through nylon 0.45µm syringe filters. 1ml filtered samples were placed in nitric acid cleaned 100ml volumetric flasks, 100 µl of 10ppm $Rh^{103}$ stock solution and 1ml nitric acid (nitric acid 70% w/w, purified by redistillation; ≥99.999% trace metals basis; Sigma Aldrich) added and made up to volume with high purity 18MΩdeionised water.

Where sufficient material was available 3 subsamples were digested and analysed but only one sample was collected from Site 10. Two replicates were prepared of each sample and the two reference clays. The tubes were randomly placed in the ICP-MS sample tray. A blank and an Instrument Calibration solution were placed after every 8th geophagy sample, allowing use of both external calibration and internal standards. Results are presented in Section 3.5.10.

#### 3.4.10.1 Adsorption/ion exchange of Fe

One of the hypotheses for geophagy is related to anti-bacterial or antiparasitic functions (Knezevich 1998). However these have either suggested no mechanism or suggested it may be a physical effect (Dominy *et al.* 2004); gastrointestinal adhesion providing a barrier.

The ability of clay minerals to exchange cations may be significant in this scenario, particularly exchange or adsorption of Fe. The importance of limiting Fe availability on the viability of several classes of microorganism and control of diseases has been extensively reported e.g. Table 3.15. Iron withholding as a potential mechanism for treating malaria has also been investigated (Weinberg *et al.* 2009) and other parasitic organisms (Glanfield *at al.* 2007). Geophagy in humans accompanied by anaemia has also been reported (Young *et al.* 2010, Young *et al.* 2011). The potential for the geophagy samples to reduce available free iron in both acidic (stomach) conditions and pH 6.88 (~intestinal pH) was explored.

Table 3.15 Publications relating to effects of Fe depletion and potential infections

| Publication | Topic |
|---|---|
| Weinberg (1975) | Hosts attempt to withhold iron from microbial invaders |
| Oppenheimer (2001) | Iron and its relation to immunity and infectious disease |
| Miethke *et al.* (2007) | Siderophore based iron acquisition and pathogen control |
| Weinberg *et al.* (2008) | Iron withholding: A defense against disease |
| Weinberg *et al.* (2009) | Iron availability and infection |
| Santos *et al.* (2012) | Effect of chelating agents on the Microorganism Development, Virulence and Pathogenesis |
| Nevitt *et al.* (2011) | Host iron and siderophore utilization for *Candida glabrata* |
| Wandersman (2010) | Heme uptake and iron extraction by bacteria |
| Nyilasi *et al.* (2005) | Iron gathering of opportunistic pathogenic fungi |
| Reniere *et al.* (2010) | Iron Uptake and Homeostasis in Microorganisms - *Staphylococci* |
| Reys-Lopez *et al.* (2001) | *Entamoeba histolytica*: Transferrin binding proteins |
| Wilson *et al.* (1998) | Iron acquisition by parasitic protozoa |
| Weinberg (1996) | Iron withholding: a defense against viral infections |
| Weinberg (1999) | The role of iron in protozoan and fungal infectious diseases |

### Method

250±5mg mg of each geophagic sample material was placed in previously cleaned (nitric acid) and dried polypropylene centrifuge tubes. 0.5ml Fe standard 1000ppm (PlasmaCAL Single Element Calibration Standards, SPCScience) was added to each tube and either:

i) 9.5 ml of 18mMΩ deionised water adjusted to pH 2.2 with HCl acid (Hydrochloric acid 37 w/w % in $H_2O$, 99.999% trace metals basis; Sigma-Aldrich).

ii) 9.5ml of 18mMΩ deionised water adjusted to pH 6.88 with (Sodium hydroxide, pellets, 99.995% trace metals basis, Sigma Aldrich).

Two replicates were prepared for each geophagic sample. Two tubes of each pH (without geophagic material) spiked with Fe standard and two tubes of 18MΩ deionised water without Fe as blanks, were prepared. Samples from Site 6, without Fe addition, as further controls, were also prepared. Results are presented in Section 3.5.10.

## 3.4.11 Microbiological Assessment geophagy samples

Soil organisms are a common source of antibiotics (Smith 2000, Ketch *et al.* 2001) and so to exclude the presence of such organisms 2-3 sub samples from Sites 1,2,4,6,9 and 10 were tested using Potato dextrose broth and agar pH adjusted to 3.5. Reduced pH inhibits the growth of bacteria but permits growth of moulds and yeasts.

### Method

Potato Dextrose Broth (P6685 Sigma Aldrich) 12g was suspended in 500ml purified water, and heated until completely dissolved. 20ml aliquots of broth was decanted into boiling tubes and was then autoclaved at 121$^0$C for 15 mins to sterilize. The broth was allowed to cool to 50$^0$C.The pH of a 20ml sample broth was measured using universal indicator paper and 250µl 10% tartaric acid sterile solution added to produce media pH 3.5. 500mg±10mg of geophagic material was added to each tube and thoroughly mixed then incubated at 28$^0$C for 48 hours.

39g Potato Dextrose Agar (70139 Sigma Aldrich) was dissolved in 1000ml purified water, heated to dissolve and then 20ml decanted into 30ml glass screw capped bottles. These were autoclaved at 121$^0$C for 15 mins to sterilize, allowed to cool and the pH adjusted in a similar manner to the broth before pouring into sterile Petri dishes to set. 0.1ml of the previously incubated broth containing the geophagy samples was aseptically spread onto the surface of the agar. Three plates were prepared for each incubated sample. The plates were then incubated at 25$^0$C for 7 days, and then examined for growth. Results are presented in Section 3.5.11.

### 3.4.12 Summary of Analyses undertaken

Detailed information relating to the samples and the analyses conducted are presented in Table 3.16.

Table 3.16 Summary of techniques and geophagy samples analysed

| | | | **Analytical Parameters measured** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample No. | Sample size (g) | Munsell Colour code | Water content | Loss on Ignition | IR | XRD Rigaku | XRD Rockwood | XRF Rockwood | Particle Size | ICP | pH | Adsorption studies | Microbiology |
| Site 1 (Control) | 1 | 82 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | ✓ | ✓ | N |
| | 2 | 8 | ✓ | N | N | N | N | N | N | N | N | N | N | ✓ |
| | 3 | 30 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N | N | N | N | N |
| | 4 | 30 | ✓ | N | N | ✓ | ✓ | N | N | ✓ | N | N | N | N |
| | 5 | 48 | ✓ | ✓ | ✓ | N | ✓ | N | N | ✓ | N | N | N | N |
| | 6 | 42 | Rock Frag. | unsuitable for detailed analysis | | | | | | | | | | |
| | 7 | 18 | ✓ | Y | Y | ✓ | ✓ | N | N | N | N | N | N | N |
| | 8 | 10 | ✓ | N | N | N | ✓ | N | N | N | N | N | N | ✓ |
| | 9 | 64 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | ✓ |
| | 10 | 15 | ✓ | N | N | ✓ | insufficient suitable material for complete analysis | | | | | | ✓ | N |
| | 11 | 26.8 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N |
| Site 3 (Control) | 17 | 46 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | ✓ | N | N | N |
| | 18 | 50 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | ✓ | N | ✓ | N |
| Site 4 (Control) | 19 | 38 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | ✓ | N | N | ✓ |
| | 20 | 12 | ✓ | N | N | ✓ | insufficient suitable material for complete analysis | | | | | | N | ✓ |
| | 21 | 46 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | 21 | 46 | ✓ | ✓ | ✓ |
| | 22 | 30 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | 22 | 30 | ✓ | ✓ | ✓ |
| | 23 | 24 | rock frag | unsuitable for detailed analysis | | | | | | | | | | |
| | 24 | 28 | ✓ | N | N | ✓ | ✓ | N | N | ✓ | N | N | N | N |
| | 25 | 76 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N | N | N | ✓ | N |

Table 3.16 (cont) Summary of techniques and geophagy samples analysed

| | | | **Analytical Parameters measured** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample No. | Sample size (g) | Munsell Colour code | Water content | Loss on Ignition | IR | XRD Rigaku | XRD Rockwood | XRF Rockwood | Particle Size | ICP | pH | Adsorption studies | Microbiology |
| Site 4 (Control) | 26 | 102 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | N | ✓ | ✓ | ✓ |
| | 27 | 34 | ✓ | N | N | ✓ | N | N | N | N | ✓ | N | N | N |
| | 28 | 20 | pumice? | unsuitable for detailed analysis | | | | | | | | | | |
| | 29 | 18 | pumice? | unsuitable for detailed analysis | | | | | | | | | | |
| | 30 | 14 | rock fragment | unsuitable for detailed analysis | | | | | | | | | | |
| | 31 | 42 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N | N | N | N | N |
| | 32 | 108 | rock fragment | unsuitable for detailed analysis | | | | | | | | | | |
| Site 6 (Control) | 39 | 86 | ✓ | N | N | ✓ | N | N | N | N | N | N | N | N |
| | 40 | 100 | ✓ | N | N | ✓ | ✓ | ✓ | ✓ | N | N | N | ✓ | N |
| | 41 | 100 | ✓ | ✓ | ✓ | ✓ | N | N | N | ✓ | ✓ | ✓ | N | N |
| | 42 | 100 | ✓ | N | N | ✓ | ✓ | N | N | ✓ | N | N | ✓ | N |
| | 43 | 24 | N | N | N | ✓ | ✓ | N | N | N | ✓ | N | N | N |
| | 44 | 15 | rock frag | unsuitable for detailed analysis | | | | | | | | | | |
| | 45 | 22 | ✓ | ✓ | ✓ | ✓ | N | N | N | N | ✓ | N | N | N |
| | 46 | 78 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | 47 | 10 | ✓ | N | N | ✓ | ✓ | N | N | N | N | N | N | ✓ |
| | 48 | 10 | rock frag | unsuitable for detailed analysis | | | | | | | | | | |
| | 49 | 6 | rock frag | unsuitable for detailed analysis | | | | | | | | | | |
| | 50 | 30 | rock frag | unsuitable for detailed analysis | | | | | | | | | | |
| | 51 | 5 | N | N | N | N | N | N | N | N | N | N | ✓ | ✓ |
| | 52 | 20 | N | N | N | ✓ | ✓ | N | N | ✓ | ✓ | N | N | N |

Table 3.16 (cont) Summary of techniques and geophagy samples analysed

| | | | **Analytical Parameters measured** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample No. | Sample size (g) | Munsell Colour code | Water content | Loss on Ignition | IR | XRD Rigaku | XRD Rockwood | XRF Rockwood | Particle Size | ICP | pH | Adsorption studies | Microbiology |
| Site 7A | 53 | 7.4 | ✓ | N | N | N | N | N | N | N | N | N | N | N |
| | 54 | 12.4 | ✓ | N | N | N | N | N | N | ✓ | N | N | N | N |
| | 55 | 34.6 | ✓ | N | N | N | N | N | N | N | N | N | N | N |
| | 56 | 70 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | N |
| | 57 | 62 | ✓ | N | N | N | N | N | N | N | N | N | N | N |
| | 58 | 60 | ✓ | ✓ | ✓ | N | N | N | N | N | N | N | N | N |
| | 59 | 31 | ✓ | N | N | ✓ | ✓ | N | N | N | N | N | N | N |
| | 60 | 53 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N |
| | 61 | 58 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | ✓ | N | N | N |
| Site 7B | 62 | 126 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | N |
| | 63 | 45.7 | ✓ | ✓ | ✓ | ✓ | N | N | N | N | N | N | N | N |
| | 64 | 27 | ✓ | N | N | N | N | N | N | N | N | N | N | N |
| | 65 | 86 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | ✓ | ✓ | N | ✓ | N |
| | 66 | 32 | ✓ | ✓ | ✓ | N | N | N | N | N | N | N | N | N |
| | 67 | 62 | crystalline tube | unsuitable for detailed analysis | | | | | | | | | | |
| | 68 | 16 | insufficient suitable material for detailed analysis | | | | | | | | | | | |
| | 69 | 20 | ✓ | N | 69 | 20 | ✓ | N | 69 | 20 | ✓ | N | 69 | 20 |
| Site 8 (*Cebus* eating) | 70 | 48 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | 71 | 34 | ✓ | N | N | N | ✓ | N | N | N | N | N | ✓ | ✓ |
| | 72 | 56 | ✓ | ✓ | ✓ | ✓ | N | N | N | ✓ | ✓ | N | ✓ | N |

Table 3.16 (cont) Summary of techniques and geophagy samples analysed

| | | | **Analytical Parameters measured** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample No. | Sample size (g) | Munsell Colour code | Water content | Loss on Ignition | IR | XRD Rigaku | XRD Rockwood | XRF Rockwood | Particle Size | ICP | pH | Adsorption studies | Microbiology |
| Site 2 *(Ateles* eating) | 12 | 34 | ✓ | N | N | ✓ | ✓ | N | N | ✓ | N | N | N | ✓ |
| | 13 | 72 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | N |
| | 14 | 94 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | N | ✓ | N |
| | 15 | 44.8 | ✓ | N | N | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | 16 | 52 | ✓ | ✓ | ✓ | ✓ | ✓ | N | N | N | N | N | N | ✓ |
| Site 9 *(Ateles* eating) | 74 | 74 | ✓ | N | N | ✓ | N | N | N | N | ✓ | N | ✓ | N |
| | 75 | 56 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | N | ✓ | ✓ |
| | 76 | 54 | ✓ | N | N | N | N | N | N | ✓ | N | N | N | N |
| | 77 | 69 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Site10 (*Ateles* eating) | 100 | 100 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

## 3.5 Results and Interpretation of Results

### 3.5.1 Munsell© Colour Analysis

Results for the colour determination of the samples in the laboratory at LJMU are listed in Table 3.17. Example Munsell chart can be seen in Appendix 1.4, Figure 1.

Table 3.17 Colour results (Sites 2,9,10 confirmed *Ateles* geophagy sites and Site 8 *Cebus* eating site). ** very pale brown describes material which could be described as cream coloured.

| Site Identity | Sample | Munsell© Colour code | Munsell© Colour description | Site Identity | Sample | Munsell© Colour code | Munsell© Colour description |
|---|---|---|---|---|---|---|---|
| Site 1 | 1 | 2.5YR 7/1 | light reddish grey | | 53 | 5YR 6/4 | light reddish brown |
| | 2 | 5YR 7/2 | pinkish grey | Site 7A | 54 | 5YR 6/4 | light reddish brown |
| | 3 | 5YR 7/2 | pinkish grey | | 55 | 10YR 7/4 | very pale brown |
| | 4 | 5YR 7/2 | pinkish grey | | 56 | 10YR 7/4 | very pale brown |
| | 5 | 5YR 7/2 | pinkish grey | | 57 | 7.5YR 7/4 | Reddish yellow |
| | 6 | N/A | rock fragment | | 58 | 7.5YR 7/4 | Reddish yellow |
| | 7 | 5YR 7/2 | pinkish grey | | 59 | 10YR 7/4 | very pale brown |
| | 8 | 10YR 7/3 | very pale brown** | | 60 | 5YR 5/6 | yellowish red |
| | 9 | 7.5YR 7/1 | light grey | | 61 | 7.5YR 7/4 | pink |
| | 10 | 10YR 7/2 | light grey | Site 7B | 62 | 10YR 7/4 | very pale brown |
| | 11 | 5YR 7/2 | pinkish grey | | 63 | 10YR 7/4 | very pale brown |
| Site 2 | 12 | 5YR 7/1 | light grey | | 64 | 10YR 8/2 | very pale brown |
| | 13 | 7.5YR 7/2 | light grey | | 65 | 10YR 8/2 | very pale brown |
| | 14 | 7.5YR 7/2 | light grey | | 66 | 10YR 8/2 | very pale brown |
| | 15 | 10YR 6/3 | pale brown | | 67 | N/A | crystalline tube |
| | 16 | 10YR 6/3 | pale brown | | 69 | 10YR 8/3 | very pale brown |
| Site 3 | 17 | 5YR 7/1 | light grey | Site 8 | 70 | 10YR 5/3 | brown |
| | 18 | 10YR 7/3 | pale brown | | 71 | 10YR 5/3 | brown |
| Site 4 | 19 | 2.5YR 7/1 | light reddish grey | | 72 | 10YR 5/3 | brown |
| | 20 | 5YR 7/2 | pinkish grey | Site 9 | 74 | 7.5YR 7/2 | pinkish grey |
| | 21 | 5YR 7/2 | pinkish grey | | 75 | 7.5YR 7/1 | light grey |
| | 22 | 5YR 7/1 | light grey | | 76 | 7.5YR 7/2 | pinkish grey |
| | 23 | N/A | rock fragment | | 77 | 7.5YR 7/2 | pinkish grey |
| | 24 | 7.5YR 7/2 | pinkish grey | Site 10 | 100 | 5R 7/1 | light grey |
| | 25 | 5YR 7/2 | pinkish grey | | | | |
| | 26 | 7.5YR 7/1 | light grey | | | | |
| | 27 | 5YR 7/2 | pinkish grey | | | | |
| | 28 | N/A | pumice? | | | | |
| | 29 | N/A | pumice? | | | | |
| | 30 | N/A | rock fragment g. | | | | |
| | 31 | 10YR 7/1 | light grey | | | | |
| | 32 | N/A | rock fragment | | | | |
| Site 6 | 39 | 5YR 7/1 | light grey | | | | |
| | 40 | 5YR 7/1 | light grey | | | | |
| | 41 | 7.5YR 7/1 | light grey | | | | |
| | 42 | 7.5YR 7/1 | light grey | | | | |
| | 44 | N/A | rock fragment | | | | |
| | 45 | 7.5YR 7/1 | light grey | | | | |
| | 46 | 10YR 8/3 | very pale brown | | | | |
| | 47 | 7.5YR 7/1 | light grey | | | | |
| | 48 | N/A | rock fragment | | | | |
| | 49 | N/A | rock fragment | | | | |
| | 50 | N/A | rock fragment | | | | |

Site 2 samples 15-16 and Site 6 samples 39-42 were parts of a single large fragment broken into suitable sized pieces for transportation purposes.

Photographs taken of samples illustrating the range of colours can be seen in Figure 3.17.

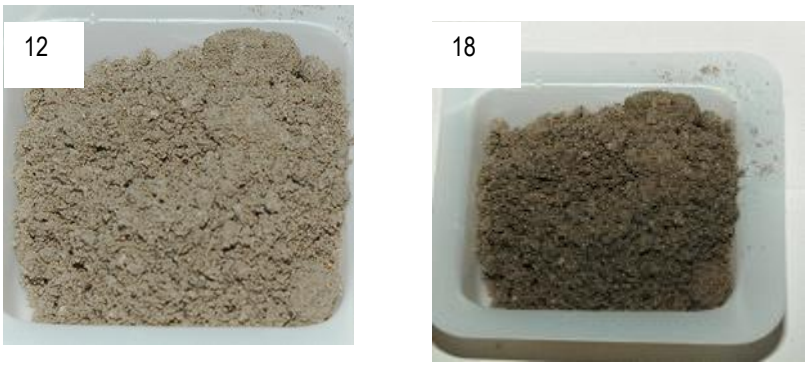



Figure 3.17 Sample 12 Light grey colour - Sample 18 pale brown colour

The samples were predominantly light coloured with hue values ranging from 2.5YR light reddish grey to 10 YR very pale brown. Most samples were in the grey - pinkish grey in ranges. Samples from Site 5 had been discarded as previously stated.

## 3.5.2 % $H_2O$ content at 105ºC

The numbers of determinations of % $H_2O$ content and Loss on Ignition were limited by sample size and the need to conserve material for subsequent analyses. Replicate determinations for each sample were not therefore possible. For the majority of sites less than five determinations were possible. Therefore it was not possible to undertake more extensive post test 1-Way ANOVA analysis comparing site data.

The results in Figure 3.18 are of determinations for sub samples selected from each site. The formula used in the calculation of water content was from Rowell (1994):

% $H_2O$ content = (mass of $H_2O$ lost/ mass of air dry material) x 100

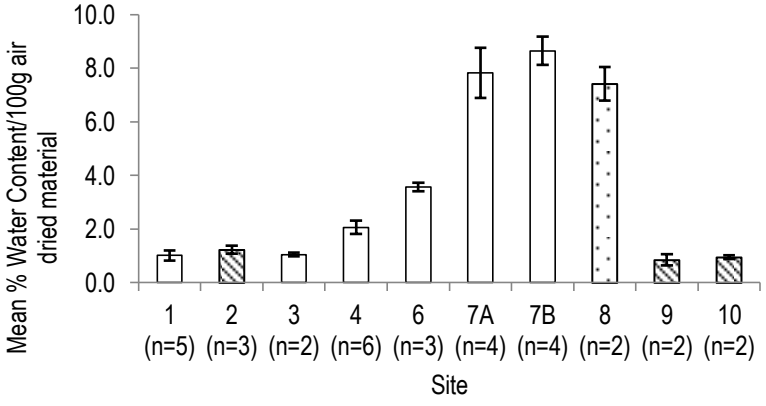



Figure 3.18 Mean % $H_2O$ content, air dried sample material (SEM), highlighting *Ateles* eating Sites 2,9,10.

The samples were dry, dusty and friable. The confirmed *Ateles* eating sites had free water content below 1.36%. Samples from Sites 7A, 7B and 8 were collected after a period of heavy rain, they had however been subjected to the developed drying protocol. One-way Analysis of Variance (ANOVA), was performed

using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com), P = 0.0012, i.e. there was significant variation among the mean free water content of the samples. It is not possible to determine the source of the water which may be due to the characteristics of the constituent minerals or to the presence of organic water.

### 3.5.3 Loss on Ignition determinations, LOI.

The results in Figure 3.19 are of determinations for sub samples selected from each site. The formula used in the calculation of water content was from Rowell (1994):

LOI = 100 x (mass of oven dry material- mass of ignited material)/ (mass of oven dry material)

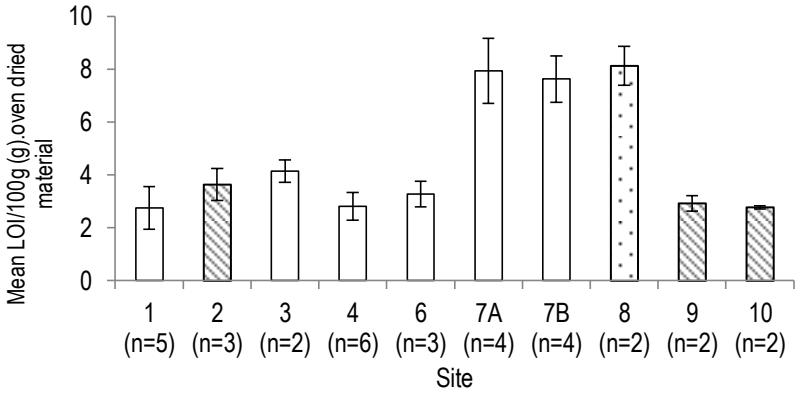



Figure 3.19 Mean % LOI, oven dried sample material (SEM), highlighting *Ateles* eating Sites 2,9,10.

One-way Analysis of Variance (ANOVA), was performed using GraphPad Prism, calculated P <0.001, i.e. there was significant variation among the mean LOI results.

Organic carbon content has been experimentally calculated as approx. half the $LOI_{550}$ (Santisteban *et al.* 2004). The loss of lattice water in clays has also been found to contribute up to 5% of $LOI_{550}$ values (Santisteban *et al.* 2004). The LOI results indicate that there is little organic material present except possibly Sites 7A, 7B and 8.

### 3.5.4 X-Ray Diffraction (XRD) Analysis

The preliminary sample data was exported as an ASCII file and plotted using Excel© (Microsoft Office 2010). A total of 37 samples were analysed (Section 3.4.12, Table 3.16) using a Rigaku Miniflex and from these 15 were selected (following examination of the Rigaku generated data) for more detailed analysis together with peak matching at Rockwood Industries, using a Philips PW1730 XRD instrument utilising DIFFRACTPLUS BASIC 5 (Brukker) software, this utilises the Rietveld method. This method, because of the whole-pattern fitting approach, is capable of much greater accuracy and precision in quantitative analysis than any peak-intensity based method. Peak positions are determined by the crystal structure. The peak intensities reflect the total scattering from the each plane in the phase's crystal structure, and are directly dependent on the distribution of particular atoms in the structure. Intensities are a function of both crystal structure and composition of the phase. Results are commonly presented as peak positions at 2θ and X-ray counts (Intensity) as x-y plot. Intensity (I) is peak height intensity, above background. A diffraction pattern shows peak positions and intensity (cps).

Determination of an Unknown - the d-spacing of each peak is obtained by solution of the Bragg equation for the appropriate value of λ. Once all d-spacings have been determined, automated search/match routines compare the d-spacings of the unknown to those of known materials. Because each mineral has a unique set of d-spacings, matching these d-spacings provides an identification of the unknown sample e.g. Table 3.18.

Table 3.18 Identification peaks degree 2θ, kaolinite mineral group; and relative intensities ($I/I_0$) from http://webmineral.com (accessed Aug 2012).

| **Identification peaks from The International Centre for Diffraction Data® (ICDD®)** | | | |
|---|---|---|---|
| | **kaolinite** | **dickite** | **halloysite 7Å** |
| principal XRD peaks degree 2θ; ($I/I_0$) | | | 11.79 (09.) |
| | 12.34 (1) | | |
| | | 12.42 (1) | |
| | 20.01 (0.6) | | 20.08 (1) |
| | 21.2 (0.45) | | |
| | 24.87 (0.8) | 24.87 (1) | 24.51 (0.9) |
| | | | 35.05 (0.8) |
| | | | 54.62 (0.8) |
| | 56.73 (0.7) | | |
| | 58.09 (0.9) | | |

A systematic procedure is used by ordering the d-spacings in terms of their intensity beginning with the most intense peak. The relative intensity is recorded as the ratio of the peak intensity from pure sample to that of the most intense observed peak (relative intensity = $I/I_0$) and is compared to values from databases e.g. The International Centre for Diffraction Data® (ICDD®) to identify matches.

Limitations which may influence the diffraction patterns obtained are: nonhomogeneous sample, degree of crystallinity, the randomness of crystal orientation, (clay minerals adopt the preferred orientation), surface adsorption which may reduce x-ray reflection and proportion of each mineral present.

### 3.5.4.1 Rigaku XRD diffractograms of CRM

XRD of both CRM (Figures 3.20-3.21) were obtained in order to provide examples to compare the diffractograms of site samples. Diagnostic peak values were identified from The International Centre for Diffraction Data® (ICDD®) database (Tables 3.18-3.19).

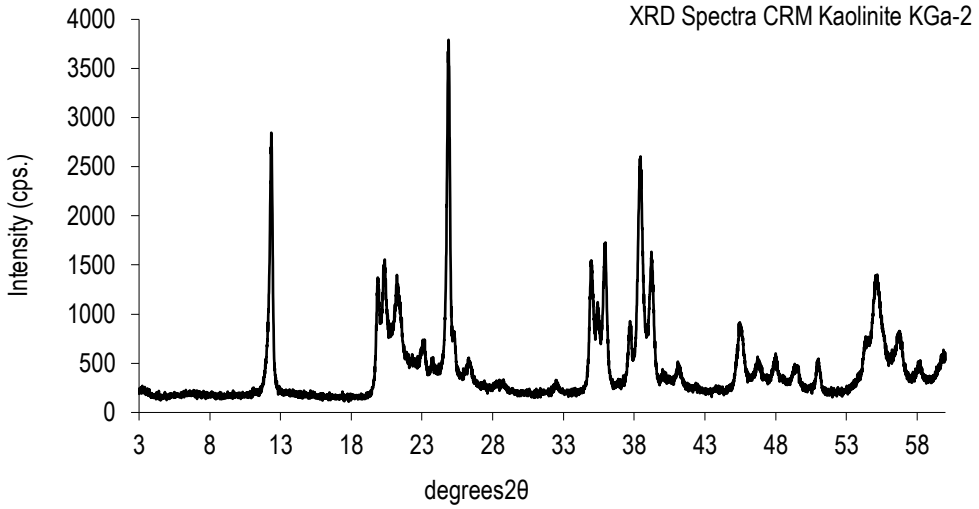



Figure 3.20 Rigaku diffractogram of kaolinite CRM, KGa-2

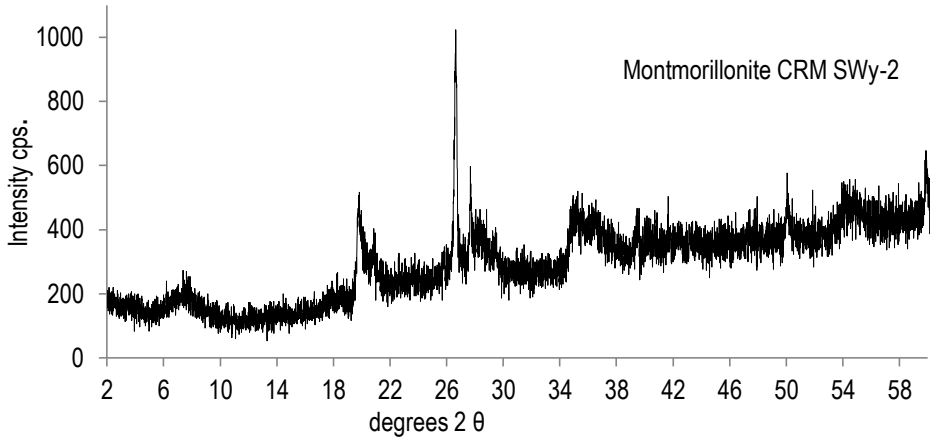



Figure 3.21 Rigaku diffractogram of montmorillonite CRM, SWy-2

Table 3.19 Identification peak degree 2θ, montmorillonite minerals; and relative intensities ($I/I_0$) from http://webmineral.com (accessed Aug 2012).

| **Minerals matched from The International Centre for Diffraction Data (ICDD®)** | |
|---|---|
| | **montmorillonite** |
| principal XRD peaks degree 2θ; ($I/I_0$) | 5.89 (1) |
| | 17.7 (0.6) |
| | 19.72 (0.8) |
| | 29.57 (0.6) |

XRD analysis of the SWy-2 CRM Chipera *et al.* (2001) reported the following content 75% montmorillonite, 8% quartz, 16% feldspar and traces of gypsum, mica/or illite, kaolinite and chlorite.

### 3.5.4.2 Rigaku XRD representative diffractograms of each site.

Excel plots for all the samples from each site were overlaid and the following examples selected as they included the representative features present in the individual plots (Figures 3.22a-b).

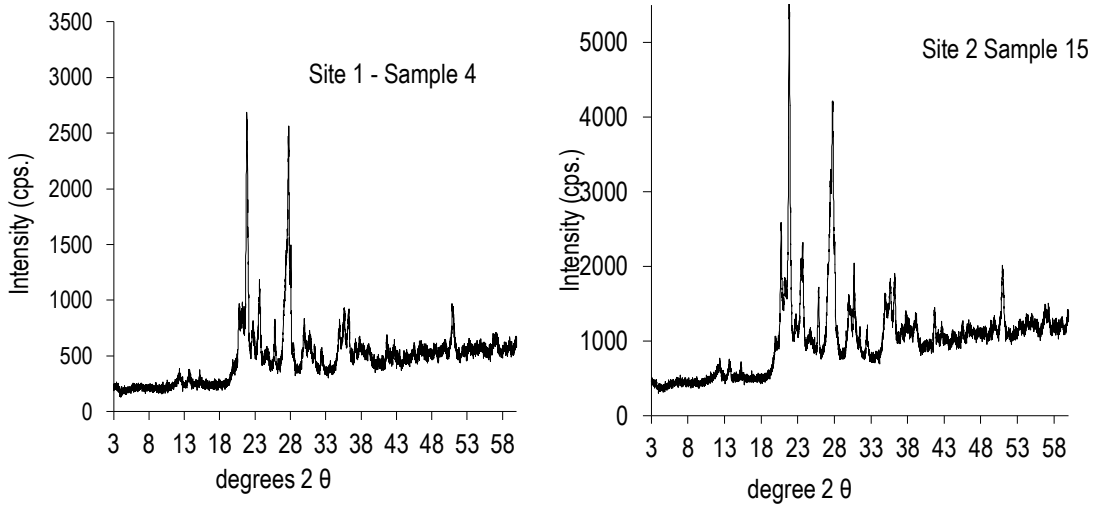



Figure 3.22(a) Representative diffractograms Site 1, Sample 4 and Site 2, Sample 15

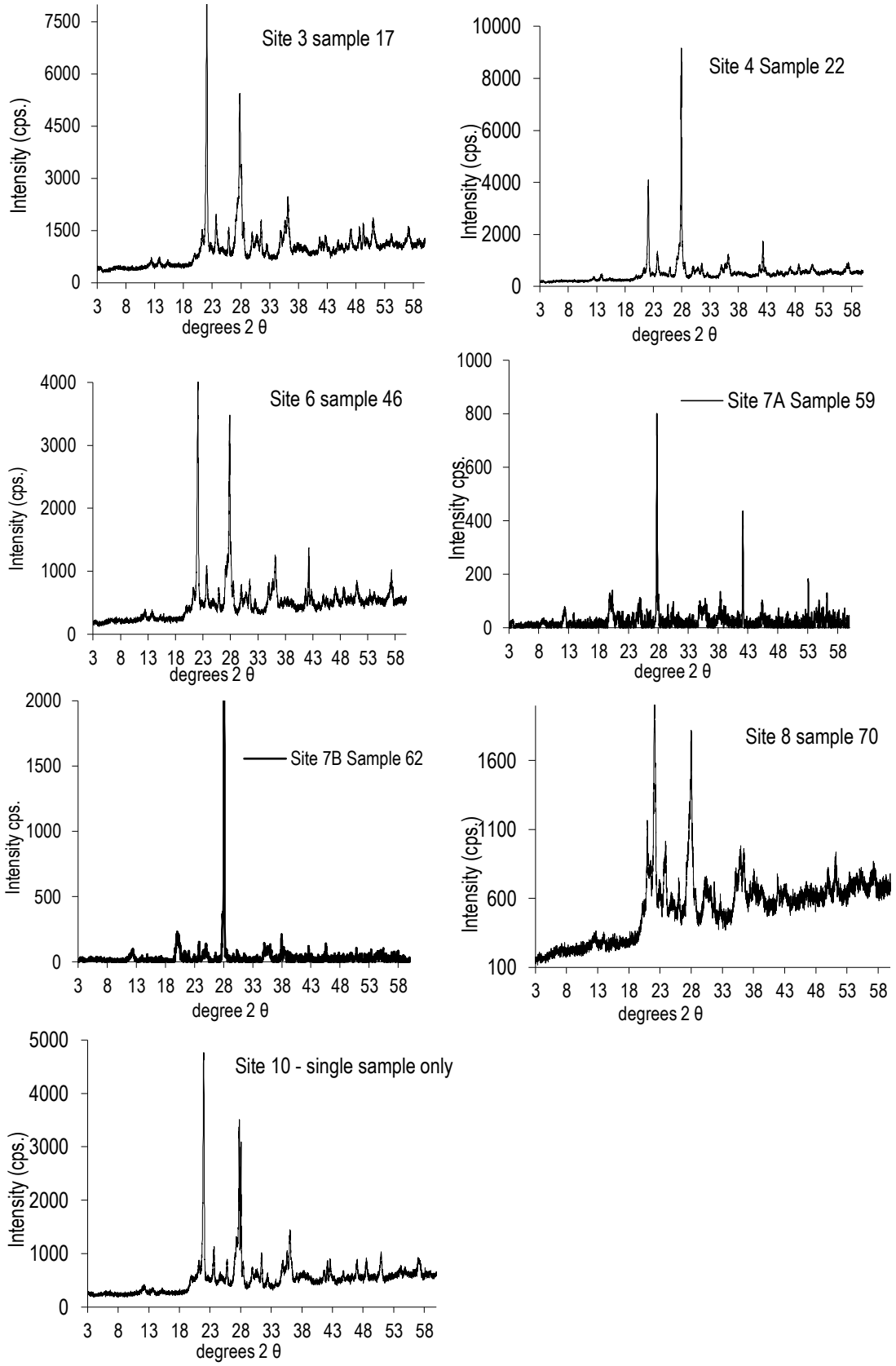



Figure 3.22(b) Representative diffractograms Sites 3, 4, 6,7A, 7B, 8 and 10.

The red colour of samples from Site 7A (Section 3.3.2, Figure 3.8) suggested there may be iron present. Figure 3.23 contains diagnostic peaks of two potential iron minerals (Table 3.20). However the peak at 35.5 degree 2θ may have a contribution from halloysite.

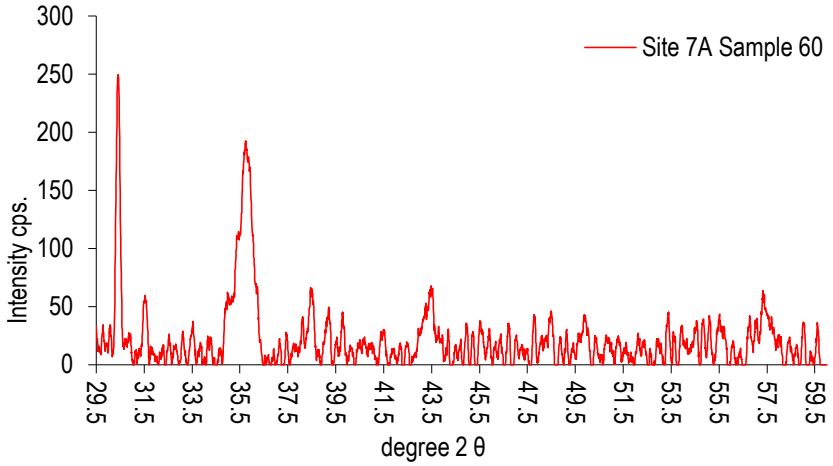



Figure 3.23 Site 7A, Sample 60, illustrating peaks related to the iron oxides maghemite and magnetite

Table 3.20 Principal diffraction peaks for iron oxide minerals, maghemite and magnetite

| **Minerals matched from The International Centre for Diffraction Data® (ICDD®)** | | |
|---|---|---|
| | **maghemite** | **magnetite** |
| principal XRD peaks degree 2θ; ($I/I_0$) | 30.29 (1) | |
| | 35.59 (1) | 35.58 (1) |
| | 57.6 (1) | 57.22 (0.85) |

Figure 3.24 highlights the similarities between the *Ateles* sites and Table 3.21 lists the similarities between the sites.

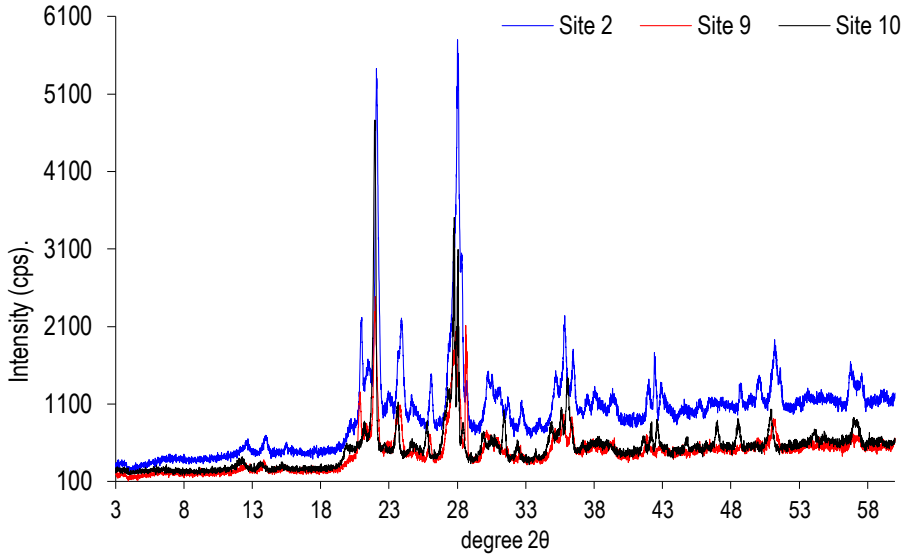



Figure 3.24 Rigaku XRD comparing *Ateles* eating sites 2,9,10.

The results Table 3.21 suggest that there is a relatively high level of homogeneity across the control sites and the *Ateles* and *Cebus* sites. The samples from sites 7A and 7B have fewer features in common with the other test sites. A Chi-sq Test was performed on the similarities of the peaks detected in the XRD data (Table 3.21). There was no significant difference between the sites Chi-Sq Statistic 7.453, df 24 p=0.9995.

Table 3.21 Examples of peaks common to multiple sites, identified from Rigaku data.

| | Control | | | | | | *Ateles* | | | *Cebus* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Approx. degree 2 θ** | **1** | **3** | **4** | **6** | **7A** | **7B** | **2** | **9** | **10** | ***8*** |
| 3.42 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 12.55 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 13.89 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 15.40 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 20.72 | | | | | ✓ | ✓ | ✓ | ✓ | | |
| 21.97 | ✓ | ✓ | | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 22.14 | | ✓ | ✓ | ✓ | ✓ | | | | | ✓ |
| 23.76 | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 25.96 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 27.85 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 28.53 | | | | ✓ | ✓ | | | | ✓ | |
| 30.00 | | ✓ | ✓ | ✓ | ✓ | | | ✓ | | ✓ |
| 30.89 | | ✓ | ✓ | ✓ | | ✓ | | ✓ | | ✓ |
| 31.64 | | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ |
| 35.02 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 35.72 | ✓ | ✓ | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ | ✓ |
| 36.22 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 41.70 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | |
| 42.44 | ✓ | ✓ | ✓ | ✓ | | | ✓ | | ✓ | ✓ |
| 42.92 | ✓ | ✓ | ✓ | ✓ | | | ✓ | ✓ | ✓ | ✓ |
| 47.09 | ✓ | ✓ | ✓ | ✓ | | | ✓ | | ✓ | ✓ |
| 48.65 | | ✓ | ✓ | ✓ | | ✓ | | | ✓ | |
| 50.00 | | | | | | ✓ | ✓ | ✓ | | ✓ |
| 54.20 | | ✓ | ✓ | ✓ | | | | | ✓ | ✓ |
| 57.16 | | ✓ | ✓ | ✓ | | | | | ✓ | ✓ |

### 3.5.4.3 Comparison of *Ateles* eating sites and CRM Kaolinite KGa-2.

Figures 3.25(a-b) are of overlaid diffractograms of the kaolinite CRM (red) and examples from the *Ateles* eating sites. The absence of the diagnostic peaks 12.33 and 24.86 (degree 2θ) is clearly illustrated.

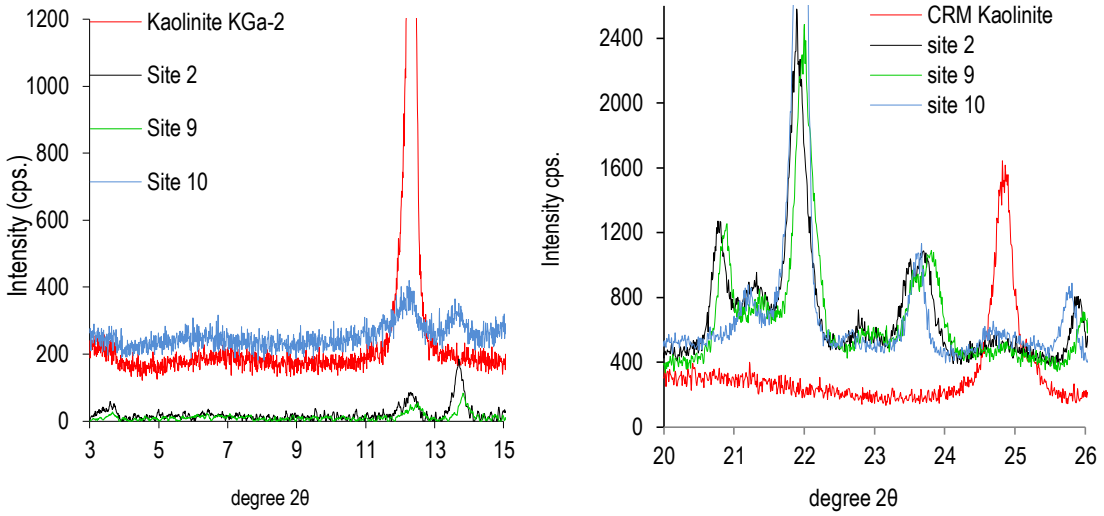



Figure 3.25(a) Comparison of the section of diffractogram showing major differences between *Ateles* eating site samples and CRM kaolinite KGa-2

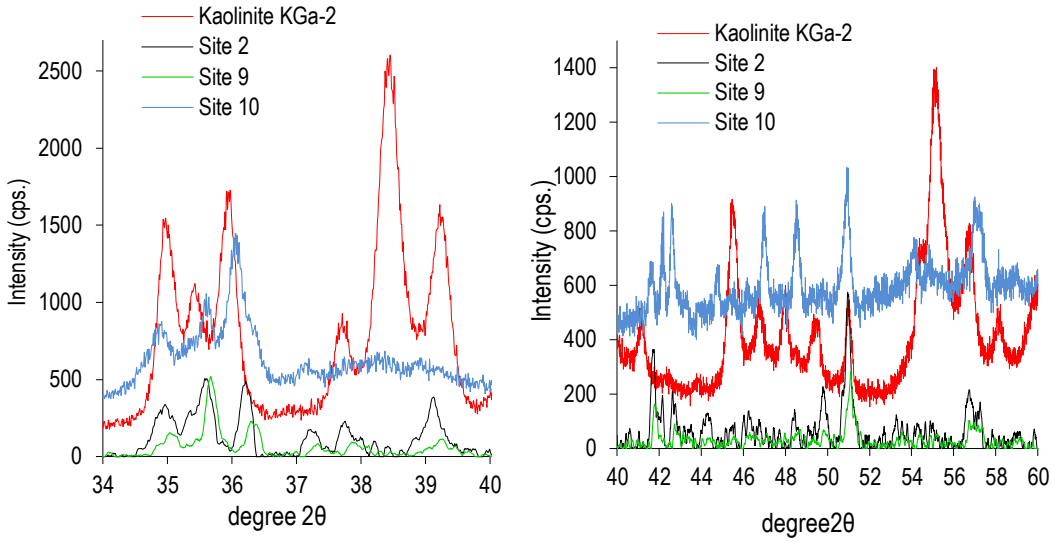



Figure 3.25(b) Comparison of the section of diffractogram showing major differences between *Ateles* eating site samples and CRM kaolinite KGa-2

The overlaid diffractograms (Figures 3.25a-b) suggest that it is unlikely that there are any significant amounts of kaolinite minerals present in the tested *Ateles* eaten samples. The peak at 35.5 (degree 2θ) may indicate the presence of the kaolinite mineral halloysite 7Å.

For mixed materials the detection limit for a component is ≥ 2% of sample (Chipera *et al*. 2001). In a mixed matrix many possible orientations are present. This causes peak overlapping which influences intensity measurements. Additionally variations in peak position may be caused by the presence of impurities and presence of polymorphs.

### 3.5.4.4 Results of Stick pattern Matched Diffractograms obtained at Rockwood Industries.

Limitations on matching include peak match data which is based on patterns produced by pure samples, with uniform crystallinity and particle size. Additional variables are due to different instruments used and the selection of Kv, and mA, scan rate and count rates (Chipera *et al.* 2001).

An example of the diffractograms generated at Rockwood Industries, identifying the presence of the mineral cristabolite can be seen in Figure 3.26. The software has suggested this mineral based on the position and relative intensities of 28 peaks. The minerals identified and the sites in which they are identified are presented in Table 3.22.

Using this multiple peak matching facility the presence of a kaolinite mineral was indicated for all the *Ateles* eating sites but there was no match for montmorillonite. The remaining minerals detected belonged to the feldspar, silicon oxide and titanium dioxide mineral groups. Montmorillonite was identified in Control sites 1 and 6 and *Cebus* Site 8 but not in any *Ateles* eating sites.

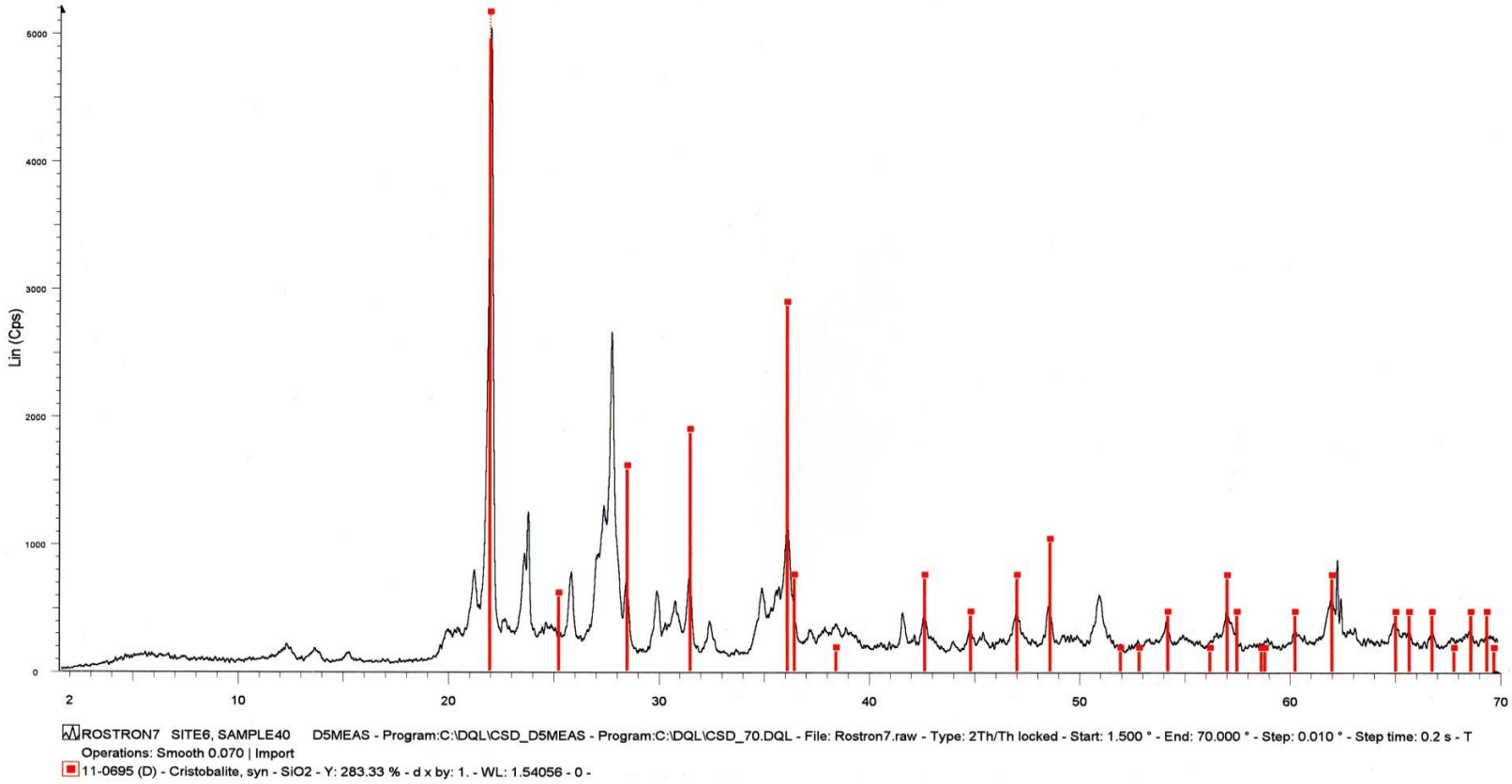



Figure 3.26 Example of Peak Matched report from Diffractplus Software; Cristabolite detected in Site 6 Sample 4

Table 3.22 List of minerals identified produced by Diffractplus software.

| **Potassium feldspar group** | **Site** |
|---|---|
| Orthoclase, barian | 1, 7A,7B |
| Orthoclase | 1,2, |
| Sanidine, potassian, disordered and Sanidine | 1, 2, 6, 8, 9,10 |
| Microcline, intermediate | 1, 2, |
| **Plagioclase feldspar minerals** | |
| Anorthoclase disordered | 1, 2, 6, 8, 9, 10 |
| Albite, disordered | 1, 2, 7A, 7B |
| Anorthite, sodian intermediate | 1, 7A, 7B, |
| **$SiO_2$ minerals** | |
| Cristabolite<br>High silica Cristabolite | 1, 2, 6, 7A,<br>1, 2, 7A, 7B, 9.10 |
| Tridymite | 1, 2, 9, |
| Quartz | 7B |
| **Al Oxide minerals** | |
| Gibbsite | 1,2,6,7A, 7B 8,10 |
| **Fe oxide minerals** | |
| Maghemite | 1, 2, 7A, |
| Magnetite | 2, 7A, 7B, 8, 9, 10 |
| **$TiO_2$ minerals** | |
| Rutile | 1, 8, 9, 10 |
| **Amorphous, paracrystalline** | |
| Halloysite | 1, 2, 6, 7A, 7B, |
| Opal | 2, 8, 9, 10 |
| **Clay minerals** | |
| Kaolinite 1Md | 1, 2, 6, 7A, 7B, 8, 9, 10 |
| Montmorillonite | 1, 6 |

In summary:

The XRD analyses from both instruments suggest the presence of a kaolinite mineral, however the intensities, all ~ 200 cps, recorded for the 12.34 degree 2θ diagnostic peak (Figure 3.35) are close to the baseline cps. If there was a significant proportion of kaolinite present the expected count valued for this diagnostic peak would be expected to have a higher intensity. This result suggests that if present kaolinite mineral there is only at a low percentage. The results also suggest that there is very little montmorillonite limited to possible identification in only two sites.

## 3.5.5 Infra Red Analysis

The crystalline structure of montmorillonites is reduced by the substitution of Al for Si. This leads to a substantial broadening of the IR spectral bands (Russell 1987). This broadening can be seen in Figure 3.27, the spectrum for the montmorillonite CRM, which lacks definition in the ~3700-3600 $cm^{-1}$. In this region band broadening can be due to overlapping bands or hydrogen bonding.

Comparative analysis of this region of the montmorillonite CRM with control sites (Figure 3.28a) and *Ateles* eating sites (Figure 3.28b) shows there is no similarity between the sites and a montmorillonite in this significant region, ~3700-3600 $cm^{-1}$.

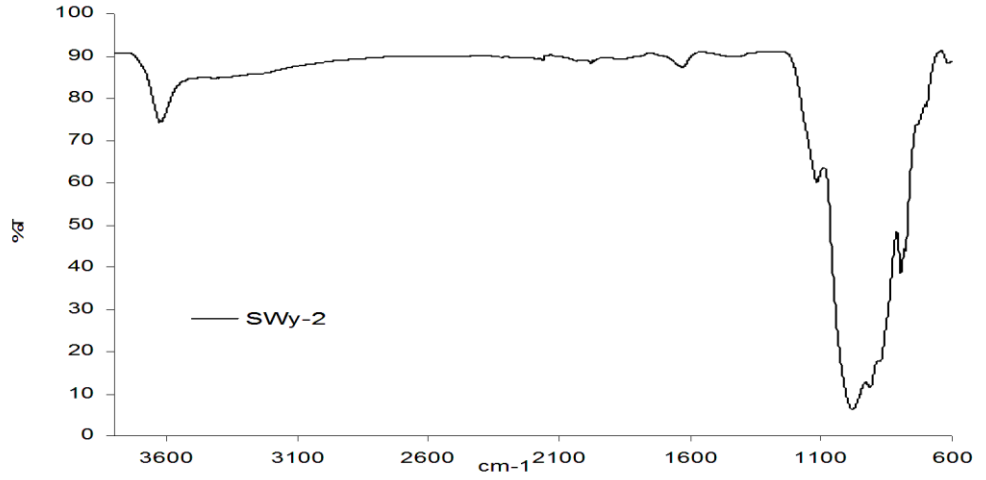



Figure 3.27 IR ATR spectrum for CRM, SWy-2.

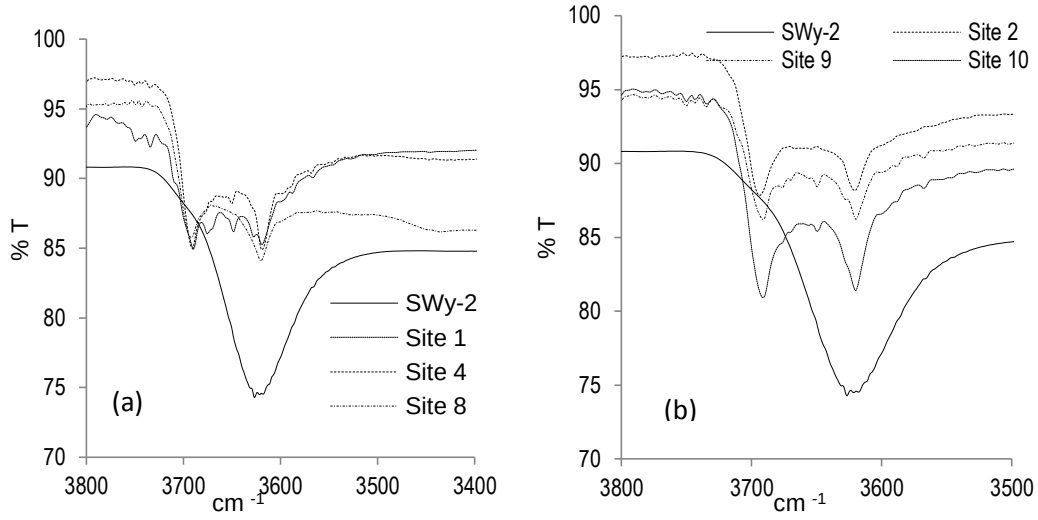



Figure 3.28 (a) Control Sites 1, 4, and *Cebus* eating Site 8, comparison with CRM, SWy-2;
(b) *Ateles* eating Sites 2,9,10, comparison with CRM, SWy-2

In contrast the spectrum for a kaolinite KGa-2, (Figure 3.29), has two major regions with sharply defined and high intensity absorbing regions ~3700-3600 $cm^{-1}$ and 1150-600 $cm^{-1}$. Characteristic diagnostic bands for kaolinite are found at 3620-3618$cm^{-1}$ (KGa-2) with a weaker band at 3651-3650$cm^{-1}$. The sharpness of the kaolinite 3620-3618$cm^{-1}$ band is an indicator of the purity (Madejova *et al.* 2001).

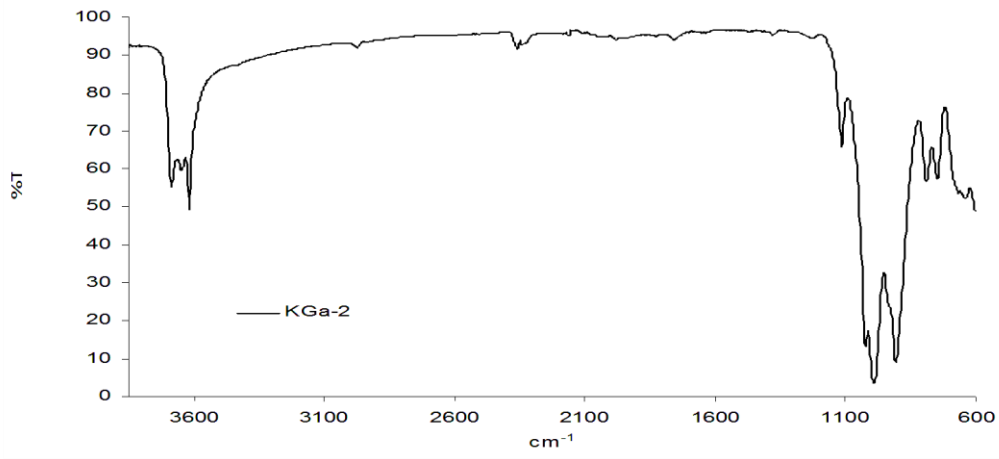



Figure 3.29 IR ATR spectrum for CRM, KGa-2

In dioctahedral minerals e.g. kaolinites, the band at approx. 3620cm$^{-1}$ has a greater intensity due to the increased substitution of Si by Al. Comparisons of these relative intensities can be seen in the spectra of the reference clay and control sites.

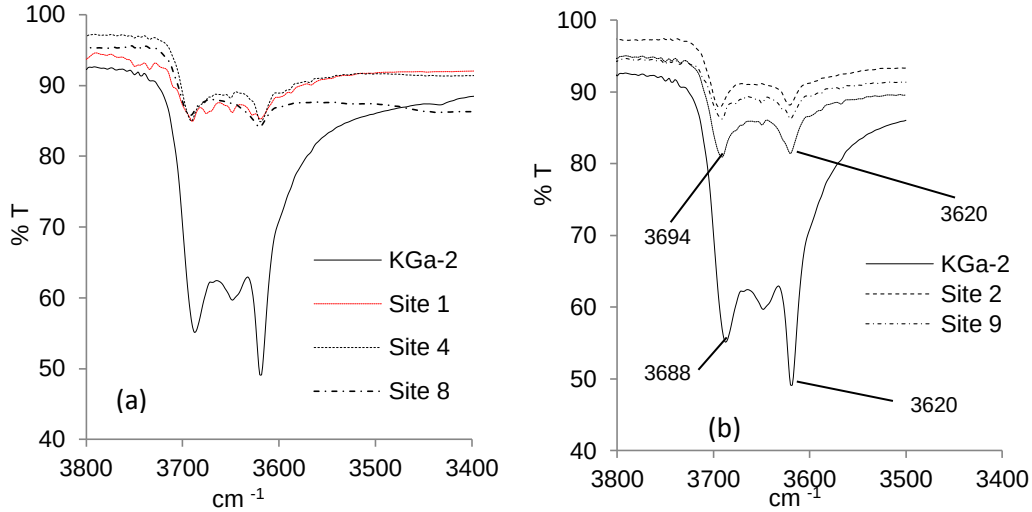



Figure 3.30 (a) Control Sites 1, 4 and *Cebus* eating Site 8; (b) *Ateles* eating Sites 2,9,10 comparisons with CRM, KGa-2.

In the Control site spectra (Figure 3.30a) and *Ateles* eating site spectra (Figure 3.30b), the two major bands at 3690cm$^{-1}$ and 3620cm$^{-1}$, are weak and similar in relative intensities, differing from the kaolinite CRM.

Semi-quantitative estimates of kaolinites have used the intensity of the feature at 3700-3690cm$^{-1}$ (Russell 1987, Kodama *et al.* 1989). The value of %T was determined at a fixed maximum at 3718cm$^{-1}$ and the band identification value at 3690cm$^{-1}$. The ratio of these values for the reference clays and samples were determined (Table 3.23). This was used as an approximation for the proportion of kaolinites present.

Table 3.23 Estimate kaolinite content - using the Ratio of % Transmission values.

| Sample/ Site No. | % T at 3690cm$^{-1}$ | % T at 3718cm$^{-1}$ | (% T 3690/ %T3718) |
|---|---|---|---|
| KGa-2 | 56.16 | 92.96 | 0.60 |
| 1 | 89.86 | 95.93 | 0.94 |
| 2 | 88.50 | 95.35 | 0.93 |
| 3 | 88.34 | 95.24 | 0.93 |
| 4 | 88.08 | 96.35 | 0.91 |
| 6 | 77.79 | 93.67 | 0.83 |
| 7A | 72.63 | 92.84 | 0.78 |
| 7B | 84.06 | 95.08 | 0.88 |
| 8 | 85.85 | 91.97 | 0.93 |
| 9 | 86.29 | 93.55 | 0.92 |
| 10 | 83.01 | 93.06 | 0.89 |

The calculated transmission ratio for the kaolinite CRM reference clay is 0.6. The higher the ratio of the %T values the lower the kaolin mineral content. The site values calculated suggest that there may be varying proportions of kaolinite present in the sites, the lowest and nearest to the kaolinite, being sites 6 and 7A with Transmission ratios of 0.83 and 0.78. The sites including the confirmed *Ateles* and *Cebus* eating sites have ratio values >0.89. These ratios cannot be converted directly to % kaolin values but are suggestive of the proportion of kaolinite which may be present (Kodama *et al.* 1989).

## Estimation of Disordered content

Disorder is a term applied to amorphous states of clay minerals and many stages of partial order and disorder occur. Disorder of various kinds can be classified under the following headings:

(i) disorder at the atomic level

(ii) disorder in layer stacking, and

(iii) disorder associated with mixed layering (Plancon 2001).

The low levels of minerals detected by the XRD may be as a result of the degree of disorder. Disorder in kaolinites is detected in the four characteristic bands of the OH stretching region.

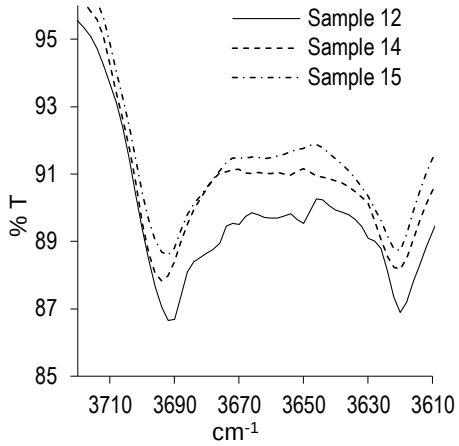



Figure 3.31 Samples from Site 2, showing some disorder

In Figure 3.31 the 3689-3691cm$^{-1}$ and 3620cm$^{-1}$ bands are unchanged, however a single broad band at 3653-3650cm$^{-1}$ replaces the doublet normally present at 3669cm$^{-1}$ and 3652 cm$^{-1}$ (Russell 1987).

Structural disorder may be produced either by geological conditions of formation, transport, or deposition, or by mechanical treatment such as grinding (Brindley *et al.* 1986). This may have effects on the properties of kaolinite minerals from different sites and so alter the spectra obtained. Kaolinites in soils are often disordered and where this occurs it makes quantification problematic (Galán 2006).

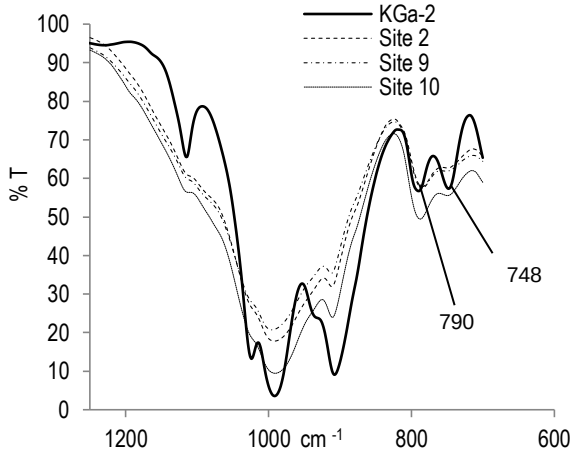



Figure 3.32 KGa-2 and *Ateles* eating sites ATR spectra 1160-700cm$^{-1}$

Spectra of Sites 2 and 9 (Figure 3.32) are representative of all the *Ateles* eating sites. They only show weak features in the range 1116 -1110cm$^{-1}$ similar to those characteristic features seen in the kaolinite CRM.

Dioctahedral minerals exhibit a diagnostic band of the AlAlOH deformation in the 937-928cm$^{-1}$ region (Russell 1987). This cannot be seen as a distinct feature in the site samples, but it may be contributing to a broad strong band between1030-910cm$^{-1}$, which is seen in all the ATR spectra for the sample sites. The lack of characteristic features again suggests very little kaolinite minerals are present or it is present in the disordered form.

The feldspar mineral IR spectra have strong bands between 800cm$^{-1}$ and 700cm$^{-1}$. The spectra for the reference clay and eaten sites (Figure 3.32) have a distinctive band at 788/4cm$^{-1}$, a feldspar characteristic. This may be due to residual unweathered feldspar in the CRM, as feldspars may weather to produce kaolinitic clays (Wada 1987, Wilson 2004). Quartz is a common constituent of most clay minerals, having sharp well defined characteristic bands at 800 and 781cm$^{-1}$. These bands are not seen in the material from any of the sites. This suggests the samples have a large degree of amorphous material.

### 3.5.5.1 IR Determination of organic content and structural water.

The IR spectra were examined for absorption bands associated with biological molecules (Table 3.26) as consumption of soil for antimicrobial products derived from microbial organisms has been suggested as a possible geophagy function (Ketch *et al.* 2001)

Table 3.24 Characteristic Infra Red absorption bands of organic groups (Madejova *et al.* 2001, Madejova 2003, Skoog *et al.* 2006).

| **Functional groups** | | **Absorption bands cm$^{-1}$** | **Functional groups** | | **Absorption bands cm$^{-1}$** |
|---|---|---|---|---|---|
| O-H | aliphatic and aromatic | 3600-3000 | CONH2 | amides | 1720-1640 |
| H-O-H | vibrations of water | 3430-3395 | C=C- | alkene | 1670-1610 |
| $NH_2$ | also secondary and tertiary | 3600-3100 | O-H | water | 1638-1627 |
| COOR | ester | 1750-1700 | Ø-O-R | aromatic | 1300-1180 |
| COOH | carboxylic acid | 1740-1670 | R-O-R | aliphatic | 1160-1060 |
| C=O | aldehydes and ketones | 1740-1660 | | | |

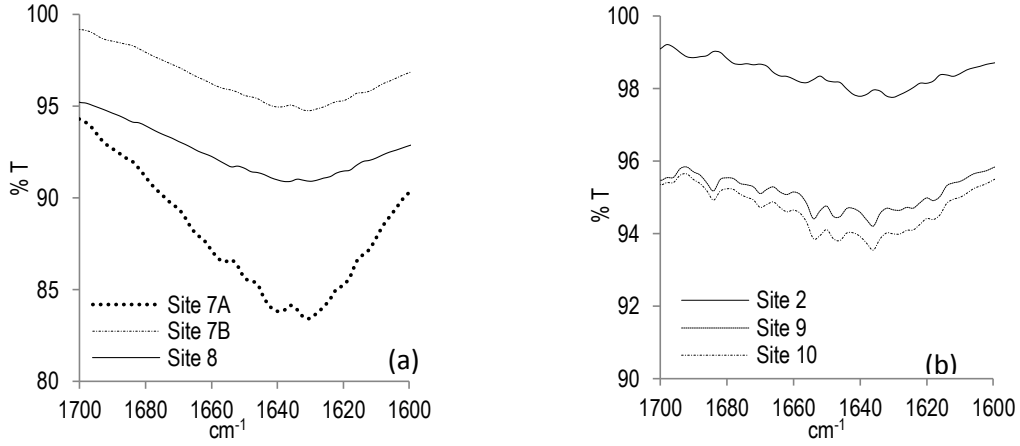



Figure 3.33 (a) Site 7A, 7B and Site 8 organic or structural water content; (b) Sites 2, 9 and 10.

The samples were dried at 105$^0$C overnight and allowed to cool in a desiccator, to remove unbound atmospheric water. Material of biological origin will have absorption bands due to amide, carboxylic acid and aromatic groups (Table 3.24).

The spectra for Sites 7A (Figure 3.33a) has a broad band from 1740-1518cm$^{-1}$ whereas 7B and 8 and spectra for Sites 2, 9 and 10 (Figure 3.33b) only shows a weak feature. There is little variation % T, over the

region 1700 -1600cm$^{-1}$. This is the region where both adsorbed water and amides would have adsorption. The spectra for all the sites show a weak broad band with a maximum % T between 1650 and 1620cm$^{-1}$. A broad peak centred on 1636cm$^{-1}$ is associated with OH deformation of $H_2O$. Peaks at 1648cm$^{-1}$ may be associated with C=O, C=C and $CONH_2$, organic groups.

Site 7A has features potentially due to organic functional groups or structural water. LOI results suggested the presence of organic carbon compounds in sites 7A, 7B and 8. Structural water may be associated with the presence of the paracrystalline short-range ordered mineraloid opal or hydrated kaolinitic mineral, halloysite. Both were identified by the Rockwood XRD stick matching analysis (Table 3.22). The halloysite form is produced from weathering of igneous rocks (Galán 2006).

In summary:

- IR spectra suggest the presence of small and variable amounts of kaolinites
- presence of feldspar and silicate minerals
- there is little evidence of the presence of montmorillonites.
- limited evidence of detectable amounts of organic material and very low structural water content.

## 3.5.6. XRF Analysis performed at Rockwood Industries

The results for analysis of the CRM standards (Table 3.25) show there is a high level of agreement between the measured and the published values, which permits confidence in the values obtained for the site samples. The differences between measured and reported was <3.5%, and may be due to the limited number of replicates. The reported values are quoted as oxides.

Table 3.25 XRF comparison of measured mean oxide results CRM, Kaolinite KGa-2 and SWy-2, Reference data* from Mermut *et al.* (2001).

| | KGa-2 Measured | KGa-2 Reference* | SWy-2 Measured | SWy-2 Reference* |
|---|---|---|---|---|
| $Na_2O$ (%) | 0.05 | 0.06 | 1.57 | 1.47 |
| MgO (%) | 0.06 | 0.04 | 2.99 | 2.94 |
| $K_2O$ (%) | 0.02 | 0.02 | 0.2 | 0.2 |
| CaO (%) | 0.04 | 0.03 | 1.96 | 1.89 |
| $TiO_2$ (%) | 1.98 | 1.91 | 0.11 | 0.09 |
| $Fe_2O_3$ (%) | 1.19 | 1.15 | 3.77 | 3.74 |
| $SiO_2$ (%) | 44.49 | 43.49 | 63.46 | 61.46 |
| $Al_2O_3$ (%) | 40.14 | 38.14 | 21.05 | 22.05 |
| SUM (%) | 87.97 | 84.84 | 95.11 | 93.84 |

Using the TAS (Total-Alkali-Silica) classification system (Figure 3.34) and the calculated values obtained from the XRF data (Table 3.26) the (%$Na_2O$+$K_2O$): The %$SiO_2$ ratio suggests the samples are close to the acidic, andesite-dacite composition junction (Figure 3.34). The mineral composition of dacite includes the plagioclase feldspars, oligoclase, andesine, quartz, silica and tridymite. Dacites are often grey in colour with dark crystal inclusions. This classification for the samples also agrees with the analyses by (Vogel *et al.* 2004), who describes the Guanacaste ignimbrite plateau as containing extensive rhyolitic and dacitic ignimbrite flows and lava flows, with small volume of andesitic pyroclastic flows.

Table 3.26 Rockwood Analysis XRF Mean % oxide results samples from Sites 1, 2, 6, 7A, 7B, 8, 9 and 10

| | **Site 1** | | | **Site 2** | | | **Site 6** | | **Site 7A** | | **Site 7B** | | **Site 8** | | **Site 10** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wt. % | **1** | **9** | **11** | **13** | **14** | **15** | **40** | **46** | **56** | **60** | **62** | **70** | **75** | **77** | **100** |
| $Na_2O$ | 3.50 | 3.57 | 3.38 | 3.25 | 2.95 | 3.69 | 3.21 | 3.27 | 2.56 | 2.06 | 2.39 | 2.49 | 3.26 | 3.36 | 2.54 |
| MgO | 0.27 | 0.27 | 0.32 | 0.2 | 0.2 | 0.17 | 0.31 | 0.25 | 0.43 | 0.43 | 0.52 | 0.63 | 0.25 | 0.25 | 0.21 |
| $K_2O$ | 3.05 | 2.96 | 3.08 | 2.95 | 2.84 | 3.23 | 2.99 | 3.29 | 2.51 | 2.1 | 0.58 | 2.36 | 3.14 | 3.09 | 3.08 |
| CaO | 1.52 | 1.63 | 1.52 | 1.33 | 1.41 | 1.27 | 1.64 | 1.32 | 1.37 | 1.23 | 2.63 | 1.67 | 1.24 | 1.33 | 1.16 |
| $TiO_2$ | 0.67 | 0.65 | 0.65 | 0.66 | 0.66 | 0.59 | 0.68 | 0.62 | 0.67 | 1.26 | 0.84 | 0.7 | 0.63 | 0.64 | 0.7 |
| $Fe_2O_3$ | 4.16 | 3.86 | 4.18 | 4.07 | 4.08 | 3.63 | 4.42 | 3.62 | 3.86 | 11.96 | 6.38 | 4.94 | 3.9 | 3.9 | 4.23 |
| $Al_2O_3$ | 13.76 | 16.92 | 17.62 | 18.2 | 18.7 | 16.97 | 18.42 | 17.1 | 19.63 | 17.96 | 25.74 | 18.03 | 17.6 | 17.49 | 18.47 |
| $SiO_2$ | 65.47 | 64.64 | 65.88 | 64.99 | 64.09 | 66.82 | 63.71 | 65.86 | 60.84 | 55.13 | 50.83 | 59.5 | 65.26 | 65.49 | 63.89 |
| SUM | 92.4 | 94.5 | 96.63 | 95.65 | 94.93 | 96.37 | 95.38 | 95.33 | 91.87 | 92.13 | 89.91 | 90.32 | 95.28 | 95.55 | 94.28 |
| $Na_2O+K_2O$ | 6.55 | 6.53 | 6.46 | 6.2 | 5.79 | 6.92 | 6.2 | 6.56 | 5.07 | 4.16 | 2.97 | 4.85 | 6.4 | 6.45 | 5.62 |

The data for the site samples Table 3.26 show a characteristic, mean $SiO_2$ 62.83% and mean %($Na_2O+K_2O$) 5.78.

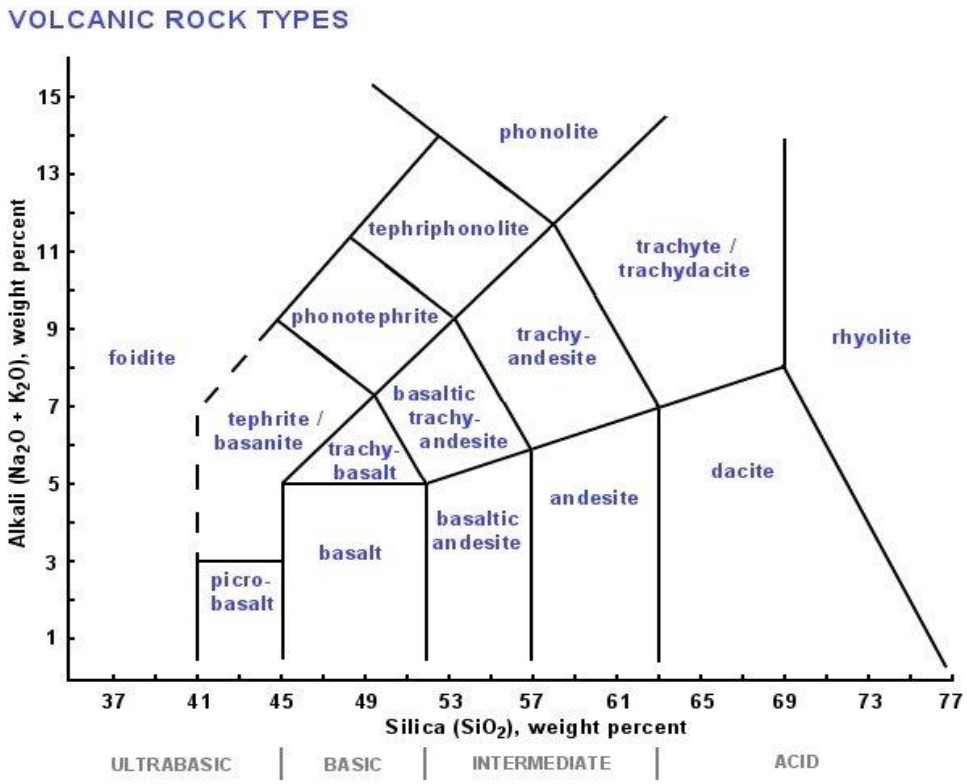



Figure 3.34 TAS (Total-Alkali-Silica) Classification of Volcanic rocks, from Streckeisen *et al* (2002).

Figure 3.35 illustrates the small difference in aluminium content of the *Cebus* (Site 9) and *Ateles* eating sites (Sites 2, 9, 10). There were insufficient data points to permit statistical analysis of the aluminium values.

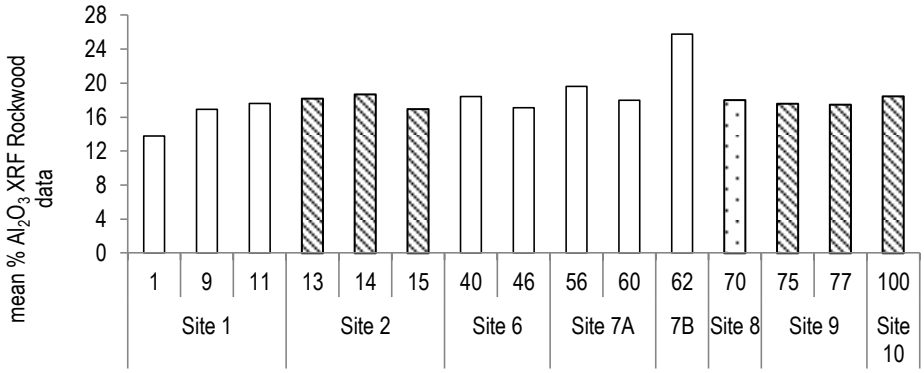



Figure 3.35 XRF mean aluminium content, calculated as oxide

## 3.5.7 Determination of sample pH

The ASTM definition for values of pH classifies the samples as predominantly acidic in character (Natural Resources Conservation Service 2004). Soil pH affects the solubility and hence availability of minerals or nutrients and mineral leaching. Most minerals and nutrients are more soluble or available in acid soils than in neutral or slightly alkaline soils.

The comparison of KCl pH with $H_2O$ pH i.e. the Δ pH provides an assessment of the nature of the net charge on the colloidal system. Aluminium, displaced by $K^+$, reacts with $OH^-$ ions and increases $H^+$ concentration. As a result, the solution pH is lowered. Commonly, exchangeable aluminium is present if the KCl pH is 5.2 or less (Natural Resources Conservation Service 2004).

Table 3.27 Results for pH determinations and value of = Δ pH

| Site | Sample | pH in deionised $H_2O$ | pH in 0.1M KCl solution | Δ pH = pH(KCl) – pH ($H_2O$) | ASTM* definition acidity pH in deionised $H_2O$ |
|---|---|---|---|---|---|
| 1 | 1 | 6.32 | 5.02 | -1.3 | slightly acid |
| 2 | 15 | 6.24 | 4.44 | -1.8 | slightly acid |
| 4 | 26 | 6.65 | 4.91 | -1.74 | slightly acid |
| 6 | 41 | 6.34 | 5.59 | -0.75 | slightly acid |
| 6 | 46 | 7.35 | 6.31 | -1.04 | neutral |
| 8 | 70 | 5.38 | 4.95 | -0.43 | strongly acid |
| 9 | 77 | 6.22 | 4.86 | -1.36 | slightly acid |
| 10 | 100 | 6.12 | 4.46 | -1.66 | slightly acid |

* American Society for Testing and Materials

When this difference is negative, the soil solution has a net negative charge, which is characteristic of acidic material. Santa Rosa samples had a negative value of ΔpH (Table 3.27). With the exception of Site 6 samples Santa Rosa samples had a pH <5.2 and therefore would provide exchangeable Al (Table 3.27). Acidic soils between pH4.0-5.0 may have high concentrations of soluble manganese which is also potentially toxic.

## 3.5.8 Laser Diffraction Particle size Analysis

For the initial determinations the sieved <2mm fractions were dispersed in deionised water/ sodium hexametaphosphate solution 0.5%w/v, with a measured pH 6.7.

Data for each site was pooled for analysis and plotting and calculations were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com). 1-Way ANOVA with Bonferroni Multiple Comparisons tests were used, where there was sufficient data. The Bonferroni method is a method for correcting for multiple comparisons. It can be used to correct any set of P values for multiple comparisons (Bonferroni was chosen as it was more conservative than Dunnett's test for multiple comparisons-Graphpad). 1-Way Anova results and Bonferroni Multiple Comparisons tests results for the comparison of individual sites are presented in Table 3.28.

### Comparison < 4 µm and 4-31µm Clay particles

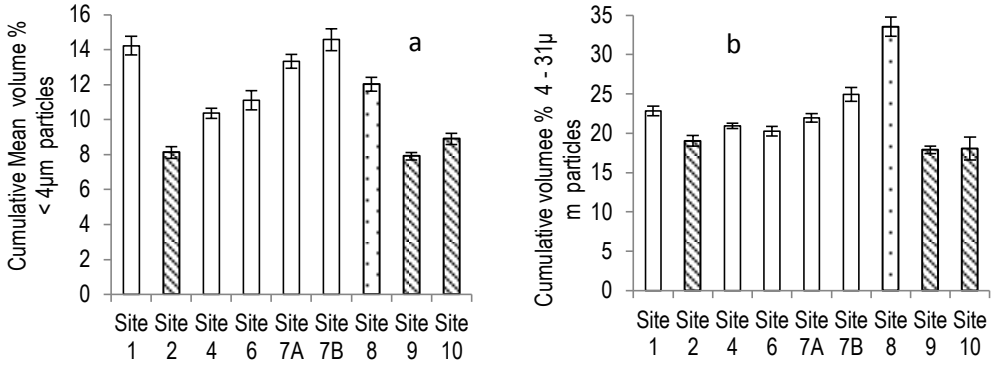



Figure 3.36 Cumulative mean volume % (SEM) < 4µm and 4-31µm, *Ateles* eating Sites 2,9,10 and *Cebus* Site 8.

There was a significant difference in the % <4µm sized particles (Figure 3.36a). There was no significant difference between *Ateles* sites but there was between *Ateles* and control sites (Table 3.28). There was a significant difference in the % 4-31µm sized particles (Figure 3.36b). There was no significant difference between the *Ateles* eating sites; however there was a significant difference between control Site 1 and *Ateles* and *Cebus* and *Cebus* and control (Table 3.28).

Table 3.28 Summary of 1-Way Anova and Bonferroni's Multiple Comparison Test results, particle size distributions.

| Particle size | Comparison | | No.of groups | P value |
|---|---|---|---|---|
| <4µm | all data sets | 1-way Anova F value = 15.94 | 9 | <0.0001. |
| 4-31µm | | 1-way Anova F value = 10.18 | 9 | <0.0001. |
| >500µm | | Non-parametric 1-Way ANOVA KW = 14.43 | 9 | <0.0001. |
| **Particle size** | **Comparison** | **Bonferroni's Multiple Comparison Test** | **t** | **P value** |
| < 4 µm | *Ateles* eating sites | 2 vs 9 | 0.3481 | NS |
| | | 2 vs 10 | 1.121 | NS |
| | *Ateles* vs Control | 9 vs 1 | 10.69 | P < 0.001 |
| | | 9 vs 4 | 4.13 | P < 0.001 |
| | | 9 vs 6 | 5.198 | P < 0.001 |
| | *Ateles* vs Cebus | 9 vs 8 | 5.526 | P < 0.001 |
| 4-31µm | *Ateles* eating sites | 2 vs 9 | 1.172 | NS |
| | | 2 vs 10 | 0.7585 | NS |
| | *Ateles* vs Control | 2 vs 1 | 2.541 | NS |
| | | 2 vs 4 | 2.187 | NS |
| | | 2 vs 6 | 1.384 | NS |
| | *Ateles* vs Cebus | 2 vs 8 | 13.18 | P < 0.001 |
| | | 9 vs 8 | 13.73 | P < 0.001 |
| | | 10 vs 8 | 10.89 | P < 0.001 |
| | *Cebus* vs Control | 8 vs 1 | 10.14 | P < 0.001 |
| | | 8 vs 4 | 11.74 | P < 0.001 |
| | | 8 vs 6 | 11.44 | P < 0.001 |
| >500µm. | *Ateles* eating sites | 2 vs 9 | 6.49 | P < 0.001 |
| | | 2 vs 10 | 8.275 | P < 0.001 |
| | *Ateles* vs Control | 2 vs 6 | 5.365 | P < 0.001 |
| | | 9 vs 6 | 11.7 | P < 0.001 |
| | | 10 vs 6 | 12.84 | P < 0.001 |
| | *Cebus* vs Control | 8 vs 2 | 10.8 | P < 0.001 |
| | | 8 vs 9 | 16.63 | P < 0.001 |
| | | 8 vs 10 | 17.22 | P < 0.001 |

Comparison of > 500µm particles

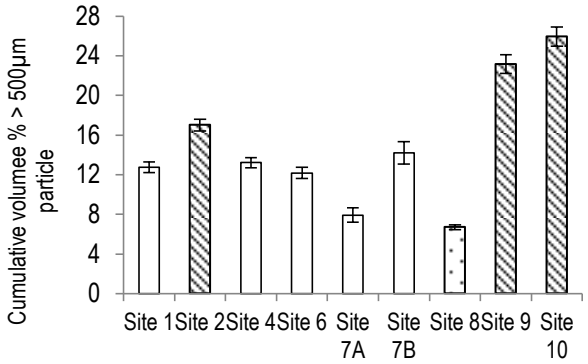



Figure 3.37 Cumulative mean volume % (SEM) > 500µm, *Ateles* eating Sites 2,9,10 and *Cebus* Site 8.

There was a significant difference between volume % >500µm sized particles (Figure 3.37). There were significant differences between *Ateles* sites; *Ateles* vs Control and *Cebus* vs control (Table 3.28).

In summary:

- There were no particles > 1µm in the reference materials and few in the 1-2µm range in any of the site sample determinations.
- *Ateles* sites had the lowest % <4µm clay sized particles and 4-31 µm, the sizes of particles involved in potential adsorption of PSM.

- *Ateles* sites had the highest % > 500µm size particles

### 3.5.8.1 Effect of pH and Gastric media on Particle Size distributions

CRM montmorillonite SWy-2 and kaolinite KGa-2

These analyses were undertaken to determine if the site samples exhibited any behaviours which may be due to trace presence of any montmorillonite minerals. The results are presented for the initial aqueous treatment (measured pH 6.7), aqueous treatment pH adjusted to pH2 and the combined gastric media (10:30) pH 2. The latter represents the conditions in the stomach where particles would initially interact with potential toxins and where the size determines the amount of adsorption possible. Analysis of the media alone did not yield size distribution data. Figure 3.38 illustrates the difference of treatments on < 31µm sized particles.

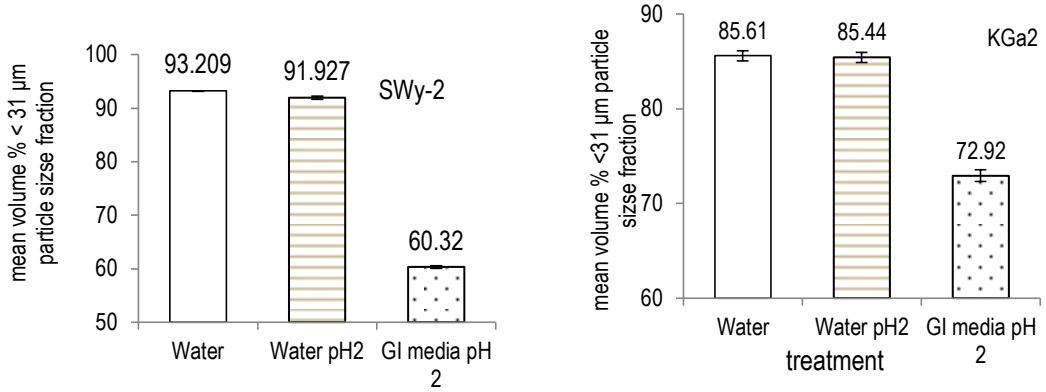



Figure 3.38 Effect of treatment on Cumulative mean volume % (SEM) <31µm, SWy-2 and KGa-2

1-Way Anova results and Bonferroni Multiple Comparisons tests results for the effect of treatments are presented in Table 3.29.

Table 3.29 Results of 1-Way Anova and post analysis test effect of pH/media < 31µm, particle size

| **CRM** | **1-way Anova F value** | **No. of groups** | **P value** |
|---|---|---|---|
| SWy-2 < 31µm | 7263 | 3 | <0.0001 |
| KGa-2 < 31µm | 162.8 | 3 | <0.0001 |
| | **Bonferroni's Multiple Comparison Test** | **t** | **P value** |
| SWy-2 < 31µm | water vs water pH2 | 4.147 | NS |
| | water pH2 vs gastric pH2 | 102.2 | P < 0.001 |
| KGa-2 < 31µm | water vs water pH2 | 0.2133 | NS |
| | water pH2 vs gastric pH2 | 15.52 | P < 0.001 |

There was no significant pH effect on either CRM on the % <31µm particles, compared to water however GI media treatments result in significant reduction in this class size (Table 3.29). These results suggest that for both CRM there is a significant interaction of the <31µm, clay sized particles beyond the potential swelling due to pH alone, due to interaction with components of the media.

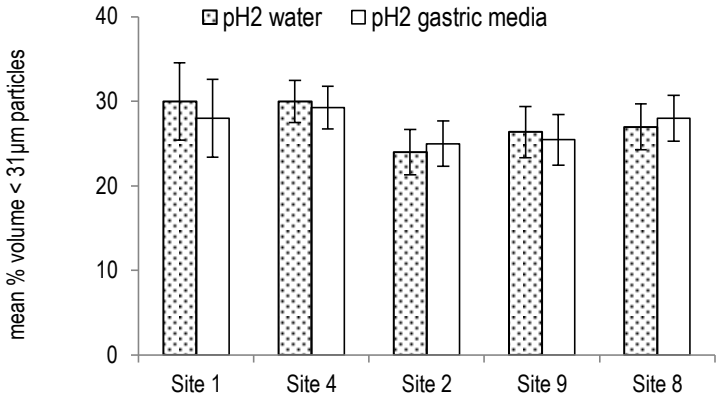



Figure 3.39 Cumulative mean volume % (SEM) <31µm in pH2 and GI media pH2.

There was no significant difference in mean volume % <31µm in pH2 water or in gastric media, Figure 3.39, 1-way ANOVA p>0.05.The geophagy samples did not exhibit the shift of particle size as seen with either CRM (Figure 3.38).

In summary:

- the site samples do not show similar changes in particle size distribution in the gastric media. This supports the IR and XRD data in suggesting that there is little kaolinite or montmorillonite present in the geophagia samples from Santa Rosa.

## 3.5.9 Adsorption of Plant Secondary metabolite test compounds

Following the method development the amount of both CRM were reduced, to enable measurement of the residual compound in the media, using the calibration data, (quinine Figure 3.14; tannic acid Figure 3.15; gallic acid Figure 3.16 and Appendix 1.5, Figures 2-5). The results were plotted (Mean and SEM) using Excel 2010®. The UV absorbance values were used to determine the residual concentration after the interaction period. This was then used to calculate the mg/g adsorbed. Kruskal-Wallis non parametric 1-Way Anova was performed, using GraphPad Prism v 4.0.

### 3.5.9.1 Adsorption of ephedrine HCl – Calibration equation y = 0.845x+0.0177.

Ephedrine HCl adsorption by the SWy-2 was 565.5±48.78 mg/g. Adsorption by KGa-2 was significantly greater than site samples (Figure 3.40) and there was also significant variation between the sites, Sites 1 adsorbing more than twice the other sites (Table 3.30).

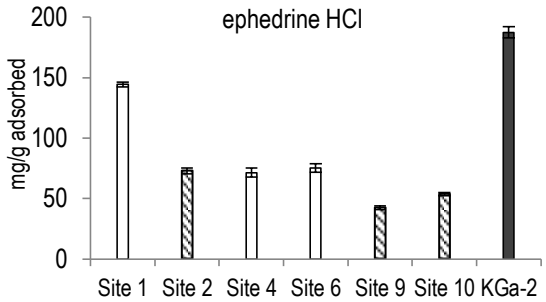



Figure 3.40 Adsorption ephedrine HCl, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6

### 3.5.9.2 Adsorption of quinine HCl– Calibration equation y = 16.334x+0.0168

Quinine HCl adsorption by the SWy-2 was 55.15±4.68 mg/g. Adsorption by KGa-2 was significantly less than site samples (Figure 3.41) there was no significant variation between the sites (Table 3.30).

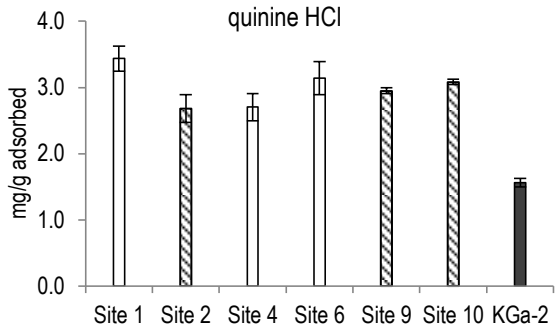



Figure 3.41 Adsorption quinine HCl, - *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

### 3.5.9.3 Adsorption of berberine – Calibration equation y = 64.8x – 0.0497

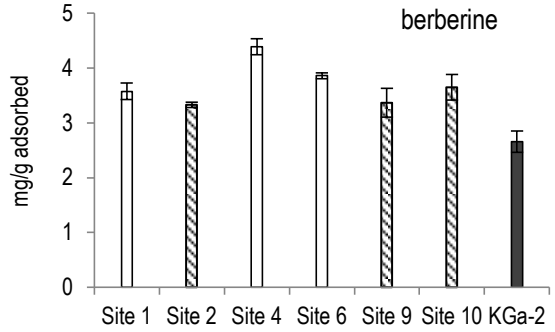



Figure 3.42 Adsorption berberine, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

The SWy-2 UV absorbance for berberine solutions when either 25mg or 10mg SWy-2 were used was below the range of the calibration plot, below the LOQ. i.e. SWy-2 was very efficient at adsorbing berberine Adsorption by KGa-2 was significantly less than site samples (Figure 3.42) and there was no significant variation between the sites (Table 3.30).

### 3.5.9.4 Adsorption of gallic acid – Calibration equation y = 48.755x – 0.0131

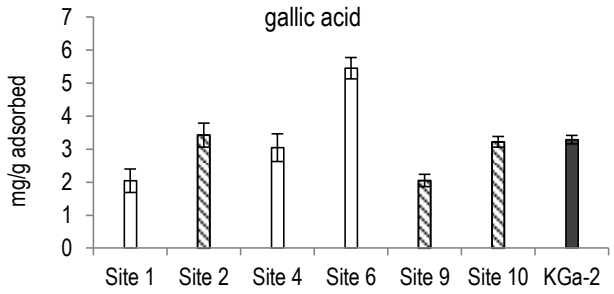



Figure 3.43 Adsorption gallic acid, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

The SWy-2 UV absorbance for gallic acid solutions when either 25mg or 10mg SWy-2 were used was below the range of the calibration plot i.e. it was very efficient at adsorbing gallic acid. Adsorption by KGa-2 was significantly different to site samples and there was significant variation between the sites also (Table 3.30) Site 6 having the greatest adsorption (Figure 3.43).

### 3.5.9.5 Adsorption of tannic acid – Calibration equation y = 36.045x + 0.0158

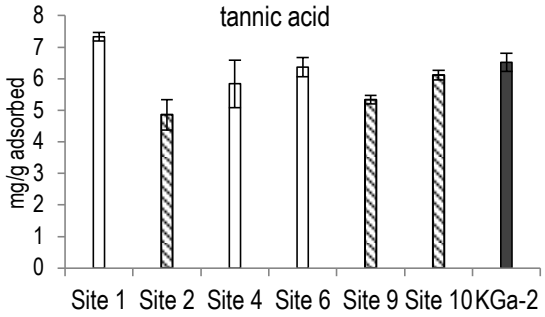



Figure 3.44 Adsorption tannic acid, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

Tannic acid adsorption by the SWy-2 was 50.64 ± 3.02 mg/g, this was higher than the site samples.. Adsorption by KGa-2 was significantly greater than *Ateles* sites, Sites 1 and 6 having the greatest adsorption (Figure 3.44) and there was also significant variation between the sites (Table 3.30).

### 3.5.9.6 Adsorption of naringin – Calibration equation y = 25.652x – 0.004

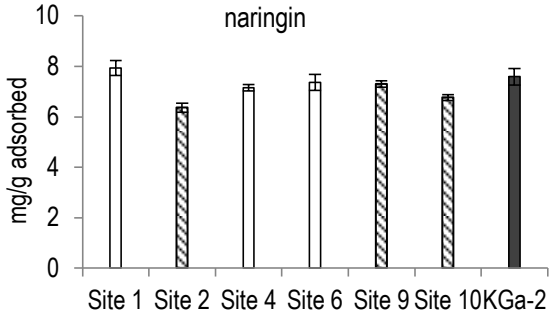



Figure 3.45 Adsorption naringin, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

The adsorption by the SWy-2 was 67.77±1.26 mg/g this was higher than the site samples (Figure 3.45). Adsorption by KGa-2 was also significantly different and there was significant variation between the sites (Table 3.30).

### 3.5.9.7 Adsorption of chlorogenic acid – Calibration equation y = 0.052x-0.0414

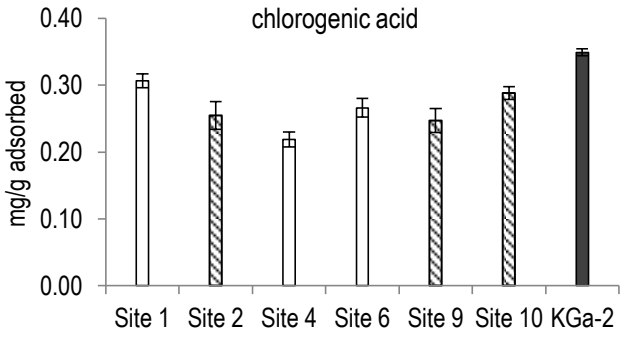



Figure 3.46 Adsorption of chlorogenic acid, *Ateles* sites 2, 9, and 10; Control 1, 4 and 6.

No value was calculated for SWy-2 as the UV absorbance from sample solutions when the amount of Swy-2 was reduced to 15mg was below the range of the calibration plot. SWy-2 had a very high affinity for chlorogenic

acid. Adsorption by KGa-2 was significantly higher than site samples (Figure 3.46). There was no significant variation between the sites (Table 3.30).

Table 3.30 Summary of Kruskal-Wallis 1-Way Anova of PSM adsorption results

| **compound** | **comparison** | **KW statistic** | **No. of groups** | **P** | |
|---|---|---|---|---|---|
| ephedrine HCl | kaolinite vs sites | 18.44 | 7 | 0.0052 | |
| quinine HCl | | 15.29 | 7 | 0.0181 | |
| berberine | | 14.89 | 7 | 0.0211 | |
| gallic acid | | 15.41 | 7 | 0.0173 | |
| tannic acid | | 14.79 | 7 | 0.022 | |
| naringin | | 14.61 | 7 | 0.0235 | |
| chlorogenic acid | | 15.36 | 7 | 0.0176 | |
| **compound** | **comparison** | **KW statistic** | **No. of groups** | **P** | |
| ephedrine HCl | inter site | 14.96 | 6 | 0.0105 | |
| quinine HCl | | 10.71 | 6 | 0.0575 | NS |
| berberine | | 10.17 | 6 | 0.0706 | NS |
| gallic acid | | 13.68 | 6 | 0.0178 | |
| tannic acid | | 12.2 | 6 | 0.0321 | |
| naringin | | 13.35 | 6 | 0.0203 | |
| chlorogenic acid | | 10.8 | 6 | 0.0555 | NS |

In summary:

- the geophagy site samples all showed some degree of adsorption for the chosen test compounds, with the highest for the simplest molecule ephedrine HCl.
- chlorogenic acid had the least adsorption of all the test compounds.

## 3.5.10 ICP-MS Determination of minerals released

Calibration plots were determined using the element/Rh ratio. The LOD and LOQ values were calculated for each element of interest, mean and SD values taken from analysis of blank. Non-parametric Kruskal-Wallis test used GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com).

The data presented in Figures 3.47-3.53 are for the only elements which reached the appropriate LOQ levels. All plots are of the calculated Mean and SEM mcg/g of dried leaf.

### 3.5.10.1 Minerals released

#### Sodium

Sites 1, 8, 9 and 10 had Na values < LOQ. Only Site 2 of the eaten sites had values > LOQ. The geophagic samples are unlikely to provide Na supplementation.

Potassium and magnesium

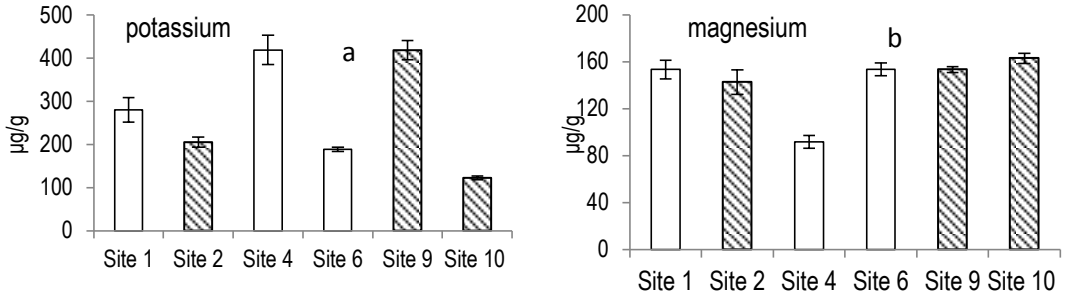



Figure 3.47 ICP- MS determined potassium and magnesium released

Sites 4 and 9 had released the highest level of K detected (Figure 3.47a). There were a significant differences in K released from *Ateles* sites, (Table 3.31). Dunn's Multiple comparison test of K released between *Ateles* sites p<0.0001.

Site 4 control site released the lowest level of Mg released (Figure 3.47b). There was a significant difference in Mg released between all of the sites (Table 3.31). However A Dunn's Multiple comparison test showed no significant difference between Mg released *Ateles* sites p>0.05.

Calcium and aluminium

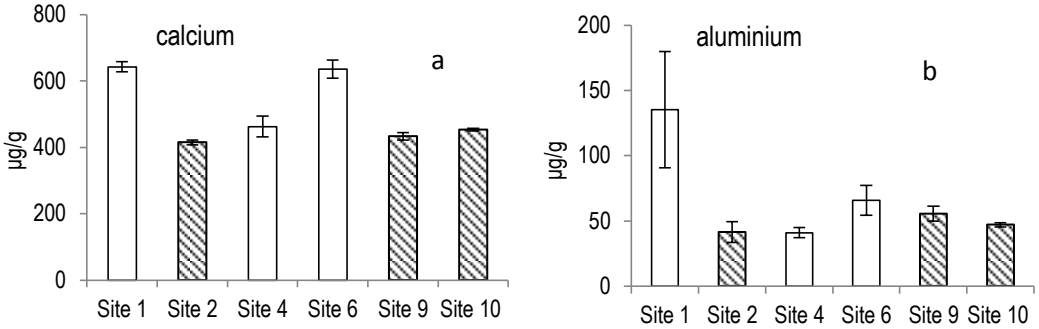



Figure 4.38 ICP-MS determined calcium and aluminium released from samples

Control Sites 1 and 6 released the highest level of calcium detected (Figure 3.48a). There were significant differences in Ca released between the sites (Table 3.31). However a Dunn's Multiple comparison test showed no significant difference for Ca released between *Ateles* sites p>0.05.

Control Site 1 released the highest level of aluminium detected (Figure 3.48b). There was a significant difference in Al released between the sites (Table 3.31). However a Dunn's Multiple comparison test showed no significant difference between *Ateles* sites p>0.05.

Barium and Manganese

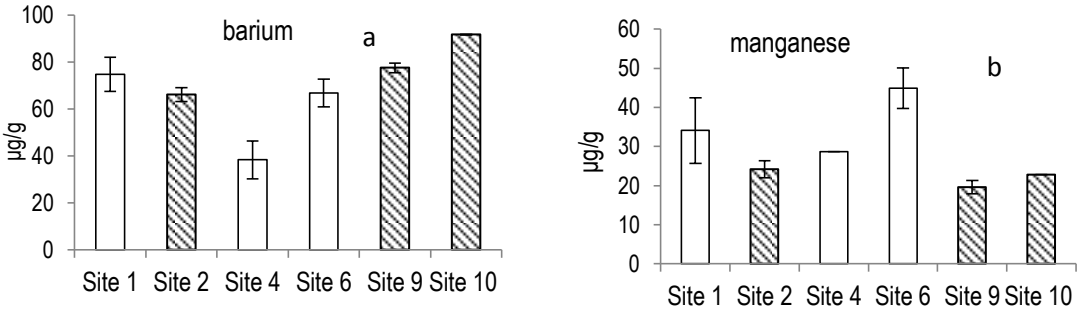



Figure 3.49 ICP- MS determined barium and manganese released from samples

Site 4 control site released the lowest level of barium detected (Figure 3.49a). There was a significant difference in the amount of Ba released between the sites (Table 3.31). In this case a Dunn's Multiple comparison test showed a significant difference in barium released between *Ateles* sites $p<0.001$.

There was no significant difference in the amount of Mn released between all the sites (Figure 3.49b and Table 3.31) and a Dunn's Multiple comparison test showed no significant difference between *Ateles* sites $p>0.05$.

Zinc

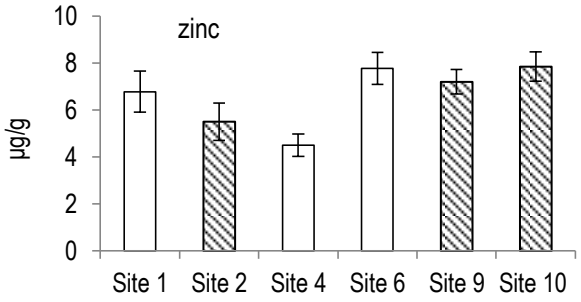



Figure 3.50 ICP- MS determined zinc released from samples

Control site 4 Site 8 released the lowest level of zinc (Figure 3.50). There was a significant difference in the Zn released between all sites (Table 3.31) and a Dunn's Multiple comparison test also showed a significant difference between *Ateles* sites $p<0.001$.

Table 3.31 Summary of Kruskal-Wallis 1-Way Anova, (all site comparisons) ICP determination of minerals released concentrations > LOQ.

| | KW statistic | No, of groups | p |
|---|---|---|---|
| potassium | 30.15 | 6 | <0.0001 |
| magnesium | 30.08 | 6 | <0.0001 |
| calcium | 28.46 | 6 | <0.0001 |
| aluminium | 19.29 | 6 | 0.0073 |
| barium | 13.25 | 6 | 0.0212 |
| manganese | 10.37 | 6 | 0.0653 |
| zinc | 11.49 | 6 | 0.0425 |

In summary:

- Only 7 elements were detected at levels above the LOQ.
- Only Site 2 had Na > LOQ
- K, Ca and Mg levels varied significantly between all the sites
- Al, Ba, Mn and Zn did not vary significantly between all sites
- K, Ca, Ba and Zn showed a significant variation between the known *Ateles* eating sites
- Mg. Al and Mn showed **no** significant variation between the known *Ateles* eating sites

### 3.5.10.2 Adsorption of Fe

The normalised calibration data was plotted using the $Fe^{58}$ data, ($y= 0.0207x + 0.0163$; $R2 = 0.9989$), the LOD and LOQ were determined.

The Fe recovery for the spiked blanks was 98.66-97.98%. The residual Fe levels were determined for each sample. Values for each sample were calculated and plotted in Excel 2010©. Due to limitations in sample available it was not possible to conduct sufficient analyses to permit statistical analysis to be undertaken.

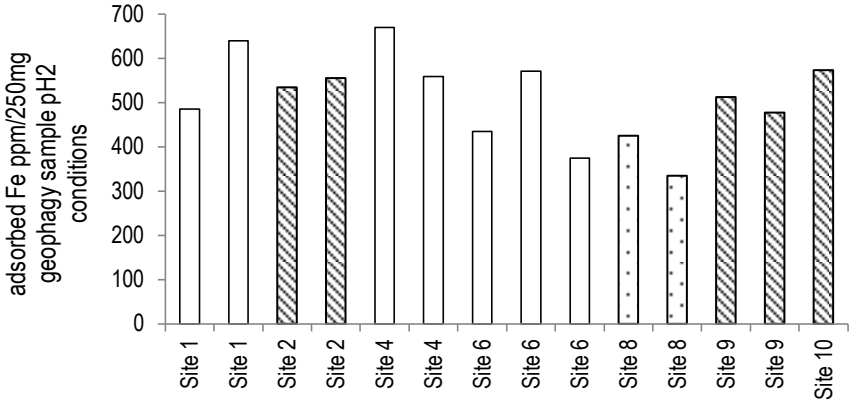



Figure 3.51 ICP- MS determined Fe adsorbed from spiked solutions, pH2 conditions, highlighting *Ateles* eating sites.

The results however suggest that there will be adsorption by the geophagy samples of Fe in both acidic/gastric (Figure 3.51) and also in the intestinal pH7 conditions (Figure 3.52).

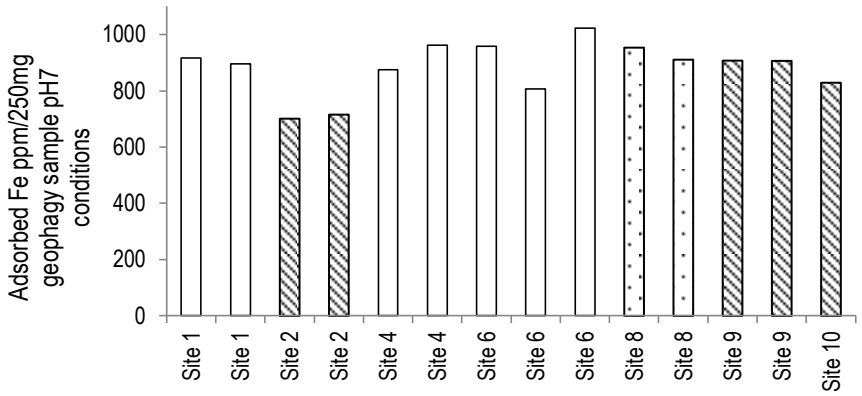



Figure 3.52 ICP- MS determined Fe adsorbed from spiked solutions, pH7 conditions.

The Fe released in samples from the sites <LOQ in previous ICP-MS analysis of geophagy samples in acidic conditions, therefore there is likely to be little contribution from the geophagy samples to the measured values remaining in solution in these spiked analyses. At low pH or in anoxic conditions the more soluble ferrous ($Fe^{2+}$) form is present in aqueous solution.

The data presented in Figure 3.52 suggests that soluble Fe is exchanged/adsorbed or lost. This may be due to formation of insoluble iron hydroxides under the pH 7 conditions (Schwertmann *et al.* 1989).

## 3.5.11 Microbiological Assessment of geophagy samples

The plates were examined after incubation for 7 days, there was no growth indicating there was no viable bacterial contamination. The plates were then returned to the incubator for a further 21 days. There was no growth after this extended period indicating that no viable fungal spores were present.

## 3.6 Discussion of Results

The results are discussed in the context of previous publications and the main functional hypotheses for geophagy. A summary of the analytical outcomes are presented in Table 3.32.

Table 3.32 Summary of analytical outcomes

| Technique- characteristic determined | Outcomes |
|---|---|
| Munsell colour characteristic | consumed samples pink/grey |
| Loss on Ignition/ presence of carbonates/organic material | very low levels organic carbon or carbonates |
| XRD –presence of clay or other minerals | quartz, feldspars, amorphous minerals minimal kaolinite absence of montmorillonites, limited magnetite and maghemite |
| IR – presence of clay minerals/organic matter/water | quartz, feldspars, amorphous minerals minimal kaolinite absence of montmorillonites |
| XRF– detectable elements present | acidic nature derived from andesite/dacite type volcanic rocks; aluminium values |
| ICP– of elements released into gastric extract | limited elements reached LOQ, variable levels across the sites |
| ICP-Effects of geophagy material on potentially available Fe | Fe levels reduced when incubated in presence of geophagy material |
| Laser diffraction particle size in variable pH-media conditions/presence of clay sized particles | 8-9% clay –very fine silts (<4µm); 18% fine, medium and coarse silts (4-32 µm)17-25% coarse, very coarse sand (500 µm) in *Ateles* eaten sites |
| Sample pH in water and KCl solution | acidic in nature |
| UV– analysis of gastric extract following exposure to example PSM in gastric conditions | *Ateles* sites 200mg samples adsorbed < 50mg kaolinite or 10-25mg montmorillonite |
| Microbiological screening of gastric digest | no organisms cultured |

### 3.6.1 Munsell Colour characterisation

A review of the literature of geophagy material published since 1999 is collated in Table 3.33. This indicated that soils consumed by primates were predominantly reddish to reddish brown in colour. The yellow red colours indicate that eaten soils were highly weathered and depleted in silica (Krishnamani *et al.* 2000) due to prolonged leaching, together with the presence of oxides high in Fe (Mahaney *et al.* 1995a, Mahaney *et al.* 1996, Mahaney *et al.* 1997). The yellow colours often indicate released hydroxides of iron or aluminium and signify high clay content (Krishnamani *et al*. 2000). Hematite, formed by dehydration of goethite (Schwertmann *et al.* 1989), is abundant in ancient red beds whereas goethite is abundant in younger yellow-brown coloured deposits.

Detection of geophagy material by colour is available to humans, Old world apes and monkeys who share trichromatic colour vision (Sumner *et al.* 2000). Trichromatic vision has both a red-green chromatic channel and blue-yellow channel. New world primates differ in that there are sex-linked polymorphisms. In many cases males are obligate dichromats and females may be either dichromatic or trichromatic colour vision (Jacobs 2007). Investigation of the polymorphism in *Ateles geoffroyi* has been undertaken at Santa Rosa (Hiramatsu *et al.* 2005, Hiramatsu *et al.* 2008). Detection and selection (based on colour alone) of red coloured geophagy samples by *Ateles geoffroyi* would therefore be limited to trichromatic females.

Geophagy reports in New World primates are predominantly behavioural and there are few reports of sample or colour characterisation (Ferrari *et al*. 2008). New World monkeys often consume soil from arboreal sources, such as termite nests and ant mounds and wet/salido material.

Table 3.33 Examples of publications relating to primate geophagy since 1999.

| Reference | Ape species | Colour |
|---|---|---|
| Aufreiter *et al*. (2001) | *Pan troglodytes schweinfurthii* | Yellow-red 10YR 5/6 bright brown-orange 7.5YR4/6** |
| Ketch *et al*. (2001) | *Pan troglodytes schweinfurthii* | Not reported |
| Klein *et al*. (2008) | *Pan troglodytes schweinfurthii* | red |
| Mahaney *et al.* (1999) | *Pan troglodytes schweinfurthii* | Yellow-brown 10YR 5/4 – 7.5YR 5/6 bright brown eaten |
| Mahaney *et al.* (2005) | *Pan troglodytes schweinfurthii* | 2.5YR 4/6 yellowish red – 10YR 4/3 yellow brown-grey |
| **Reference** | **Old world species** | **colour** |
| Mills *et al.* (2007) | *Papio ursinus* | Bright yellow brown (10YR 7/6) bright red brown (5YR 5/6) grey yellow (2.5YR 6/2) |
| Pebsworth *et al.* (2012) | *Papio cynocephalus ursinus* | White pink; ochre/pink no hue/chroma classification |
| Pebsworth *et al.* (2013) | *Papio cynocephalus ursinus* | White, no hue/chroma classification |
| Voros *et al.*(2001) | *Macaca radiata* | 5YR 4/8 reddish brown 5YR 5/8 bright red brown** |
| Wakibara *et al.* (2001) | *Macaca fuscata* | No colour reported |
| **Reference and New world primate species** | | |
| **No reports of colour in these publications**<br>Adams *et al*.(2011) *Pithecia irrorata*; Blake *et al*. (2010, 2011, 2013) *Ateles belzebuth* Campbell *et al*. (2005), *Ateles* spp.; De Souza *et al*.(2002), *Alouatta belzebul*; Dew (2005) *Ateles belzebuth belzebuth*; Molina Gonzalez (2010) *Alouatta seniculus*; Link *et al*. (2006, 2011a, 2011b) *Ateles hybridus*; Molina *et al*. (2014) *Alouatta seniculus*; Montenegro (2004) *Ateles paniscus.* | | |

The results for the Santa Rosa samples (Section 3.5.1, Table 3.17) are the first colour characterisation of geophagy materials for New World primate species. The eaten material was light grey-pinkish grey colours.

Pebsworth (2013) reported an example of consumption in an Old World primate of white coloured material which had lower Fe content than a rejected pink soil. The report also showed that there was a low bioavailability of Fe from both eaten and non-eaten samples. This illustrates the importance of not relying on total element content when suggesting a function. Colour can be used to infer presence but not bioavailability (Fontes *et al.* 2005). The colour of the Santa Rosa samples suggests that there is unlikely to be significant amounts of Fe in the sites chosen by *Ateles*.

**In summary**:

The Santa Rosa sample colours were markedly different from those in the bulk of the published literature for primates.

## 3.6.2 Mineral Content

The results from these analyses: XRD, Section 3.5.4, IR Section 3.5.5 and XRF Section 3.5.6 were used to characterise the mineral content.

The XRF data from Rockwood suggest the materials from Santa Rosa belong to the andesite/dacite groups of minerals (Appendix 1.2 Glossary of Geological terms). This is based on the TAS diagram classification (Figure 3.34). Andesites have $SiO_2$ content 52%-63% and dacites 63-68%, this is consistent with the Santa Rosa data (Table 3.26). The feldspar constituents of andesites produce pink grey coloured material (McGraw-Hill 2003). XRD results indicate the presence of quartz, feldspars and traces of halloysite, kaolinite and the iron minerals maghemite and magnetite in the *Ateles* eating sites. These results agree with the minerals of andesitic volcanic lavas in Costa Rica identified by van Dooremolen *et al*. (1990) who describe poorly sorted lahars composed of kaolinite, illite, both 0.7 and 1.0nm halloysite, goethite and gibbsite. Halloysite, metahalloysite are species of kaolin minerals with similar structure to kaolinite. Kaolinites are

produced in dryer conditions. Halloysite is produced by weathering of volcanic igneous rocks and halloysite in dry conditions degrades to metahalloysite (Nieuwenhuyse *et al.* 2000).

IR analysis did not identify the presence of montmorillonites in the samples (Figure 3.28a), however XRD data from Rockwood suggested montmorillonites were present in a limited number of samples but were not in the *Ateles* eating sites and also identified small amounts of both kaolinite and halloysite in some of the samples Table 3.22. IR analysis of Site 7A 7B and 8, Figure 3.33a, have features which may be due to organic functional groups or structural water. The water identified may be associated with the presence of the paracrystalline short-range ordered mineraloid opal, or hydrated kaolinitic mineral, halloysite. The Rigaku data (Figures 3.25a-b) and the IR data (Figure 3.30 and Table 3.23) also suggested that kaolinite was only present in small amounts.

South American terrestrial termitaria samples contained 80% kaolinite, 10% gibbsite ($Al(OH)_3$) and trace amounts of goethite $Fe^{3+}O(OH)$, feldspar, plagioclase and quartz (Setz *et al.* 1999), whereas Asian termitaria samples contained halloysite, metahalloysite and kaolinite as the major clay minerals with minimal montmorillonite together with quantities of the feldspars, orthoclase and plagioclase (Voros *et al.* 2001). The latter, when exposed to a magnet, identified magnetic minerals ilmenite ($Fe^{2+}TiO_3$) and magnetite. During the author's preliminary investigations a magnetic stirrer was used to aid dispersion of the Santa Rosa samples. This resulted in large amount of dark coloured needle shaped material becoming attached to the magnet. Ferromagnetism is the strong attraction to magnetic fields and it is exhibited in few minerals, e.g. magnetite and maghemite. These were amongst those identified in XRD from Rockwood (Table 3.24). XRD analysis of African samples detected (in order of abundance): quartz, illite, kaolinite, gibbsite, paragonite, siderite, halite, and magnesite (Pebsworth *et al.* 2012). All these soil samples had swelling properties, which implies that some form of smectite was present in the soils; however it had not been detected in their analysis. Santa Rosa samples did not show any swelling properties, nor did the samples exhibit the shift in particle size in media comparable to the montmorillonite or kaolinite CRM (Figures 3.38a-b).

Variation in constituents is widely reported with ingested and uneaten soils dominated by metahalloysite (Mahaney *et al*. 2005). The <2µm fraction also contained quartz, plagioclase feldspar, and orthoclase. This differs from their other publications where consumed soil samples contained higher levels of halloysite and kaolinite than to non-eaten samples (Mahaney *et al.* 1995b, Mahaney *et al.* 1995c, Mahaney *et al.* 1997). Soils eaten by human populations had high levels of hydrated halloysite and kaolinite in eaten samples but material also available locally containing montmorillonites was avoided (Mahaney *et al*. 2000).

**In summary:**

The Santa Rosa samples contained relatively little kaolinite. In contrast the literature analyses of consumed soils and termitaria material consumed by humans and a range of primates reported the principal mineral present was kaolinite.

### 3.6.3 Particle size distribution properties

*Ateles* geophagy sites were those with the lowest volume % of clay sized < 4µm (Figure 3.36a) and there was no significant difference between the *Ateles* eating sites mean volume % <4µm sized particles, however there was a significant difference between *Ateles* and the remaining sites with the site chosen by the *Cebus* having larger amounts (Table 3.28). For the particles classed as silts i.e. 4-31 µm particles (Figure 3.36b) there

were significant differences in the mean volume % 4-31µm sized particles (Figure 3.36b and Table 3.28) but there was no significant difference between the *Ateles* eating sites. Both the <4 and 4-31µm particle size fractions representing the clay sized and silts are those which would be expected to be involved in potential adsorption of PSM or toxins.

*Ateles* sites had higher values for the largest particle size class > 500µm (Figure 3.37) however there was a variation between all the sites (Table 3.28). Needle like particles may, depending on their cross-sectional presentation, may be recorded as either smaller or larger sized particles, and therefore potentially distort the data, for the particle sizes. There were no particles > 1mm in the reference materials and few in the 1-2mm range in any of the site determinations. These limited results suggest that the two monkey species use geophagic material with different characteristics. The *Cebus* Site 8 had the largest proportion of very fine, fine and medium silts, *Cebus* are more folivorous and also consume up to 50% insect and invertebrates (Fragaszy *et al.* 2005).

Table 3.34 Published clay particle proportions relating to primate geophagy since 1999.

| **Reference** | **Ape species** | **Eaten samples Particle sizes** |
|---|---|---|
| Aufreiter *et al*. (2001) | *Pan troglodytes schweinfurthii* | ≥50% clay sized < 2µm |
| Ketch *et al*. (2001) | *Pan troglodytes schweinfurthii* | 30-68% <2µm |
| Klein *et al*. (2008) | *Pan troglodytes schweinfurthii* | 15-44% <2µm |
| Mahaney *et al.* (1999) | *Pan troglodytes schweinfurthii* | 34.3-57.3% <2µm |
| Mahaney *et al.* (2005) | *Pan troglodytes schweinfurthii* | 39-91% <2µm |
| **Reference** | **Old world species** | **Eaten samples Particle sizes** |
| Mills *et al.* (2007) | *Papio ursinus* | 37.5% < 2µm |
| Pebsworth *et al.* (2012) | *Papio cynocephalus ursinus* | 25-34% < 2µm |
| Pebsworth *et al.* (2013) | *Papio cynocephalus ursinus* | 42% < 2µm |
| Voros *et al.*(2001) | *Macaca radiata* | 26-31% < 2µm |
| Wakibara *et al.* (2001) | *Macaca fuscata* | 4-18% < 2µm and silts 42-61% |

For New World species the analysis of geophagy samples at Cayo Santiago found that the macaques consumed material with clay (<2µm) fraction 52-88% compared to control samples 19-46% (Mahaney *et al.* 1995c). There was no further published data for New World primates to permit a comparison. Table 3.34 lists size fraction data for Old World primate species.

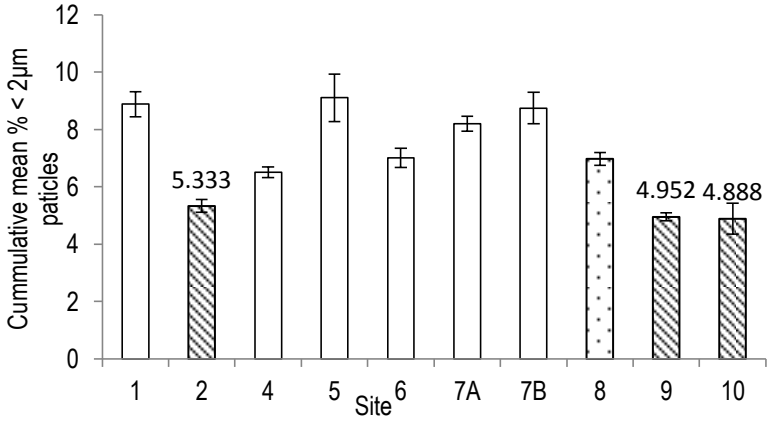



Figure 3.53 Cumulative mean volume % < 2µm sized particles

Several of these determinations were made using the dry sieving methods, and whilst not directly comparable the differences in scale when compared to the data from Santa Rosa are obvious in Figure 3.53. The nearest in character is the data from Wakibara (2001) which is 8-14% < 2µm.

**In summary**:

The measured particle size distribution is a further instance of a major difference in the material chosen by the *Ateles* compared to that reported for other primate species.

### 3.6.4 pH characteristics

Table 3.37 shows the range of published values for other primate species. There was no published data for New World primates to permit a comparison.

Table 3.37 Published pH characteristics relating to primate geophagy since 1999.

| **Reference** | **Ape species** | **samples pH** |
|---|---|---|
| Aufreiter *et al*. (2001) | *Pan troglodytes schweinfurthii* | eaten 6.09-7.41 control 5.01-5.97 |
| Ketch *et al*. (2001) | *Pan troglodytes schweinfurthii* | eaten 6.1-6.7 control 5.0-5.3 |
| Klein *et al*. (2008) | *Pan troglodytes schweinfurthii* | N/D |
| Mahaney *et al.* (1999) | *Pan troglodytes schweinfurthii* | eaten 6.02-8.57 control 5.01-6.15 |
| Mahaney *et al.* (2005) | *Pan troglodytes schweinfurthii* | eaten 5.44-6.12 control 5.4-6.16 |
| **Reference** | **Old world species** | **Eaten samples Particle sizes** |
| Mills *et al.* (2007) | *Papio ursinus* | N/D |
| Pebsworth *et al.* (2012) | *Papio cynocephalus ursinus* | eaten 9.4-9.8 |
| Pebsworth *et al.* (2013) | *Papio cynocephalus ursinus* | eaten 10.1 non eaten 10.3 |
| Voros *et al.*(2001) | *Macaca radiata* | N/D |
| Wakibara *et al.* (2001) | *Macaca fuscata* | N/D |

The Santa Rosa samples consumed by the *Ateles* have pH ranges 6.12-6.24, little different from the control sites (Section 3.5.7 Table 3.27) whereas the single site eaten by the *Cebus* was pH 5.28. All of the Santa Rosa samples have a negative ΔpH value. Δ pH = pH(KCl) – pH ($H_2O$), suggesting that the particles have a net negative charge. This would allow them to behave as a buffer (Ngole *et al.* 2010). The ΔpH value of -0.43 the *Cebus* site is categorised as strongly acidic, suggesting it would have a lower buffering capacity. Folivore stomach pH needs to be at a relatively high pH to sustain their symbiotic bacteria and mitigate the effects of over production of volatile free fatty acids (Krishnamani *et al.* 2000).

Commonly, exchangeable aluminium is present if the KCl pH ≤ 5.2 (Natural Resources Conservation Service 2004). This is the case for Sites 2, 4, 9 and 10 Table 3.29. Antacid properties are related to the ability to neutralise acidity, reduce acid secretion and to interactions with the glycopeptides of the mucous layer at acid pH. The measured $pH_{KCl}$ suggests that there will be $Al^{3+}$ ions released from clay minerals or Al oxides in the Santa Rosa geophagy samples. $Al^{3+}$ ions have been shown to increase synthesis of protective prostaglandins which increase mucus gel viscosity and bicarbonate secretion by gastric mucosa (Lacy *et al.* 1982). Aluminium salts are found in many pharmaceutical antacid preparations (Krishnamani *et al.* 2000). However Tennant *et al.* (2008) established a clear role for gastric acid in reducing susceptibility to infection with ingested bacterial pathogens, therefore elevating pH, by neutralising HCl in the stomach may be deleterious under some circumstances.

There may also be disadvantages to buffering/pH changes caused by ingesting such materials. Cation solubility, including Fe, falls as pH rises. Any rise in pH will therefore reduce the dissolution of Fe and so reduce bioavailability even where samples levels are high (Young *et al.* 2008). Too little acid can interfere with the absorption of iron, calcium, vitamin B12, as well as predispose to enteric infection, bacterial overgrowth, and gastric malignancy (Schubert 2007).

**In summary**:

The samples may, through the release of $Al^{3+}$, have potentially beneficial or deleterious effects for the monkeys, depending upon the amount consumed and the circumstances of consumption.

### 3.6.5 Antibacterial activity

Recently there have been several publications citing the properties of clay minerals as alternatives to antibiotics in treating various different types of bacterial infections (Haydel *et al.* 2008, Vondruskova *et al.* 2010, Williams *et al.* 2010, Williams *et al.* 2011, Otto *et al.* 2013). Alternatively soil organisms are a common source of antibiotics (Smith 2000, Ketch *et al.* 2001). Antimicrobial screening of geophagy material did not detect the presence of any bacteria or fungi which may have produced antibacterial growth inhibiting compounds. The results for the LOI analysis (Figure 3.19) together with evidence from the IR analysis (Figures 3.33a-b) suggest that there is minimal if any organic material present in the samples. With the exception of Sites 7A, 7B and 8 the LOI values are all below 5%. A Low level of organic carbon introduces errors which may prevent reliable quantitative comparisons (Santisteban *et al*. 2004). The very small sample size available together with the effect of sample variation errors may have introduced errors in LOI determinations.

A direct antibacterial activity of aqueous leachate through the activity of exchangeable ions has been reported (Otto *et al.* 2010, Williams *et al.* 2010, Williams *et al.* 2011, Otto *et al.* 2013).

The ability of clays to bind to siderophores and other iron-chelating agents has been reviewed by Siebner-Freidbach *et al*. (2004) and Maurice *et al*. (2009). The interaction of the geophagy samples with Fe (Figures 3.70-3.71) show that samples have the potential to lower Fe levels in the GI tract. There are two principal mechanisms involved in the Fe losses, ion exchange and formation of Al/Fe insoluble complexes.. Bacteria have several mechanisms for Fe uptake which facilitates survival at different life stages e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp. and *Klebsiella pneumoniae* use secreted siderophores to scavenge host iron. Siderophores are low molecular weight catechol or hydroxamate compounds with a high affinity for iron, which effectively compete for host Fe (Wilson *et al.* 1998). In order to adhere to mucosal surfaces *Trichomonas* synthesise surface adhesins and cyto-adherence molecules. Production is increased in the presence of a high level of iron. Virulence is increased by several mechanisms in the presence of iron (Wilson *et al.* 1998) and Trichomonads grown in iron deficient media lose their virulence (Glanfield *et al.* 2007). Limitation of availability of Fe is a possible mechanism for reducing bacterial growth and subsequent colonisation or infection by prevention biofilm formation (Maurice *et al.* 2009). These mechanisms could reduce the local production and prevent diffusion and subsequent adsorption of enterotoxins and hence systemic or local toxicity and so potentially reduce the impact of an infection.

**In summary:**

The IR results in combination with the lack of fungal or bacterial growth makes it unlikely that there would be any source of potential antibiotic effects. The possibility of Fe adsorption may assist in limiting establishment of infective organisms or reduce infection load.

### 3.6.6 Antiparasitic activity

It has been suggested that geophagic material may reduce internal parasite load e.g. geohelminths by reducing colonization of hosts (Krishnamani *et al.* 2000). The larger particles present in the *Ateles* site samples, if sand like, may exert an abrasive function on the lining of the GI tract possibly detaching or damaging the surface of attached infecting organisms, in a similar manner to the possible function for hairy leaf swallowing by chimpanzee.

Particle fractions <4μm in geophagic material may become bound into the mucosal layer effectively increasing its depth and so increasing its effectiveness. Clay minerals have been shown to increase the thickness of the mucous layer due to the interaction of mineral particles and mucous glycopeptides, increasing gastrointestinal glycopeptide polymerisation (Leonard *et al.* 1994, Reichardt *et al.* 2009). Geophagi has also been shown to induce a change in the morphology of the mucosal epithelial layer (Sayar *et al.* 1975). Such changes may also reduce infection by preventing the attachment and penetration into the epithelial cells of the gut wall of microorganisms and parasitic protozoa, as attachment is often the critical primary stage in infection (Nobuko *et al.* 2011).

The viability of infective organisms may also be reduced by interactions with clays as is reported for *Histoplasma capsulatum* (Lavie *et al.* 1986b, a). Clay and fine silt sized particles may coat the surface of infective organisms. Tapeworms, a cestode, lack an alimentary canal and so nutrients must be absorbed through the tegument The external cuticle contains negatively charged pores (15Å radius) through which it obtains water, ions and other nutrients and excretes its waste products (Thompson *et al.* 1995). The tegument of trematodes i.e. a fluke is metabolically active and is involved in adsorption of nutrients, osmoregulation and excretion (Smyth *et al.* 1987). Physical blocking of such pores by particles adhering to the cuticle would have a significant effect on viability. In this manner the smaller sized fractions may reduce the viability and hence virulence of infecting organisms and regulating parasite load. This may be the mechanism responsible for the observations reported by Knezevich (1998).

**In summary**:

There are several potential mechanisms related to physical effects or related to particle size which may potentially influence establishment of infections and subsequently parasite load.

### 3.6.7 Antidiarrhoeal activity

A reduction of fluid loss and antidiarrhoeal activity could be related to the swelling properties of clay minerals. Montmorillonite clay minerals exhibit swelling of the crystal lattice due to interlayer expansion caused by the adsorption of water. There may also be interparticular swelling which involves an increase in volume due to adsorption of water molecules between individual clay sized particles. Kaolinites, whilst not exhibiting swelling, are also capable of adsorbing water onto their exterior surface layer (Baeshad 1955).The lack of montmorillonite and low kaolinite content of the Santa Rosa samples does not preclude the geophagy samples from being used in mitigation of diarrhoea symptoms but it may have limited antidiarrhoeal activity against watery diarrhoea.

Enterotoxins are produced by many bacteria and those from organisms such as *Escherichia coli*, *Staphylococcus aureus, Salmonella enterica,* and *Listeria monocytogenes* may potentially have serious

impacts on health. Toxins from these organisms can cause gastrointestinal distress (diarrhoea), dizziness, and muscle pains; at sufficiently high levels they can be mutagenic, carcinogenic, or fatal (Young *et al.* 2011). Changes in the properties and thickness of the mucous layer will reduce the direct effects of enterotoxins and by reducing absorption mitigate the systemic effects.

A further mechanism for reducing toxicity results from the binding potential of toxins/micro-organisms directly to the clay material and the subsequent elimination of these from the body. The mycotoxin T2 toxin (Fioramonti *et al.* 1987b) and toxins produced by *Vibrio cholera* (Fioramonti *et al.* 1987a) are adsorbed by clay minerals thus reducing the increased gastric motility caused by these toxins. This reduction in motility would have a beneficial effect on fluid balance.

Geophagy as an antidiarrhoeal agent has been suggested as a potential function for controlling diarrhoea induced by diet change in the predominantly folivorous mountain gorilla (Mahaney *et al.* 1995a). Ingestion of food items high in soluble carbohydrates and protein and low fibre may cause digestive upsets. Wakibara (2001) reported that the provisioned foods given to the Japanese macaques met this characteristic and suggested this may be one of the uses in this situation. *Rhesus macaque* ate soil at the same time on 64 occasions, with 84% of events taking place close to supplemental feed stations (Knezevich 1998). Often the monkeys still had feed in their cheeks when they ate the soil. Monkey chow traditionally has high carbohydrate and protein and low fibre (LabChows Purina® and Mazuri® Zoo feed data sheets). A reduction in geophagia was reported in lemurs when they reduced their intake of provisioned foods (Ganzhorn 1987).

*Ateles* at Santa Rosa had changed the major dietary fruits at the time of their geophagy (Filippo Aureli personal communication Chapter 1). During September-November the monkeys' principal fruits are *Spondias mombin* and *radikoferi* (Table 1.1). *S. mombin* has a higher fructose level than other commonly eaten fruits at Santa Rosa for which published data was available (Riba-Hernandez *et al.* 2003). Fructose malabsorption (Andersson *et al.* 1978) and high fructose content of the diet have been linked to bacterial metabolism in the colon producing free fatty acids and gases causing bloating, cramp and osmotic diarrhoea (Ledochowski *et al.* 2010). Clay minerals and clay sized particles can adsorb free fatty acids (Theng 2012).

**In summary**:

Whilst the geophagy material had little potential for reducing watery diarrhoea by the adsorption of water it retains the potential to adsorb toxins or free fatty acids which are possible causes of diarrhoea. The response to altered dietary items appears to have more merit but warrants further investigation before any clear connection can be established.

### 3.6.8 Mineral or micronutrient supplementation

Figure 3.54 is a comparison of some nutrient elements of interest; which had values > LOQ. There is little difference between the sites suggesting the areas sampled are reasonably uniform in their composition. Whilst the highest levels are for calcium, they are only at the µg/g level.

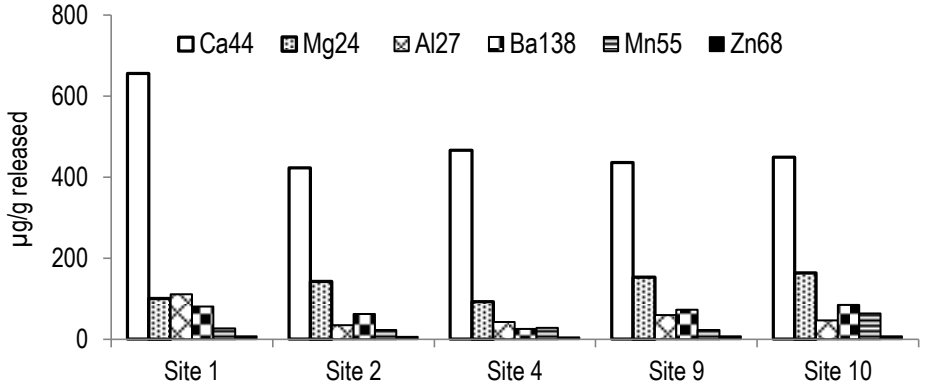



Figure 3.54 Intersite comparisons of elements released in GI digest conditions

New World primates at Twycross Zoo (working on the basis of a max 2% weight/day based on 6kg adult) are provided with 42g/day Mazuri Primate® and 138g/day Mazuri Browser Breeder® pellets to supplement the fresh fruits and vegetables (Zak Showell, Personal communication).

As can be seen from Table 3.36, geophagy samples provided insignificant amounts compared to those listed in the two purchased supplements. In order to benefit from the levels of minerals released in pH2, a large amount of geophagic material would need to be consumed to have any significant contribution. This would involve eating a relatively large volume of material which would impact on food intake.

Table 3.36 Comparison of nutrient minerals in geophagic samples and commercial primate feeds used for feeding *Ateles fusciceps* and *Ateles paniscus* at Twycross Zoo, UK.

| | **Mazuri primate® 42g/day/6kg monkey** | **Mazuri Browser Breeder ®138g/day/6kg monkey** | **max value geophagy samples µg/g** |
|---|---|---|---|
| calcium | 1.11 (g) | 1.76 (g) | 450 |
| phosphorus | 449 (mg) | 676 (mg) | < LOQ |
| sodium | 130 (mg) | 483 (mg) | <LOQ |
| magnesium | 92 (mg) | 524 (mg) | 163 |
| copper | 0.69 (mg) | 3.27 (mg) | 22 |

This lack of support for nutritional function is consistent with much of the published literature for primate species (Mahaney *et al.* 1995c, Aufreiter *et al.* 2001). Commonly, only minor differences in minerals and trace elements were detected between eaten and control samples (Voros *et al.* 2001). Material from termitaria were reported as being high in Ca, Mg, K and P (Krishnamani *et al.* 2000) Mn, Fe, Al, Na, Co, Zn, Cu and Ni (Setz *et al.* 1999). Leaf cutter ant material was reported as being high in Ti, Al, Fe, K, Zn, Ni, Cr and P but the bioavailablity was not determined (Müller *et al.* 1997). Pebsworth *et al.* (2013) reported that eaten samples were lower in Fe than rejected but that Fe had low bioavailability.

Dividing minerals into macro, micro and trace categories Krishnamai *et al.* (2000) suggested that macromineral levels were sufficient in primate diets and that micro and trace elements were likely to be more significant due to their essential function in enzymes and DNA and RNA synthesis. General trace element sourcing was hypothesised by (Heymann *et al.* 1991) and specifically Zn and Cu (Kikouama *et al.* 2009a, 2009b). Mills *et al.* (2007) reported that the largest of the lick sites analysed were enriched in micronutrients and concluded it is possible that the need for micronutrients was driving the geophagy. As previously stated the availability of the trace minerals in Santa Rosa samples of Bo, Cu, Co, Se, Mo, Se and I, did not meet LOD/LOQ criteria.

Rode *et al.* (2003) conducted analysis of leaves and fruits available to two colobine monkeys and concluded that whilst the fruit content of Zn, Ca, Fe and Mg was less than that of available eaten leaf and that there may only be deficiencies in Na and Fe in the diet. *Ateles* at Santa Rosa consume a large proportion of *Ficus* fruits, particularly strangler figs (Table 1.1). Wendeln *et al.* (2000) analysed 14 fig species and reported high levels of Ca and K and the figs were not deficient in Na. It is unlikely that there would be a deficiency of Mg where the monkeys ate green unripe fruit as chlorophyll is a potential source of Mg (Bolton *et al.* 1998).

**In summary**:

The quantities released from Santa Rosa samples in acidic conditions do not support nutrient supplementation hypothesis.

## 3.6.9 Adsorption of PSM

This hypothesis has been suggested for many species (Johns 1986, Gilardi *et al.* 1999, Krishnamani *et al.* 2000, de Souza *et al.* 2002, Brightsmith *et al.* 2008, Arrigo-Nelson *et al.* 2010, Young *et al.* 2011), for both PSM and other toxins.

Adsorption to soil/clay minerals occurs through several different mechanisms. Physical attraction due to London-Van der Waals forces is important in external surface reactions. This is particularly important for large organic cations (Hendricks 1941). Uncharged organic molecules are adsorbed mainly by Van der Waals interactions and this is important in larger molecules where there is larger area of contact.

Hydrogen bonding may arise where an organic molecule has an N-H or OH group and it is important at external and intermicellar surfaces. Electrostatic forces occur where organic cations are attracted to –ve charged surfaces and organic anions to the +ve charged edge surfaces (Tan 1998).

The pH of the solution will determine the charge on clay mineral suspensions. The pHs at which the clay has its isoelectro point is called the $pH_{ZPC}$. At this point the clays will have no exchange capacity. The $pH_{ZPC}$ kaolinite is pH 2-4.6 and montmorillonite pH <2-3. Below these pH clays will have anion exchange capacity. Bonding to Al and Fe oxides is more variable as Al and Fe oxides have high $pH_{ZPC}$ 5-9 values. Consequently the surface charges may be either -ve or +ve. At low pH, adsorption of $H^+$ ions results in a net +ve charge and this favours anion adsorption (Tan 1998).

Strong bonds between the layers of kaolinites limits adsorption to the outer surfaces and edge layers. Montmorillonites have active inner and outer layers, the inner layer being the greater. Inter layer adsorption depends upon molecular size, polarity and polarsability of the organic compounds (Tan 1998).

### 3.6.9.1 Adsorption of tannins

Johns *et al.* (1991) reported tannin adsorption by clays used to make acorn bread ranged from 5.6-23.7 mg/g, and to two CRM clays a kaolinite 2.5mg/g and a montmorillonite 21.2mg/g. They also reported tannin adsorption by African clays of 3.9-9.9mg/g. In both cases the utilised clays had kaolinites as their major mineral constituent with the acorn bread clays also containing montmorillonites. Adsorption of tannic acid by the Santa Rosa samples ranged from 4.86-7.43mg/g, and the CRM clays kaolinite (KGa-2) 6.52mg/g and montmorillonite (SWy-2) 50.64mg/g. This is a similar pattern to the acorn bread clays.

Tannic acid exhibits a strong affinity for Al. This leads to the formation of hydroxy-Al-organic complexes and/or ill-defined aluminosilicate complexes (Inoue *et al.* 1986). DL-tartaric, citric, and DL-malic acids behave in

a similar manner and so if present in physiological situation may compete with tannic acid binding. In addition to the chemical bonding between hydrox-Al-tannate complexes and clay particles the physical adsorption forces are additive in high molecular weight complexes. The aggregation of hydroxy-Al-clay particles is promoted by the tannic acid which is then retained within the complex (Huang 1995).

There are variable results for tannin adsorption in the literature. A high binding capacity for tannins by the geophagy material was reported using the tannin precipitation method (Setz *et al.* 1999). Wakibara *et al.*(2001) reported low affinity for tannins at 32-58% of extracted oak tannin in test solutions. Adsorption by the Santa Rosa samples was tannic acid (4.86-7.43mg/g) > gallic acid (2.05-3.60mg/g). Aluminium released in acidic conditions can form aluminium-tannate complexes with tannins which can then be adsorbed by montmorillonite clay minerals (Goh *et al.* 1986, Buondonno *et al.* 1989). Negligible amount of tannate binds to the clay surfaces in the absence of aluminium, whereas in the presence of Al, hydroxy-Al-tannate species easily adsorbed on clay surfaces. There are positively and negatively charged sites present in hydroxy- Al-organic acid complexes, (Goh *et al.* 1986). Tannic acid binding was highest for Santa Rosa Sites 1, 6 and 8, Figure 3.44, these were the sites identified as potentially containing traces of montmorillonite clays by the XRD performed at Rockwood Industries (Table 3.22). Tannins adsorption to the Al/Fe oxides rather than to the kaolinite mineral has been reported by (Siebner-Freibach *et al.* 2004). They found that adsorption of organic cations to montmorillonites occurs rapidly reaching a constant value < 1hr, however kaolinites had a very poor affinity and there was no significant adsorption after 3 days. This may relate to molecular size and structure.

Brightsmith *et al.* (2004) reported that the %< 4µm particles contributed 39.02% adsorption of tannic acid and 38.41% gallic acid adsorption. Pearson Rank correlation for Santa Rosa samples showed no correlation of tannin adsorption and particle sizes less than 31µm. However a weak correlation was found for adsorption vs Al released in the ICP-MS determinations. The results indicated that adsorption of 39.46% (tannic acid) and 47.05% (gallic acid) was in part related to the amount of Al detected but there are other factors involved.

### 3.6.9.2 Adsorption of alkaloids

Santa Rosa samples adsorbed 2.68-3.08mg/g quinine (Figure 3.41) and this was similar to the results of Mahaney *et al.* (2000) and Aufreiter *et al.* (2001) shown in Table 3.37.

Table 3.37 Published values of alkaloid adsorption by geophagy samples

| Reference | species | alkaloid | adsorption | Main mineral type |
|---|---|---|---|---|
| Aufreiter *et al.*(2001) | chimpanzee | quinine | 1.85-3.03mg/g | Kaolinite (1:1) clays, illite and significant levels of (2:1) montmorillonite type clays |
| | | atropine | 1.55-2.56mg/g | |
| | | lupanine | 1.41-2.34mg/g | |
| | | sparteine | 1.25-2.08mg/g. | |
| Mahaney *et al.* (2000) | humans | quinine | 3.07-3.41mg/g | mainly kaolinite minerals illite and minimal montmorillonite |
| | | atropine | 2.33-3.04mg/ | |
| | | lupanine | 1.34-2.77mg/g | |
| | | sparteine | 1.76-2.45mg/g | |
| Johns (1986) | humans | tomatine | 490 mg/mg pH2 | predominantly montmorillonite clays |
| Wakibara *et a*l (2001) | Japanese macaque | quinine | 9.1-11.33 mg/g | Moderate levels of kaolinites and illite |
| | | atropine | 8.1-10.13 mg/g | |
| | | lupanine | 6.93-8.69 mg/g | |
| | | sparteine | 6.56-8.20 mg/g | |
| Brightsmith *et al.* (2008) | birds | quinine | 27mg/g | kaolinite, montmorillonite |

Brightsmith *et al.* (2008) suggest that the montmorillonite was responsible for the much higher binding of quinine (Table 3.37) stating that montmorillonites bind 25 times more quinine than kaolinites. Brightsmith *et al.* (2008) also reported that proportion of clay sized particles accounted for 46% of adsorption of quinine. This suggests that in this case both types of clay mineral present and % clay sized particles both contribute to adsorption of quinine. Heat treating montmorillonite collapses the interlayer structure and heat treated material only adsorbed a similar amount to a kaolinite clay, suggesting most of the quinine binding occurs in the interlayer. The lack of inter-layer space is responsible for the lower binding in kaolinites.

The author's adsorption data shows the montmorillonite (SWy-2) adsorbing >30 times more quinine than the kaolinite (KGa-2) in accordance with the Brightsmith data. Sites 1 and 6 had the highest adsorption and these were tentatively identified as the only potential montmorillonite containing sites (Table 3.22).

Pearson rank correlation found no significant correlations for Santa Rosa sample adsorption of alkaloids and particles sizes below 31µm. Montmorillonites were not detected in the *Ateles* eating sites.

#### 3.6.9.3 Adsorption of flavonoids and phenylpropanoids

Adsorption of naringin was higher than that of chlorogenic acid. There were no determinations available in the literature to enable any comparisons to be made. However effective rapid removal of naringin, responsible for the bitter taste in citrus juices, has used modified organo-clay minerals (Arellano-Cardenas *et al.* 2012) adsorbing 60-72% at 25$^0$C and pH3. Ferulic acid, a phenylpropanoid closely related to chlorogenic acid, has been intercalated with an anionic montmorillonite like clay using ion-exchange mechanism but there were no data for a kaolinite mineral in the literature.

**In summary**:

The results suggest that the Santa Rosa geophagy samples would be of potential use in reducing exposure to PSM to varying degrees according to class of PSM and constituents in the samples. Whilst not undertaken there is much literature relating to the mitigation of other classes of toxins e.g. bacterial endotoxins (Brouillard *et al.* 1989), the mycotoxins aflatoxin (Phillips 1999) and zearalenone toxin (Sprynskyy *et al.* 2012) and the toxic lectin ricin (Jaynes *et al.* 2005). Ricin and mistletoe lectins have similar structure, belonging to the same class of compounds.

### 3.6.10 Summary of the findings and Conclusions

- Santa Rosa sample colours were markedly different from those in the bulk of the published material.
- XRD and IR results indicated the samples contained relatively little kaolinite. In contrast the literature analyses of consumed soils and termitaria material consumed by humans and a range of primates reported the principal mineral present was kaolinite.
- The measured particle size distribution is a further instance of a major difference in the material chosen by the *Ateles* compared to that reported for other primate species.
- The quantities of minerals released from Santa Rosa samples in acidic conditions do not support nutrient supplementation hypothesis.
- The samples may, through the release of Al3+, have potentially beneficial or deleterious effects for the monkeys, depending upon the amount consumed and the circumstances of

consumption.

- The results suggest that the Santa Rosa samples would be of potential use in reducing exposure to PSM to varying degrees according to class of PSM and constituents encountered in the diet.
- The IR results in combination with the lack of fungal or bacterial growth makes it unlikely that there would be any source of potential antibiotic effects. The possibility of Fe adsorption may assist in limiting establishment of infective organisms or reduce infection load.

## In Conclusion:

The physicochemical characteristics of the Santa Rosa samples showed a marked difference from the majority of the primate geophagy literature in the following areas: colour, absence or low detection levels of the clay minerals kaolinites or montmorillonites, and the low % of clay sized particles. There was a high level of consistency across the selected control sites and the confirmed eating sites.

With regard to the current hypotheses the remaining results indicated that the samples had little potential for detoxification for a range of example PSMs, were unlikely to provide nutritional support or act as an antacid. There remains the potential for the adsorption of antimicrobial toxins and free fatty acids both classes of compounds which may be linked to diarrhoeal symptoms. There was no detectable direct activity against bacterial organisms however there are several other mechanisms which may have an impact on establishment of infection in the GI tract. The possible abrasiveness of the larger particles may increase the rate of mucous secretion in effect reducing number of infecting organisms. Smaller sized particles may stimulate changes in the endothelial tissues and increase the viscosity of mucous secretions - limiting attachment of organisms to GI tissues. Finally there is the potential of a reduction in available Fe which may retard the growth of infecting organisms reducing virulence and hampering the establishment of infection.

# Chapter 4 Investigation of *Phoradendron* leaf

## 4.1 Introduction

This chapter relates to Specific Aim 2 (Section 1.11) to determine whether differences in the mineral content and the phytochemistry of the *Phoradendron* influence the selection of the *Phoradendron* species consumed. Additionally if such a selection suggests a possible function for the *Phoradendron* consumption and Specific Aim 3 (Section 1.11): To determine whether there is any interaction when geophagy follows *Phoradendron* consumption.

Several important factors led to the investigation of *Phoradendron*, and not any other leaf consumed. It has already been stated, (Sections 1.3.1 and 1.5), that the consumption required route adjustments, had seasonality, and was not related to fruit scarcity. Nor was it likely to be an important source of proteins as only small amounts (1-2 leaves) were consumed. The **regular** consumption of **mature** mistletoe leaves (>5 times/year) was also considered significant, as only one other example of **regular** leaf eating had been observed, and this was of young leaf (Table 4.1). The remaining examples of **mature** leaf consumption were occasional (<5 times/year). A further consideration was the nature of the mistletoe as a small parasitic plant species with a patchy distribution (Aukema 2003), being a very limited resource when compared to leaf eaten from the tree species. There is also the widespread perception that mistletoe leaf and berries are poisonous (Hall *et al.* 1986, Perez 2011).

Prior to the identification of the mistletoe species, it was thought that there were three mistletoe species occurring on three main host trees. The use by the monkeys mainly of a single host/mistletoe (*Manilkara chicle/Phoradendron quadrangulare*) combination was therefore considered highly interesting. Following identification (Section 1.4) two mistletoe species and three main host tree combinations were identified. There was more of the non-consumed second species potentially available and the leaves of non eaten mistletoe species (Figure 4.1, A and B) were larger than the eaten species (Figure 4.1, C).

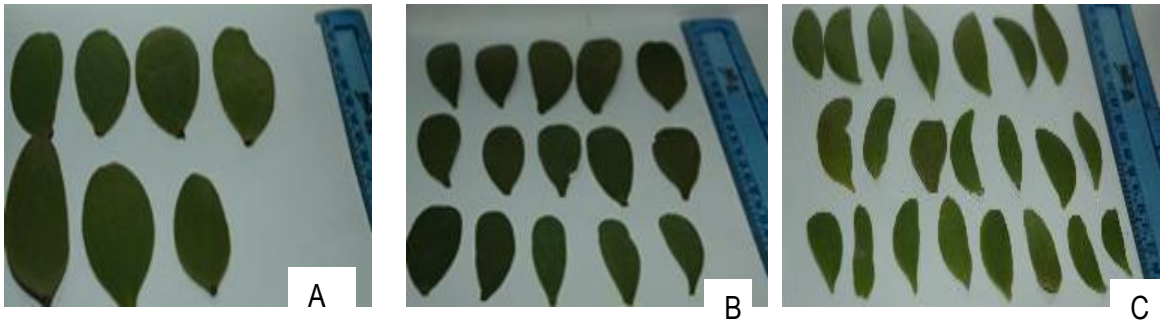



Figure 4.1 (A) *P. robustissimum* host tree *T. ochracea* (B); *P. robustissimum* from host tree *L. speciosa* and (C) *P. quadrangulare* host tree M. chicle (Author's photograph).

The differences between the species is illustrated by collection data as approx. 16g fresh weight mistletoe leaves required 45 leaves grown on host *Manilkara chicle* (MC), 18 leaves grown on host *Luehea speciosa* (LS) and 8 leaves grown on host *Tabebuia ochracea* (TO*).*

Table 4.1 lists the reported phytochemical classes of the **mature** or **regular** consumed leaves (i.e. >5 times/year) together with examples available from the literature of their ethnomedical or medicinal uses.

Table 4.1 Activities of leaf regularly consumed or mature leaf consumed by *Ateles* geoffroyi at Santa Rosa.

| Family: species | Leaf / frequency | Literature relating to constituents |
|---|---|---|
| Fabaceae: *Hymenaea courbaril* | young - **regular** | Reported to contain sesquiterpenes, tannins and flavonoids. (Welker *et al.* 2007). |
| Apocynaceae: *Marsdenia engleriana* | **mature** - occasional | No specific publications; flowers have sweet scent like jasmine (Jürgens *et al.* 2010). There are over 100 *Marsdenia* spp. in Asia, America and tropical Africa. Plants used to treat GI upsets, reduce fever, pain and treat diabetes and infections (Wiart 2007). |
| *Araceae: Syngonium angustatum* | **mature** - occasional | Leaf high in carotenoid lutein (Chandrika *et al.* 2010) and lutein. This linked to visual acuity (Stringham *et al.* 2010). |
| *Boraginaceae: Cordia panamensis* | **mature** - occasional | No specific publications but extensive worldwide use (Thirupathi *et al.* 2008). Review of Indian *Cordia* spp. indicated the presence of flavonoids, triterpenes, tannins, alkaloids and fatty acids with analgesic, anti-inflammatory, antimicrobial, antiviral, antifertility, hypoglycemic and antihyperglycemic activities (Jamkhande *et al.* 2013). *Cordia* leaves contain flavonoid artemetin which has proven anti-inflammatory activity (Sertie *et al.* 1990). *Cordia* leaf from Panamanian species contain naphthoquinones with antifungal activities against *Cladosporium cucumerinum, Candida albicans* and toxicity against two species of mosquito larvae. Larvicidal activity has been reported from leaves of the Brazilian species *Cordia globosa* (de Menezes *et al.* 2006). Other constituents in *Cordia* include: the triterpenes - cordialin A and B, and cordiaquinones (substituted naphthoquinones). Naphthoquinones activities include: antibiotic, antiviral, anti-inflammatory, antipyretic, antiproliferative and cytotoxic effects (Babula *et al.* 2009). No alkaloids (José *et al.* 1971). |
| *Fabaceae: Samanea saman* | **mature** - occasional | Multiple activities of anti-oxidant, antimicrobial, free radical scavenging, (Prasad *et al.* 2008, Ferdous *et al.* 2010, Chew *et al.* 2011). No alkaloids present in leaf (Chew *et al.* 2011). Traditional remedy for colds, diarrhea, headache, intestinal ailments and stomach ache (Duke *et al.* 1981, Duke 2008). The leaf infusion is used as a laxative in the West Indies (Ayensu 1981). |
| *Malvaceae: Apeiba tibourbou* | **mature** - occasional | Antioxidant (Souza 2012); no alkaloids (José *et al.* 1971). |

Table 4.2 highlights the potential medicinal/ beneficial properties of the range of **young leaves** that are eaten only **occasionally** by the *Ateles geoffroyi* at Santa Rosa (SR).

An important consideration for the investigation of the *Phoradendron spp.* was the availability and use of licensed *Viscum album* medicinal products. These products are used for supplementing surgical and pharmaceutical treatments e.g. Iscador®, Isorel® and Helixor® (Kovacs *et al.* 1991, Zarkovic *et al.* 2001, Enesel *et al.* 2005, Elluru *et al.* 2006, Urech *et al.* 2009, Weissenstein *et al.* 2014) in Europe and Asia. Ethnomedical literature for mistletoe use (Section 1.8.1) includes: European (Büssing 2000), African (Deeni *et al.* 2002), South American (Fernandez *et al.* 1998), Central American (Rivero-Cruz *et al.* 2005) the Indian subcontinent (Islam *et al.* 2004) and Asian mistletoes (Lee *et al.* 1999). In Germany mistletoe products including Helixor are included in an Arzneimittelrichtline (AMR) directive for therapeutic medication for cancer.

Worldwide, mistletoes are considered a keystone resource (Watson 2001), providing nectar, fruit and foliage and nesting sites to hundreds of faunal species. Three years after experimental removal of mistletoe there was a significant effect on bird species (Watson *et al.* 2012). Woodlands lost approximately 20.9% of the previous total species richness, 26.5% of woodland-dependent bird species and 34.8 % of the woodland-dependent residents, compared with moderate increases in bird species at control sites where no removal took place (Watson *et al.* 2012). *Ateles geoffroyi* are classified as an endangered species (Section 1.3) therefore the Santa Rosa National park authorities closely regulate any project which may have an impact on the wellbeing of the species present in the park. Arruda *et al.* (2012) reported an important interdependence between *P. quadrangulare* (Kunth) and birds of the species *Euphonia* (Fringillidae). *P. quadrangulare* is a major food for

and is almost exclusively dispersed by *Euphonia* spp. These types of factors were taken into account when the permissions to collect and export mistletoe were granted. The sample size and the duration of collection were controlled and sampling had to have minimal impact on each source plant.

Table 4.2 Activities of **young** leaf consumed **occasionally** by *Ateles geoffroyi* at Santa Rosa.

| **Family: Species** | **Leaf / frequency*** | **Literature relating to constituents** |
|---|---|---|
| *Anacardiaceae: Astronium graveolens* | young – occasional | Antioxidant antibacterial volatile oils, mainly monoterpenes.*(Rodríguez-Burbano et al.* 2010, Hernández *et al.* 2013, Hernandez *et al.* 2014). Activity against *Trichomonas* sp., *Giardia muris* and *Hexamita muris* (free living flagelate) (Martínez-Esquivel 2010). |
| *Apocynaceae: Forsteronia spicata* | young - occasional | No specific publications but Apocynaceae family has anti cancer activity (Wong *et al.* 2011a) and antiplasmodial activity (Wong *et al.* 2011b), constituents include chlorogenic acid (Wong *et al.* 2014). |
| *Fabaceae: Diphysa americana* | young - occasional | Historical use for snake bite, stings of the black widow, washing of wounds (Leonti *et al.* 2003). Mayans use leaf extract for treating wounds or ulcers and bloody dysentery (Duke 2008) and also used to induce sweating . |
| *Lauraceae: Ocotea veraguense* | young - occasional | This genus contains benzyl-isoquinoline alkaloids (Castro 1993, López *et al.* 1996). The major constituents in leaf oil were α-copaene (triterpene), δ-cadinene (sesquiterpene), spathulenol (a sesqiterpene), globulol and β-caryophyllene (sesquiterpene). Herbal uses of the Lauraceae include anti-inflammatory, antibiotic, antioxidant, anticarcinogenic and local anaesthetic activities (Chaverri *et al.* 2005). Spathulenol has insect repellant properties (Cantrell *et al.* 2005); globulol antibacterial activity; β-caryophyllene has anticancer activity (Legault *et al.* 2007). Veraguensin, a ligan (Crossley *et al.* 1962) has also been identified; it has activity against osteoclasts (Asai *et al.* 2012). |
| *Moraceae: Brosimum alicastrum* | young - occasional | Leaf is low in total polyphenols; total tannins; (Hernández-Orduño *et al.* 2012). No alkaloids (José *et al.* 1971). Activity against *Trichomonas* sp., *Giardia muris* and *Hexamita muris* (Martínez-Esquivel 2010). |
| *Moraceae: Ficus cotinifolia* | young - occasional | *F. cotinifolia* leaf extracts are used to treat wounds and bruises in Mexico and Central America; no antioxidant properties are exhibited (Ruiz-Terán *et al.* 2008). In Honduras used to treat worms and intestinal parasites (Lentz *et al.* 1998). Activity against *Trichomonas* sp., *Giardia muris* and *Hexamita muris* (Martínez-Esquivel 2010). |
| *Moraceae: Ficus hondurensis* | young - occasional | Similar to *F. cotinifolia* |
| *Urticaceae: Cecropia peltata* | young - occasional | Chlorogenic acid present, exhibits antidiabetic activity (Andrade-Cetto *et al.* 2005, Andrade-Cetto *et al.* 2010) |

Following consultation with Professor J. Kuijt, (University of Victoria, Victoria, Canada), Dr. G. Kite (Royal Botanic Gardens Kew, London) and Dr. S. Larsson (University of Uppsala, Sweden) it was realized that there would be insufficient material collected at any single time/plant to perform a typical phytochemical analysis and isolation of identified compounds. This project was therefore designed to attempt to detect any differences in classes of PSM between the eaten and non-eaten species that may/may not influence the choice by the monkeys. The impact of host tree on mistletoe phytochemistry was highlighted (Section 1.8.6). Should any difference in classes be detected this could be then be investigated further beyond this project, perhaps using material sampled from outside the boundaries of the national park, with reduced impact on the endangered species resident in the park.

The presence of several known classes of biologically active PSM in the Viscaceae (Sections 1.8.2-1.8.6) together with the possible concentration of certain minerals (Section 1.8.7) suggests that the consumption of the mistletoe may potentially be beneficial to the monkeys. Consumption may be an example of self-medication or nutrient supplementation.

The possibility that *Phoradendron* was being used as self-medication was supported by the limited

published information regarding the leaf chemistry of *Phoradendron* spp. This relates to the presence of phoratoxin, ligatoxin and mistletoe lectins (Sections1.8.2-1.8.3). Larsson (2007) reported the presence of phoratoxins in *P. quadrangulare* (the consumed species). Flavonoids and phenylpropanoids have also been identified (Section1.8.4) as have proanthocyanidins (Varela *et al.* 2004).

## 4.2 *Phoradendron* Leaf drying

### 4.2.1 Background

The drying method would need to be suitable for use with the limited on-site resources available for processing and subsequent storage of leaf material. The field site conditions precluded the use of any electrically powered drying ovens. The method also needed to be easily followed by local field research assistants. The method was required to provide rapid low temperature drying of the leaf to reduce degradation and prevent fungal or bacterial spoilage of the samples. It had to be sufficiently robust to cope with conditions during the rainy seasons when the relative humidity reaches >85% and temperatures in the dry season >33$^0$C (Table 1.8). A literature search for appropriate methods was undertaken. Chase *et al.* (1991) investigated the use of silica gel in tropical field site conditions concluding that silica gels were an inexpensive and reliable material to preserve field-collected leaves for molecular studies of variation in DNA. This paper had more than 200 citations and it was therefore decided to trial the use of silica gel.

### 4.2.2 Method Development leaf drying

The European mistletoe, *Viscum album,* is reported to contain the same classes of compounds as the *Phoradendron* spp. (Büssing 2000) and so was used for method development. Freshly harvested *Viscum album* was obtained from Tenbury Mistletoe Enterprise, Teme, Gloucestershire.

The leaves were removed from the stems and checked for obvious signs of damage and were then weighed using an analytical balance (Mettler AE 163 4dp). Orange-Green self-indicating silica gel, 0.5-1.00mm granules (GeeJay Chemicals Ltd., Bedfordshire England) was added. Two commonly used container types were trialled (Table 4.3).

Table 4.3 Drying conditions trialled

| **Container** | | **Silica gel (g)** |
|---|---|---|
| 1 | 20ml glass jar with lid | 20 |
| 2 | 20ml glass jar with lid | 50 |
| 3 | Unwaxed brown paper bag 21cm x 21cm* | No gel |
| 4 | Unwaxed brown paper bag 21cm x 21cm* | 20 |
| 5 | Unwaxed brown paper bag 21cm x 21cm* | 50 |

*Commonly used for drying and storing plant samples.

The containers were placed in a thermostatically controlled oven at 30$^0$C, examined and weighed daily and the silica gel replaced if there was any visible colour change. This was continued until a constant weight was achieved. The dried samples were then placed in airtight containers and stored in a cool dark cupboard at room temperature. Results are presented in Section 4.13.1.

### 4.2.3 Transfer to Santa Rosa of drying protocol

The protocol using glass containers was adopted for use on site, based on the trials with *Viscum album*. The materials necessary were shipped to the site and onsite a wooden box was constructed. It was found possible to maintain a temperature of $28^0$C $\pm 3^0$C within the box for the whole of the collection period. This also permitted storage of the leaf material in the dark thus reducing the possibility of light-induced deterioration of the samples.

During July 2007, 31 batches of leaf samples were collected by the author and processed on site using a sample to gel ratio of approx. 1:10. The samples were examined daily and silica gel replaced if it had changed from orange to green. If there was no green colour after 4 days, the leaves were removed and weighed and the silica gel replaced. This was repeated until dried to a constant weight was achieved. Once this was reached the dried leaf material was reweighed and transferred to Zip Lock Freezer bags (purchased locally) containing 10g silica gel, the bags rolled gently to remove air, sealed and placed in airtight storage boxes in the wooden box. Results are presented in Section 4.13.2.

Samples were examined regularly, by the author, during 4 weeks at Santa Rosa and the silica gel replaced if there was any change in colour. The samples were transported in the airtight containers to LJMU and examined upon arrival. The samples were monitored regularly and the sample bags only opened if it was felt that silica gel needed to be replaced i.e. if there was any colour change. Leaf was discarded during the project where it showed signs of colour change e.g. yellowing or blackening.

### 4.2.4 Selection of *Phoradendron*/Host tree

Lists of samples indicating host tree regularly sampled are presented in Table 4.4. The host trees were identified by the long-term research assistant Elvin Murillo-Chacon who also undertook the subsequent sampling and drying. In some cases, identification was a number and for others only a position within the trail system was available. The host tree selection included trees already identified as sites of *Phoradendron* consumption. A further two individual trees were identified for each of the three main hosts from within the normal range of the *Ateles.* In the case of the host *Tabebuia ochracea* (TO), samples were taken from two separate mistletoe infestations on a single tree. This was due to the height of the TO trees and subsequent difficulties of access to the *Phoradendron.*

It was hoped to collect mistletoe samples monthly for a 12-month period from *Luehea speciosa* (LS); *Manilkara chicle* (MC) and *Tabebuia ochracea* (TO) and occasional samples from a fourth tree used rarely *Guazuma ulmifolia* (GU), however local circumstances (availability of staff and their work load) prevented this from being achieved. In addition to the monthly collections, *ad hoc* samples were taken where the *Phoradendron* eaten was accessible for sampling. Lists of samples indicating host tree are presented in Table 4.5. The following sample numbers were host leaf; 112, 118,124,126 and 128. Samples 149, 190, 193, 218 had evidence of fungal growth and were discarded.

Table 4.4 Collection Date, Host tree and Sample Identification number

| ***Phoradendron* collected from Host Tree** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Date** | **MC 1** | **MC 21** | **MC76** | **LS 75** | **LS 20** | **LS 49** | **TO 50** | **GU 77** | **Ad hoc** |
| 13.02.07 | 101 | 103 | | | 106 | 105 | 108 | | |
| 20.03.07 | | | | | | | 110 | | |
| 19.04.04 | | | | | 117 | 120 | 111 | | |
| 12.05.07 | 125 | 127 | | | | 119 | | | |
| 12.06.07 | | | | | | | 123 | | |
| 18.06.07 | 130 | | | | | | | | |
| 21.07.07 | 132 | 133 | 139 | | | | 131 | | |
| 28.07.07 | | | | 135 | 134 | 138 | 136 | 137 | 140 |
| 07.08.07 | | | | | | | 144/145 | | |
| 14.08.07 | 147 | 146 | 148 | 142 | 143 | 141 | | | |
| 20.09.07 | 155 | 156 | 157 | 158 | 159 | 160 | 161 | 162 | |
| 22.10.07 | 165 | 166 | 167 | 168 | 169 | 170 | 171 | 172 | |
| 18.11.07 | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 185 | |
| 28.11.07 | | | 186 | | | | | | |
| 08.01.08 | | 191 | | | | | 189 | | |
| 09.01.08 | | | | | | | | | 192 |
| 28.02.08 | 195 | | | | | 194 | 196 | | 197 |
| 26.03.08 | | 198 | | | 199 | | 200 | | |
| 27.04.08 | 201 | | 202 | 203 | | 204 | 205 | | |
| 30.05.08 | | 207 | | | 208 | | 209 | | |
| 02.07.08 | 210 | | 211 | 212 | | 213 | 214 | | |
| 22.09.08 | 221 | | | | | | | | |
| 23.09.08 | 222 | | | | | | | | |
| 06.10.08 | 223 | | 226 | | | | | | |
| 16.10.08 | 225 | | | | | | | | |

Table 4.5 *Ad hoc* collections of eaten *Phoradendron* samples, Collection Date, Host tree and Sample Identification

| | **Tree Identifier** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Date** | **MC 2** | **MC 4** | **MC 5** | **MC 9** | **MC Q Trail WP6** | **MC CM WP6** | **MC Wpt 3** | **MC Wpt 2** | **MC Q trail BH** | **MC Junct. T & M trails** | **GU Aad** | **LS CM Wpt 6** | **LS Wp4** | **LS CM Wpt 5** |
| 15.04.07 | | | | 115-116 | | | | | | 113 | | | | |
| 16.04.07 | | | | | | | | | | 114 | | | | |
| 06.06.07 | | | | | 121 | 122 | | | | | | | | |
| 15.06.07 | | 129 | | | | | | | | | | | | |
| 29.08.07 | | | | 150 | | | | | | | | | | |
| 05.09.07 | 151 | | | | | | | | | | | | | |
| 06.09.07 | | 153 | 152 | | | | | | | | | | | |
| 19.09.07 | 154 | | | | | | | | | | | | | |
| 24.09.07 | | | | | 163 | | | | | | 164 | | | |
| 16.10.07 | | | | | | | | | | | | | | |
| 25.10.07 | | | | | | | | | | | | 173 | | |
| 08.11.07 | | 175 | | 174 | | | | | | | | | | |
| 13.11.07 | | | | | | | | | | | | | 176 | |
| 15.11.07 | | | | | | 177 | | | | | | | | |
| 28.11.07 | 187 | | | | | | 186 | 187 | | | | | | 188 |
| 28.05.08 | 215 | 216 | | | | | | | 206 | | | | | |
| 28.08.08 | | 217 | | | | | | | | | | | | |
| 11.09.08 | | | | 219 | | | | | | | | | | |
| 17.09.08 | 220 | | | | | | | | | | | | | |
| 02.10.08 | 224 | | | | | | | | | | | | | |

## 4.3 Method Development – preparation of digests

### 4.3.1 Digestion

The presence of any phoratoxin (a thionin) or lectins in the samples would contribute to any possible biological activity (Sections 1.8.2 - 1.8.3). In order to determine if either would potentially be present in the gastric digests a literature review of extraction protocols was undertaken.

Thionins were first extracted and described as thionin-lipid complexes (Balls *et al.* 1940). Subsequently it was reported that thionins themselves are insoluble in the majority of organic solvents (Garciá-Olmedo *et al.* 1989, Bohlmann *et al.* 1991, Florack *et al.* 1994, García-Olmedo 1999). Aqueous acetic acid extraction of mistletoes was the reported standard method for detection and isolation of mistletoe toxins (Samuelsson 1961, Samuelsson *et al.* 1967, Samuelsson *et al.* 1970b, Mellstrand *et al.* 1973, Samuelsson 1973, Thunberg *et al.* 1977, 1982, Samuelsson *et al.* 1997). A revised improved extraction protocol for thionins was developed which resulted in a concentrated extract free of large polysaccharides, polyphenols, enzymes and other large proteins (Claeson *et al.* 1998). This required multiple steps and large sample sizes. It was subsequently refined by several other authors (Broussalis *et al.* 2001, Tonevitsky *et al.* 2001, Tabiasco *et al.* 2002). Larsson (2007), using *Viscum album* simplified this to a two stage process: acetic acid extraction followed by polyamide (PA) filtration that produced a high yield of viscotoxin. PA has a strong affinity for tannins at acidic pH. This method produced extracts which were still rich in compounds other than thionins (S. Larsson personal communication).

Initial method development for HPLC and UV analyses in this study was undertaken using gastric extracts of the European mistletoe, *Viscum album* prepared under simulated gastric conditions (Section 2.3.5).

#### Method

Dried *Viscum album* leaf was examined to eliminate any with signs of deterioration or insect damage, leaves were cut into small slices and 500mg±10mg of cut leaf was added to a 15ml conical polypropylene centrifuge tube. 2ml of warmed ($37^0$C) of freshly prepared simulated saliva media was added to the leaf and allowed to stand for 3 mins then 6ml of warmed simulated gastric media was added (Sections 2.3.2 - 2.3.5). The tubes were capped, placed on an orbital mixer in an incubator at $37^0$C for 60 mins. The sample tubes were removed and placed in a refrigerator to cool and permit sedimentation of particles. The digest was centrifuged, 3,000rpm for 5 mins (Sigma-2-6E), the supernatant collected and filtered through 0.45 µm nylon filter (Whatmann) and divided into 8 x 250µl aliquots (1.5ml microcentrifuge tubes), 1ml was placed in an amber chromatography vial and the remaining approx. 1.5ml placed in an amber glass vial. The samples were then placed in $-80^0$C freezer for 6-8 hours before lyophilizing overnight.

### 4.3.2 HPLC method development

Following literature reviews relating to mistletoe analyses and information provided by Dr. G. Kite, Royal Botanical Gardens, Kew, initial HPLC gradients using variable proportions of acetonitrile/water/acetic acid mobile phases, flow rates and temperatures were trialled. The chromatograms of the digests were poorly resolved (resolution is a measure of the separation of compounds) using multiple different conditions and required run times >120 min. Hydrolysable tannins (HT) when analysed using RP-HPLC usually appear as poorly resolved humps (Mueller-Harvey 2001). The isomeric forms of condensed tannins (CT) have similar

polarities and consequently have overlapping retention times so elute as a poorly resolved hump with RP-HPLC. CTs are best separated by normal phase HPLC (Schofield *et al.* 2001, Romani *et al.* 2006). Ghost peaks (peaks caused by incomplete elution during prior runs) were found to occur in subsequent chromatograms. These occurred randomly during both column equilibration runs and sample runs. These peaks increased over time and were unpredictable in retention time had the potential to mask peaks of interest.

It became apparent during method development that it was not possible to perform HPLC analysis on the proposed gastric media digests due to the organic content (mucin, pepsin and amylase). Even after centrifugation, the solutions were viscous and could not easily be filtered. Injection of centrifuged but unfiltered digests caused blocking of the guard column in less than 10 analysis runs leading to increased pump pressures. This was not resolved by increasing the centrifugation speed or duration. Additionally the relatively high concentrations of inorganic salts in the media created 'salting out' problems in the HPLC pump and HPLC column, and the UV signal from residual organic content overwhelmed detection of the sample signal.

Phoratoxin had successfully been extracted using aqueous acidic conditions, as stated previously. Digests were prepared of *Viscum album* in 0.2M acetic acid and water adjusted to pH2 with HCl. The two types of mistletoe digests were then analysed using the previously trialled HPLC acetonitrile/water gradient conditions. This resolved the issues with pump pressure, blocking of the guard cartridges, signal strength from the samples but did not significantly improve the resolution and the results were not reproducible. The previously prepared *V. album* digests were light brown coloured when produced and became darker after less than 6 hours out of the freezer or whilst in the chromatography vials stored at $4^0$C. This darkening, thought to be due to oxidation of polyphenol compounds present may be responsible for the lack of reproducibility. HT in aqueous acidic conditions oxidise to produce brown colour and CT polymerise producing red/brown colours (Mueller-Harvey 2001).

All these problems resulted in the decision to digest the mistletoe leaves omitting both organic and electrolyte content. There was no detectible difference in the UV spectra or HPLC chromatograms between the acetic acid and pH2 aqueous digests. It was decided to use deionised water adjusted to pH2 with HCl, warmed to $37^0$C for the incubation media, this achieved a similar pH to that of the stomach. The chromatograms remained complex and poorly resolved.

A standard procedure for analysis of complex plant materials is to clean up the digests before HPLC analysis using Solid Phase Extraction techniques (Winter *et al.* 1984, Lorimer *et al.* 1996, Claeson *et al.* 1998, Collins *et al.* 1998). HPLC analysis using this two stage method which included PA- SPE identified phoratoxins as being present in *P. quadrangulare* (Larsson 2007), the mistletoe species eaten at Santa Rosa.

### 4.3.3 Solid Phase Extraction (SPE) - Method development

Polyamide resin (PA) is used to adsorb polar compounds (containing multiple -OH and -COOH groups) from aqueous or methanolic solutions. Polyamide is an effective tannin remover; HT type tannins are irreversibly adsorbed however non tannins (< 3 phenolic OH groups) are easily eluted (Wall *et al.* 1969). Polyamide Solid Phase Extraction (PA-SPE) has been used by previous researchers to remove tannins from mistletoe extracts (Samuelsson *et al.* 1967, Mellstrand *et al.* 1973, Mellstrand 1974, Collins *et al.* 1998, Larsson 2007). Elution is typically with water followed by 50% methanol and then 100% methanol. The methanol washing elutes polyphenols with 2-3 OH groups i.e. most flavonoids (Collins *et al.* 1998, Hostettmann *et al.*

1998). Removal of tannins has however been shown to eliminate certain types of enzyme inhibitory activity associated with plant extracts (Wall *et al.* 1996).

## Method

Work was undertaken to produce a standardised method to clean-up the samples, using Chromabond® 50mg PA (polyamide) cartridges and HPLC grade solvents. The following is an example of the preliminary procedure:

Step 1 - The PA cartridge was conditioned with 1ml deionised water. The water was added and allowed to drip through until it was just above the top frit, (Figure 4.2).

Step 2- Previously freeze-dried digest (approx. 6.ml) was reconstituted with 3ml of water, added to the conditioned cartridge and the liquid allowed to flow down the cartridge. A yellow front was observed migrating down the column. Elution was stopped once the yellow front reached the bottom frit, the collected eluent was analysed by UV, ferric chloride test, gelatine test and examination of the fluorescence of sample spots. Further 3ml quantities of water were added and fractions collected until the eluent was no longer yellow.

Step 3 – Methanol: water 50:50 was then added to the column. The collection was resumed until the top of the liquid layer was level with the top frit; the eluent was then tested as previously described. This was then repeated several times, collecting several fractions.

Step 4 - Once the UV of the eluent decreased substantially 100% methanol was introduced and the fractions collected and tested as previously; collections stopped once no UV spectrum was obtained.

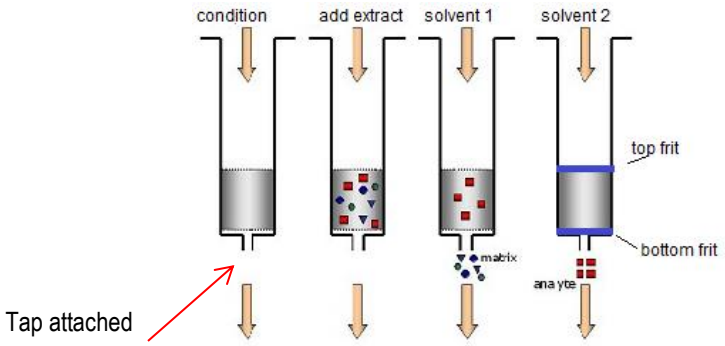



Figure 4.2 SPE typical procedure for sample clean up and fractionation (modified from Chromabond® product information)

The digests filtered extremely slowly and the polyamide in the cartridge became dark brown and blocked relatively quickly. A colourless eluate was obtained which showed only limited improved stability when stored at $4^0$C; however the eluate was more stable after lyophilisation. PA 500mg cartridges with greater capacity were obtained. Information from the manufacturer indicated that it was not necessary to pre-wet the PA when using aqueous solutions provided it was allowed to drip through slowly, < 1ml/min. Use of these improved filtration rates and reduced subsequent browning, suggesting an improved removal of phenolic/tannin compounds. The SPE process was applied to trial *Phoradendron* digests.

## Final procedure

4ml freshly prepared digest was added to a 500mg PA SPE cartridge and the liquid allowed to drip

through, until the solvent front reached the top frit. The eluent was collected in pre-weighed amber containers and measured (F1). 4ml MeOH added to column and allowed to elute, collected and weighed (F2). Two further 2ml volumes of MeOH added (F3) and (F4) were collected. 10µl original solution and of each fraction was analysed by UV spectroscopy, ferric chloride and gelatine tests were also performed. The fractions were also tested against *Bacillus subtilis* and *Staphylococcus aureus* Section 4.6. Results are presented in Section 4.13.3 and 4.13.4.

### 4.3.4. HPLC Instrument and conditions

Columns:

Optimal ODS-L, 25cm x 4.6 cm, 5µm particle size. (Capital HPLC Ltd, Broxburn, West Lothian, Scotland, UK. EH525NN). Optimal ODS-L has a slightly lower carbon loading (15%) than other C18 columns and is useful for the analysis of a wide range of hydrophilic compounds which are normally not retained by other C18 based packing material. It can also be used with 100% water and at low pH conditions.

Agilent Zorbax, SB C18 300Å column. This column is suitable for separation of peptides around 5000Da and for use in aqueous, low pH conditions.

HPLC Instrument:

Agilent 1100 Series HPLC, connected to UV Detector. Chromatograms were obtained using UV detection at wavelengths: 254, 280, 325 and 350nm. These can then be used to identify similarities and differences in chromatograms of the leaf samples.

Solvents and Mobile Phases:

Acetonitrile (ACN) 99.8%, for HPLC gradient analysis, glacial acetic acid, HPLC grade (Obtained from Fisher Scientific); High purity 18Ω deionised water. All mobile phases were filtered through 0.2µm nylon filter and degassed with helium, and maintained in amber containers.

Several different ACN/water gradient conditions were trialled and the results for both acetic acid and pH2 water digests were compared. An ACN:$H_2O$/0.1% acetic acid, HPLC gradient of 5 to 95% ACN, run over 70 min. with a 10 min hold at 95% ACN was found to have the better resolution of the peaks. Three samples of SPE treated *Phoradendron* digests from each host tree were then analysed under these conditions. The chromatograms of the mistletoe from host trees LS and TO remained less well resolved than those from MC or the *Viscum album.* The *Phoradendron* chromatograms showed little similarities to those of the *V. album* and there were obvious differences between the *Phoradendron* species.

At this time there were several problems worldwide in production and availability of acetonitrile (ACN) which made it impossible to continue use of ACN for further work. There was insufficient *Phoradendron* sample material available to permit further extensive method development using the alternative solvent methanol and therefore the developed gradient was modified using standard equal solvent strength substitution proportions form methanol (MeOH) in place of acetonitrile (ACN), (Table 9.7, p 426 in Snyder *et al.* 2002). It was not possible using MeOH:$H_2O$ to resolve or identify the potential thionin peptide peaks using the Zorbax column. There were many potential compounds with absorbance at 280nm remaining in the samples.

## 4.4. EDXRF analysis of *Phoradendron* leaf mineral content

### 4.4.1 Introduction

Non-destructive methods of assessing the health of crops and other vegetation are used in agriculture and in ecological assessment (Marguí *et al.* 2009). XRF has been used for many years to determine nutritional status and mineral tissue distributions (Swain *et al.* 2012) and detect mineral deficiencies (Khan 1970, Miah *et al.* 1999). The analysis of medicinal plants using Energy Dispersive X-Ray Florescence (EDXRF) has been used by many researchers including (Majid *et al.* 1995, Ekinci *et al.* 2004, Queralt *et al.* 2005, Behera *et al.* 2010). As a technique it is suitable for elements with atomic number >10 (Queralt *et al.* 2005) and is suitable for small samples as it avoids the complex acid digestion techniques required by other techniques such as Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) and Atomic Absorption Spectrometry (AAS). It is suitable for determinations in the range of µg-mg/g. XRF methods have shown high levels of reproducibility when compared against both ICPAES and AAS e.g. for the determination of K, Ca, Mn, Fe, Cu, Zn in *Anisum vulgare,* liquorice root *Glycyrrhiza glabra*, and *Artemisia herba alba* (Khuder *et al.* 2009).

The main advantages of EDXRF include:

i) the possibility of performing analysis directly on solid samples

ii) multi-element capability

iii) the possibility of performing qualitative, semi-quantitative and quantitative determinations

iv) relative speed and it is non-destructive

v) quantitative analysis in the absence of suitable reference standard

### 4.4.2 Instrument and Conditions

Analyses were performed using a Shimadzu EDX720. Table 4.6 lists the conditions and settings used. Both of the available scan ranges were utilised i.e. Ti-U and Na-Sc. These ranges cover the elements in the periodic table with atomic number > 11 capable of being analysed using EDXRF.

Table 4.6 Shimadzu EDX720 Conditions and setting

| | |
|---|---|
| **Collimator** | 10mm |
| **Count time/element** | 400 sec. |
| **Scan Range** | Ti-U |
| | Na-Sc |
| **Sample holder** | Universal 31mm open ended cups with Prolene® 4µ film support. |
| **Condition** | vacuum |

### 4.4.3 Sample Preparation

Leaf material was selected such that it was as flat as possible and had no visible areas of damage/discolouration. Leaf material was cut into suitably sized pieces to cover the majority of the surface film area. The dimensions and mass of each sample was recorded. Multiple layers of leaf were placed in the cups in order to produce a vegetal layer in the optimal range of range 0.8–2.5 mg/cm$^2$ (Marguí *et al.* 2009). Layers of intermediate thickness are used because uncertainties in values of the mass attenuation coefficients have less effect on the analysis results, the sensitivity is improved for low-Z elements, and secondary enhancement effects are less significant (Shimadzu EDX 720 Manual).

### 4.4.4 Quantitative Analysis

Quantification was performed utilising the Shimadzu FP Fundamental Parameter method available in the software. X-ray intensity is measured and quantitative analysis is performed using a theoretical calculation. This method is best suited to quantitative analysis where no Reference Standard is available, (Dr. Johannes Hesper, Shimadzu Technical Assistance, personal communication). The FP method of quantification has been used in plant analysis by several authors F, Na, Mg, Si, Al, P, Ca, Ti, U;(Omote *et al.* 1995). Mn, Fe, Cu, Zn, Br, Rb, Sr, Zr, Pb, Na, Mg, Al, Si, P, S, K, (Anjos *et al.* 2002). Na, Mg, Al, Si, F, S, K, Ca, Ti, Mn, Fe, Cu, Zn, As, Rb, Sr, and Pb (Queralt *et al.* 2005). Stephens *et al.* (2004) reported good comparisons with certified reference standards of plant materials for 30 elements, however with light elements e.g. Mg and Al there were large analytical errors in the 0.1-10mg/kg ranges. Results for elements (≥ 1ppm) are presented in Section 4.14.

## 4.5 Phytochemical Analysis of *Phoradendron* digests

### 4.5.1 Potential *Phoradendron* phytochemical classes

The tests undertaken were determined by the known classes of phytochemicals present in the Viscaceae (Sections 1.8.2-1.8.6) and which are potentially soluble in the aqueous pH conditions of the simulated gastric digestion process. The constraints imposed by sample collection size limited the investigation to distinguishing between the eaten and non-eaten *Phoradendron* species in the classes that were detected in digests under 'simulated gastric' conditions.

#### 4.5.1.1 Thionins

The presence of a phoratoxin (a thionin toxin) had previously been reported in *P. quadrangulare* (Larsson 2007). Dried *Phoradendron* samples from Santa Rosa, collected during 2006, had been analysed, (HPLC-MS) at Royal Botanic Gardens, Kew on behalf of Professor Aureli (Appendix 1.7, Table 2.1). The mass ions identified relating to phoratoxin, are listed in Table 4.7.

Table 4.7 Phoratoxin m/z ions identified (✓ < 1000 chromatography peak area units; ✓✓ > 1000 peak area units). Data supplied by Dr. G Kite, RBG/F. Aureli.

| | **Host Tree** | | | |
|---|---|---|---|---|
| **Phoratoxin m/z ion** | **MC** | **LS** | **TO** | **GU** |
| 1607 | ✓✓ | ✓ | | ✓ |
| 1593 | ✓✓ | ✓ | | |
| 1603 | ✓ | | | |
| 1610 | | | | ✓✓ |
| 1561 | | | | ✓✓ |

The method used by Larsson (2007) to detect phoratoxin required > 4g/extraction and whilst it enabled HPLC resolution there were no certified standards available to aid identification. Nor would it provide sufficient material for identification of individual thionins present using fractionation and the required subsequent Edman degradation and trypsin enzymatic digestion; and electrospray ionization tandem mass spectrometry sequencing (S. Larsson personal communication).

No characteristic phoratoxin *m/z* ions were detected for the two samples from host tree TO but both had been dried in an oven without temperature control. A mixture of oven drying and blue/pink silica gel had been

used for the drying methods with silica gel for the samples from host tree MC (prior to the involvement of the author). Therefore, the absence of phoratoxin cannot be assumed. It may be due to sample size and/or drying conditions. Lectins were not analysed at this time.

All chemical structures following were drawn using ChemBioDraw Ultra 13®

#### 4.5.1.2 Lectins

Lectin extraction methods range from water, TRISS buffer pH7.2 (Park *et al.* 1999, Oliveira *et al.* 2008), sodium chloride solution (Kumar *et al.* 1993), phosphate buffer (Luther *et al.* 1980, Lee *et al.* 1999, Pfüller 2000) and 20mM acetic acid (Peumans *et al.* 1996). The iso-electric points for mistletoe lectins I-III vary between pH5.8-7.9 (Pfüller 2000) and will therefore be soluble in acidic aqueous conditions.

#### 4.5.1.3 Alkaloids

Typical alkaloids found in plants contain one or more nitrogen atoms, usually in a heterocyclic ring, (Figure 4.3 a-b) and are basic. Alkaloid freebases are usually sparingly soluble in water and alkaloid salts are readily soluble in water. However this is not always the case as the freebase caffeine is readily soluble in water (Evans 2009a). Alkaloids commonly have significant physiological effects.

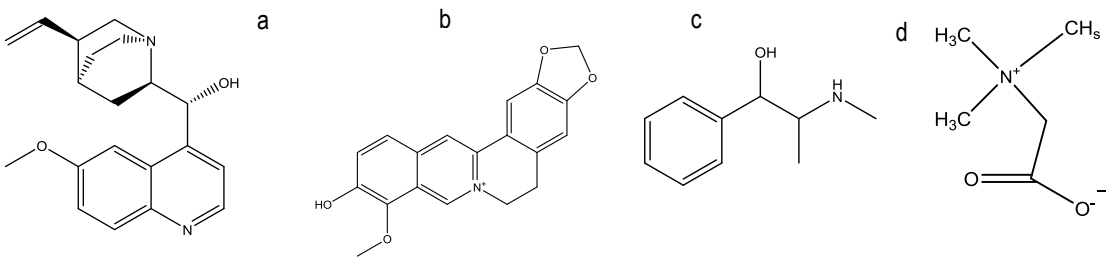



Figure 4.3 Structures (a) quinine, (b) berberine (c) ephedrine (d) rubrine-c.

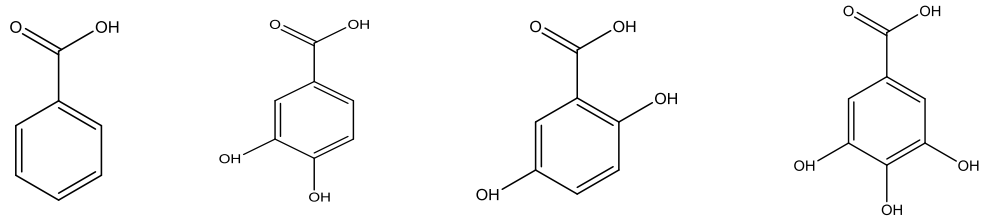
The true alkaloids have not been reported in mistletoes; however two amino-alkaloids have been reported by (Amer *et al.* 2012). It is unlikely that any alkaloids would be derived from the host trees (Section 1.8.5). The name 'proto-alkaloid' or 'amino-alkaloid' is sometimes applied to compounds such as ephedrine (Figure 4.3 structure c) and colchicine which lack one or more of the properties of the major alkaloids. Unlike true alkaloids the nitrogen is not part of the heterocyclic ring and these compounds are named for the amino acid from which they are derived (Evans 2009). Rubrine-C an alkaloid like compound (Figure 4.3 structure d) has been reported in *P. rubrum,* growing on a magnolia species (West *et al.* 1967).

#### 4.5.1.4 Phenolic compounds

The solubility of phenolic compounds is determined by the type phenols are present and how they are incorporated into the plant structure, e.g. complexity and occurrence within the plant as well as the polarity of the solvents used (Dia *et al*. 2010). Plant materials may contain phenolics varying from simple phenols, phenolic acids and anthocyanins to highly polymerized substances e.g. tannins all in differing quantities. Phenolics may also be combined with organic acids, sugars, amino compounds, lipids, structural components and other phenolic compounds (Bell *et al.* 1980).

Simple phenols are rare in plants but phenolic acids are universally distributed in plants. Phenolic

compounds may polymerise into diphenols and both HT and CT.



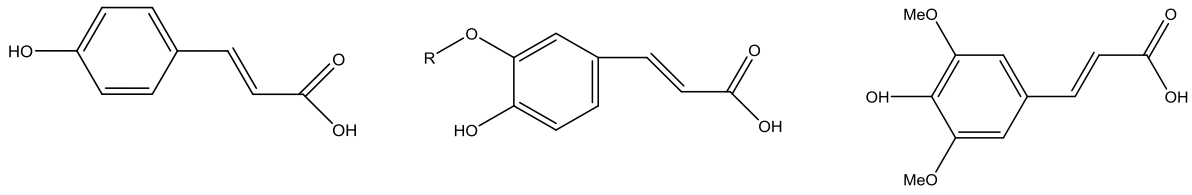
a) hydroxybenzoic acid b) protocatechuic acid c) genisic acid d) gallic acid

Figure 4.4 Structures of common phenolic acids,

The following phenolic acids are universal in the angiosperms; *p*-hydroxybenzoic, vanillic, syringic and protocatechuic acids (Bell *et al.* 1980) and the following (Figure 4.4) p-hydroxybenzoic, protocatechuic, gentisic and gallic acids have been identified in mistletoes (Vicas *et al.* 2012).

#### 4.5.1.5 Phenylpropanoids (PP)

Phenylpropanoids comprise three distinct groups: the hydroxycinnamic acid derivatives (HCA), the coumarins and the chromones (Ibrahim *et al.* 1989). The most widely distributed HCA are p-coumaric, caffeic ferulic and sinapic acids. PP are universal in plants and are the precursor for many other molecules. The most common PP example found in higher plants is caffeic acid. It is usually present as chlorogenic acid found as the depside (an ester formed from two or more phenol carboxylic acid molecules) but many other combined forms e.g. the methylated ferulic and sinapic acids are also found (Bell *et al.* 1980). These acids are commonly found in combinations with organic acids, sugars, amino compound which have increased solubility in aqueous conditions (Bell *et al.* 1980).



p-hydroxy-cinnamic acid caffeic and ferulic acids sinapic acid
R= H caffeic acid
R= $CH_3$ ferulic acid

Figure 4.5 Examples of phenylpropanoid structures

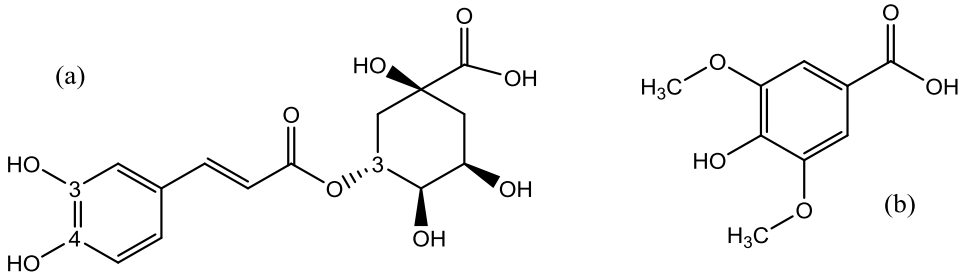


Figure 4.6 Structure of (a) chlorogenic acid, 3-(3,4-dihydroxycinnamoyl)quinic acid
(b) syringic acid, 3,5-Dimethoxy-4-hydroxybenzoic acid

Caffeic, syringic, ferulic, sinapic and chlorogenic acids, have all been identified in mistletoes (Panossian *et al.*

1998, Deliorman *et al.* 1999, Deliorman *et al.* 2000, Vicas *et al.* 2012), (Figures 4.5-4.6). PPs have a wide range of functions in the plants from anti fungal/antibacterial inhibition of plant growth and seed germination (Ibrahim *et al.* 1989).

### 4.5.1.6 Flavonoids

Flavonoids are polyphenolic secondary metabolites widely dispersed throughout higher plants, the most widespread being the anthocyanins, flavones and flavanols (Bell *et al.* 1980). Anthocyanins comprise the water-soluble plant pigments ranging from bright orange-pink-violet-blue. Flavonols and flavones are synthesized in plant tissues from phenylpropanoid precursors. The major flavonol aglycones found in plants are quercetin, myricetin, kaempferol and isorhamnetin, together with the flavones, apigenin and luteolin (Figure 4.7).

Flavonols and flavones are found conjugated to sugars, primarily glucose, rhamnose and rutinose (Markham 1989). The flavones identified in *Phoradendron* include apigenin and luteolin, (Dossaji *et al.* 1983, Varela *et al.* 2004) as C-6 and C-8-glycosylflavones and in the Argentinian mistletoe *Ligaria cuneifolia* 3-O-glycosylated quercetin was isolated (Wagner *et al.* 1998). The flavanone sakuranetin (Figure 4.8) has been isolated from Mexican mistletoe *P. robinsonii (Rivero-Cruz et al. 2005).*

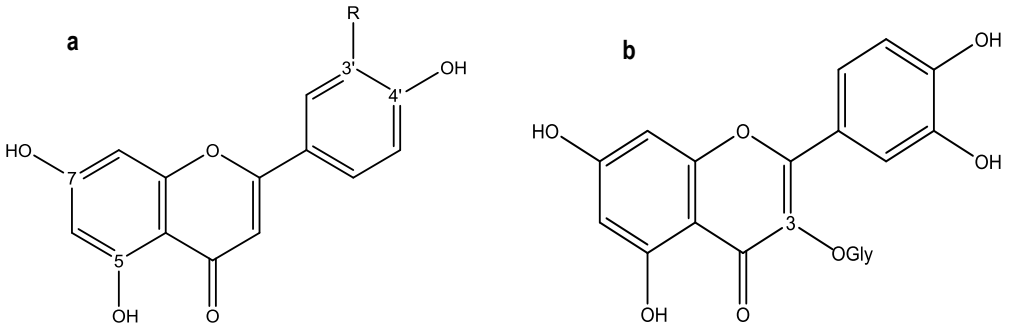



Figure 4.7 Flavones a) apigenin R= H; (5,7,4'-Trihydroxyflavone) b) quercetin 3-O-glycoside luteolin R= OH (5,7,3',4'-Tetrahydroxyflavone)

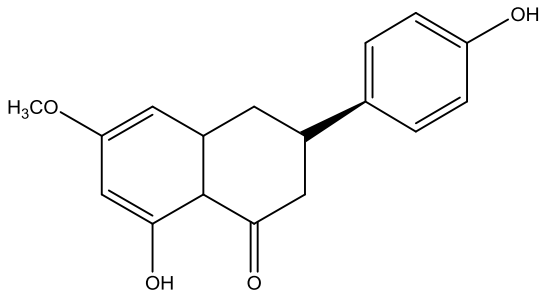



Figure 4.8 Structure sakuranetin (naringenin 7-methyl ether; 4',5-dihydroxy-7-methoxyflavanone).

### 4.5.1.7 Tannins

Tannins are oligomers with MW ranging from 500-20,000 with free phenolic OH groups**.** With the exception of some of the largest molecules, tannins are water soluble and have the ability to bind to proteins.

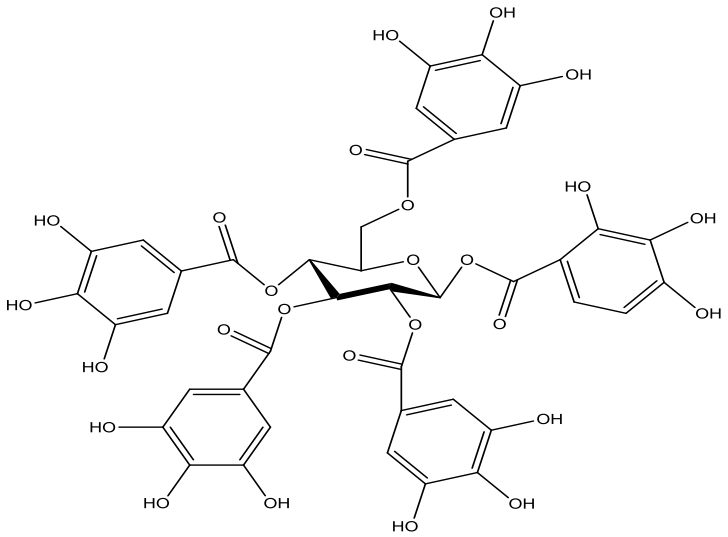


Figure 4.9 Structure β-1,2,3,4,5,6-pentagalloyl-O-D-glucose (PGG), hydrolysable tannin

Two classes, the hydrolysable tannins (HT) and condensed tannins (CT) are recognised in higher plants. The simplest HT usually consists of a central d-glucose core with at least 3 of the OH groups esterified with phenolic groups e.g. gallic acid (gallotannins) or ellagic acid (ellagitannins). The structure, Figure 4.9, is of a gallotannin, β-1,2,3,4,5,6-pentagalloyl-O-D-glucose (PGG). PGG can exist in many isomeric forms however this means that structural dependent properties including ease of hydrolysis and precipitation of proteins are also variable (Hagerman 2002). HT are considered potentially toxic when consumed in large quantities whereas CT may have beneficial effects (Waghorn 2008). Proanthocyanidins or condensed tannins (CT) are polyhydroxy-flavan-3-ol oligomers, flavonol subunits Figure 4.10, linked by C-C bonds (Figure 4.11). CT are one of the most abundant plant polyphenolics (Naumann *et al.* 2013).

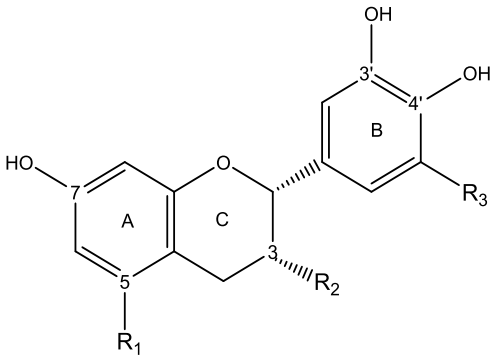


Figure 4.10 Example of the cis-orientation repeating unit in condensed tannins (CT). $R_2$ may be O-galloyl grouping

The combinations of groups at $R_1$ and $R_2$ are responsible for the different classes of CT, all of which respond differently to screening reagents (Schofield *et al.* 2001). The stereochemistry of CT may be either cis or trans depending on the orientation of the functional group located at the C-3 and C-4 positions relative to the C ring (Figure 4.10). Condensed tannin monomer units exhibiting cis stereochemistry are prefixed epi- (Naumann *et al.* 2013).

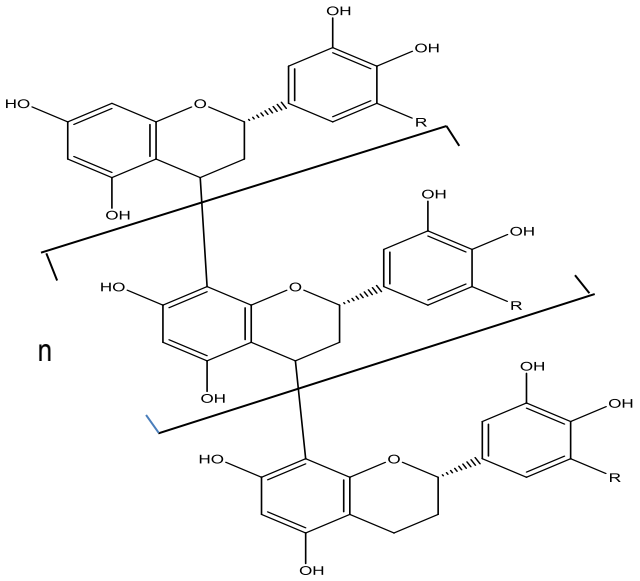



Figure 4.11 Example of a condensed tannin, where R= H or OH structure is a procyanidin, n = replicate structures.

The cis (epi) stereochemistry exists more commonly than that of trans (Foo *et al.* 1980). The 3-deoxyanthocyanidin, apigeninidin (Figure 4.12) has been isolated from *P. liga* (Varela *et al.* 2004).

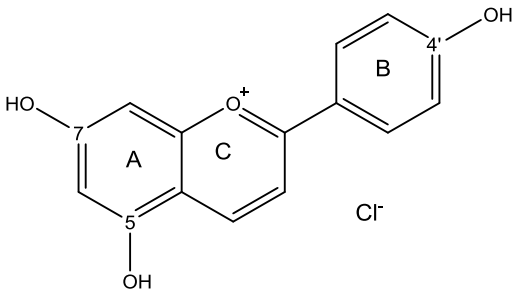



Figure 4.12 Structure apigeninidin chloride (4',5,7-trihydroxyflavylium chloride)

## 4.6. Summary of Analysis of *Phoradendron* digests

Figure 4.13 provides the proposed schematic of the analyses to be undertaken, following the non-destructive EDXRF analysis and determination of lectin content. This enabled the maximum number of tests to be performed on each individual sample, whilst providing comparative information.

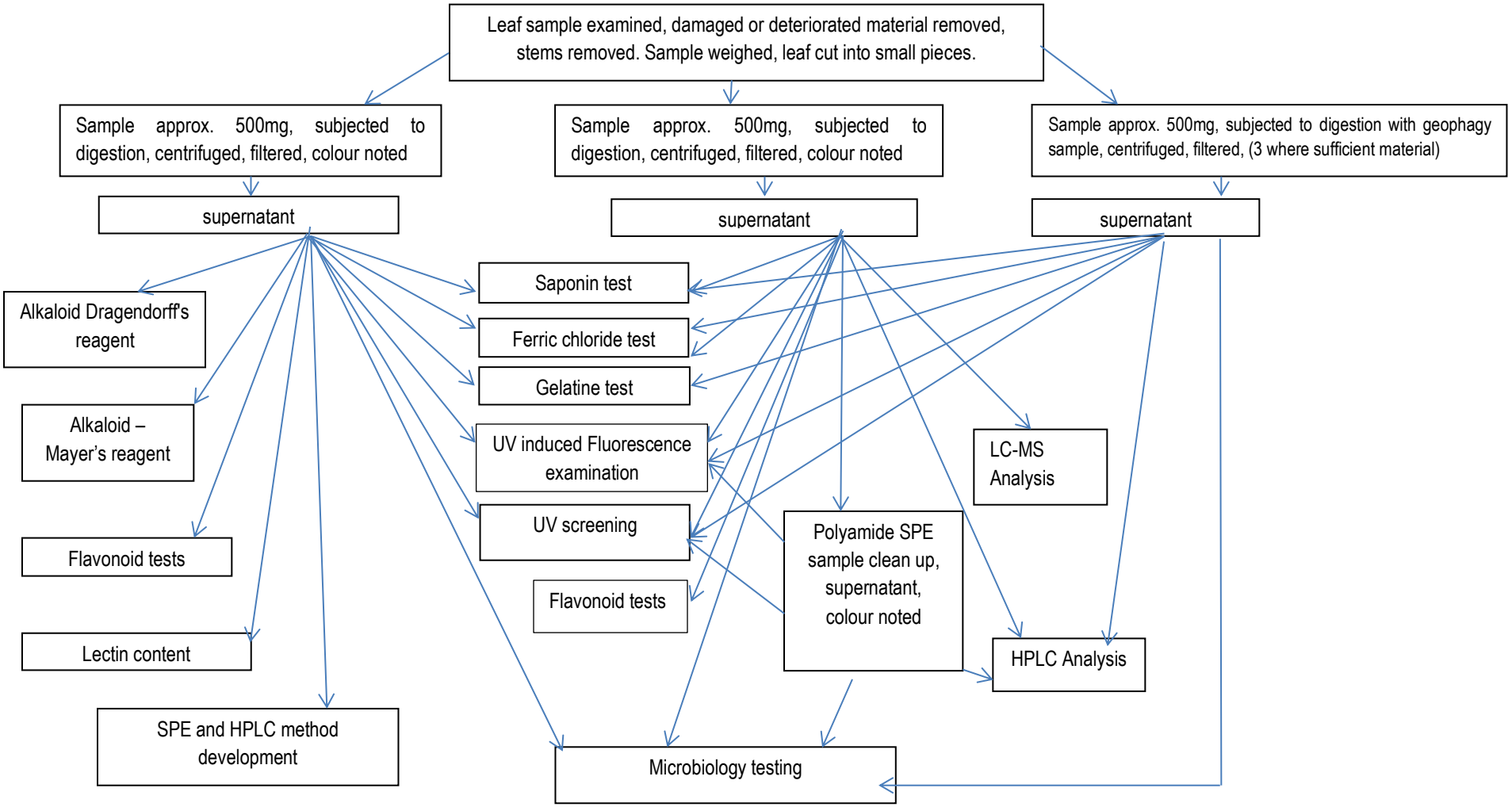



Figure 4.13 Schematic of digest analyses undertaken



134

### 4.6.1 Mistletoe lectin activity

The presence of lectins in *Phoradendron* spp. has been reported by (Endo *et al.* 1988, Endo *et al.* 1989, Varela *et al.* 2004). *V. album* lectins are glycoproteins with molecular mass approx. 60,000, composed of two sub units 34,500 and 29,000 connected by 5 disulphide bonds (Luther *et al.* 1980). Three mistletoe lectins (MLI, MLII and MLIII) with differing sugar specificities have been isolated and characterised together with a chitin binding lectin (Franz *et al.* 1981, Franz 1986, Lee *et al.* 1999, Franz *et al.* 2004). Mistletoe lectin extraction and purification from 100g *Viscum album* dried leaf produced total lectin yields MLI 99mg; MLII 18mg; and MLIII 45mg (Eifler *et al.* 1993). The amounts of *Phoradendron* material available made it impracticable to attempt identification of the specific types of lectin present or individual concentrations. However as these peptides are highly toxic, differential toxicity to mouse BD (brain derived) cells, MLI 4.1± 2.8ng/ml and MLII and III at 206-630 ± 20.3ng/ml (Eifler *et al.* 1993), it was felt necessary to attempt to confirm presence in the samples.

The sugar specificities are MLI – galactose (d-Gal), MLII - α-N-acetyl-D-galactosamine (GalNAc) and MLIII – both galactose and α-N-acetyl-D-galactosamine. All three lectin types bind to the sugar groups on the surface of human erythrocytes (rbc) causing agglutination. Lectins with high specificity for α-N-acetyl-D-galactosamine (GalNAc) agglutinate human group A cells and aggregation is not inhibited by d-galactose (d-Gal) only by (GalNAc). Lectins which are specific for d-galactose cause agglutination of human group B erythrocytes and aggregation is not inhibited by α-N-acetyl-D-galactosamine only by (d-Gal) (Franz *et al.* 1981).

#### 4.6.1.2 Agglutination Assay Method

White Wistar rat erythrocytes were selected for use as rats share human blood groups A and B determinant types (Vos *et al.* 1979). Rat erythrocytes show lectin agglutination which is inhibited by d-galactose and α-N-acetyl-D-galactosamine (Ali *et al.* 2004).

Fresh whole blood harvested from White Wistar rat heart was suspended in a solution of 1mg/ml K-EDTA (Potassium-EDTA), 1ml of whole blood suspension was removed and diluted with 1ml phosphate buffered saline (PBS) and centrifuged at 2500 rpm/1000g in a Thermo Cri3 centrifuge at $4^0$C for 5 minutes. The supernatant was removed and discarded, fresh PBS was added and the pellet gently re-suspended then centrifuged again. This process was repeated two further times until the supernatant was clear. The weight of the pellet was then determined and sufficient PBS added to produce a 1% w/w suspension (modified from assay in Magnusson *et al.* 1995). Following trials with *Viscum album* the erythrocyte concentration was reduced to 0.25%w/w and the amount of leaf digested was increased to 1.00 g ± 20mg. After filtration, 6ml was freeze-dried to concentrate. The freeze-dried digests were reconstituted with1.5ml freshly prepared PBS, and added to a 96 'U' shaped well plate according to the schedules (Table 4.8).

Table 4.8 Well plate schedule for lectin agglutination testing

| | **Positive control** | | | **Negative Control** | | | **Sample** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| plant igest | 50µl | 50µl | 50µl | | | | 50µl | 50µl | 50µl |
| PBS | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | | | |
| rbc in PBS | | | | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |

A lid was placed over the tray and the samples were examined at 30 min, 60 mins and 90 mins with the aid of a

light box. In order to limit the number of animals used only a small sample of digests were tested, these included leaf from host trees, TO, LS and MC, *Viscum album* and two *Phoradendron* samples from the three host trees. Two replicates were performed. Results are presented in Section 4.15.2

#### 4.6.1.3 Inhibition of agglutination

Minimum Inhibitory Concentrations (MIC) of sugars for mistletoe lectin was reported as 3-12μM/ml (Franz *et al* 1981) for a standardised lectin activity preparation. 36μM/ml solutions of d-galactose, α-N-acetyl-D-galactosamine (GalNAc), d-mannose in PBS were prepared and 50μl added to the 96-well plate as per schedule, (final concentration 12μM/ml) and either 50μl of aqueous leaf digest or PBS (Table 4.9). The plates were agitated to mix and left to incubate for 15 mins (modified from Magnusson *et al.* 1995). Freshly prepared rat red blood cells, prepared as previously detailed Section 4.6.1.2 were then added to the plates and the plates incubated for 90 mins as before. The plates were examined at 30, 60 and 90 mins. Results are presented in Section 4.15.3.

Table 4.9 Well plate schedule for inhibition of lectin agglutination

| | **Control** | | | | | **Sample** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| plant digest | | | | | | | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl |
| d-galactose in PBS | 50μl | 50μl | | | | | 50μl | 50μl | | | | |
| GalNAc in PBS | | | 50μl | 50μl | | | | | 50μl | 50μl | | |
| D-mannose in PBS | | | | | 50μl | 50μl | | | | | 50μl | 50μl |
| PBS | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | | | | | | |
| rbc in PBS | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl | 50μl |

### 4.6.2. Screening for presence of the remaining classes of PSM

Reagents or constituents used in preparing the reagents were analytical standard and were purchased from Sigma-Aldrich UK or Fisher Scientific UK.

#### 4.6.2.1 Alkaloids

A common method to isolate and identify alkaloids involves moistening powdered leaf with water then mixing with lime releasing any alkaloid salts then extracting with organic solvents, followed by partitioning with acidified water. This results in any alkaloids present in the plants being in the aqueous phase. Alternatively leaf is extracted with acidified $H_2O$/alcohol, shaking the aqueous phase with chloroform/organic solvent followed by the precipitation of free alkaloids with sodium bicarbonate or ammonia (Evans 2009a). Routine screening methods include the precipitation of alkaloids from neutral or slightly acid solution by Mayer's reagent, Wagner's reagent and Dragendorff's reagent. The screening tests are performed on the aqueous plants extracts (Coe *et al.* 1996, Deeni *et al.* 2002, Makkar *et al.* 2007, Evans 2009a, Fawole *et al.* 2009).

Mayer's Test - Mayer's reagent (mercuric-potassium Iodide TS US Pharmacopeia) was prepared as follows:

Solution A -1.358g mercuric chloride was dissolved in 60ml distilled water, Solution B - 5g potassium iodide was dissolved in 10ml distilled water. A and B solutions were mixed and diluted to 100ml with distilled water.

Reaction: Alkaloid + $K_2[HgI_4] \rightarrow$ [Alkaloid-H+][$HgI_3$]$^-$ ↓ (or [$HgI_4$]) salt

Method

500µl of freshly prepared filtered acidic digest was placed in a semi-micro test tube and a few drops of reagent added. The tube was shaken to mix. A positive response for the presence of alkaloids is the formation of a creamy white precipitate.

Dragendorff's Test - Dragendorff's reagent- (US Pharmacopeia) was prepared as follows:

Solution A- 850mg bismuth sub nitrate was dissolved in 40ml distilled water and 10ml glacial acetic acid added. Solution B – 8g potassium iodide was dissolved in 20ml distilled water. A stock solution was prepared by mixing 20ml solution A and B. The stock solution was stored in an amber bottle. Reagent was prepared by mixing 10ml stock solution, 20ml glacial acetic acid diluting distilled water to 100ml.

Reaction: $\text{Alkaloid} + BiI_4 \rightarrow (\text{alkaloid}^{+})_n(BiI_4^{-})_n$

Method

500µl of freshly prepared filtered acidic digest was placed in a semi-micro test tube and a few drops of reagent added. The tube was shaken to mix. A positive response is the formation of an orange-red precipitate. The colour may vary from red/black to pink and purple with different plant genus (Coe *et al.* 1996).

#### 4.6.2.2 Saponins

Following filtration the aqueous digests were shaken in centrifuge tubes and the formation of a foam/froth which persisted more than 10 min noted (Harborne 1998b).

#### 4.6.2.3 Phenolic compounds

##### 4.6.2.3.1 Gelatine precipitation test

This screening test detects the presence of tannins using the irreversible formation of a precipitate, due to formation of covalent bonds between the gelatine and the polyphenol (Le Bourvellec *et al.* 2012). The ability of proteins to bind polyphenols depends on protein size and amino acid composition. The proteins which most readily bind to tannins are relatively basic, large, hydrophobic with high proline content (Le Bourvellec *et al.* 2012). Mistletoe thionins and lectins are hydrophilic, tightly coiled peptides with relatively few proline residues (Soler *et al.* 1996, Soler *et al.* 1998, Johansson *et al.* 2003) therfore unlikely to contribute to precipitation.

Gelatine precipitation is greater for hydrolysable tannins than condensed tannins (Schofield *et al.* 2001). Proanthocyanidin/protein binding interactions are due to hydrogen bonding between the carbonyl functional group of the proline residues and both the phenol and catechol OH of the condensed tannin (Hagerman *et al.* 1981). Protein precipitation increases with molecular weight, degree of polymerisation and increases with increased hydroxylation of CT e.g. the number of galloyl groups. It is also influenced by stereochemistry and interflavanic linkage and the degree of aqueous solubility (Haslam 2007, Le Bourvellec *et al.* 2012). The method of Thomas *et al.* (1923) which is still widely used for screening of tannin content, was adapted to account for the small sample sizes.

Method

1g Granular Gelatine for Laboratory Use (Fisher Scientific), 10g NaCl (Analar grade Fisher Scientific) was dissolved in 100ml of sterile water. This was then divided into 5ml samples, and autoclaved at $105^0$C. The preferred proportions for the test are gelatine:tannin 1:2. It was not possible to use these proportions due to the unknown nature of the leaf tannin content. Several trials were undertaken and the following gave readable results: 500µl gelatine solution was placed in a clear glass bottle and 200µl of the reconstituted freeze-dried digest added, the bottles were allowed to stand for 15 mins and then the results recorded.

4.6.2.3.2 Ferric chloride Test

This is a traditional colorimetric test for phenolic compounds (Wesp *et al.* 1934, Soloway *et al.* 1952). The equation describes the reaction: $3ArOH + FeCl_3 \rightarrow Fe(OAr)_3 + 3HCl$

The optimal pH for flavonoid/metal ion complex formation, although strongly dependent on the features of the metal ion, is around pH 6. Complex formation at pH < 3.0 is difficult because the flavonoids are predominantly present in the undissociated form (Malešev *et al.* 2007). Solvent and pH may also determine the metal complexation site as either 3'-4' (B ring) or 3-4 (C ring) (Ren *et al.* 2008), Figure 4.10.

A transient or permanent coloration indicates the presence of a phenol or enol (an unsaturated compound with an OH group attached to one of the C atoms). Most water-soluble phenols give a red, blue, purple or green colour. The reagent gives a blue colour with pyrogallol groups and a green colour with catechol groups, therefore hydrolysable tannins produce a blue colour and condensed tannins a green-brown colour. In a digest the variable proportions of individual constituents will impact upon the colour formation e.g. where both types of tannin are present the initial colour may be blue which then changes to dark green (Evans 2009b).

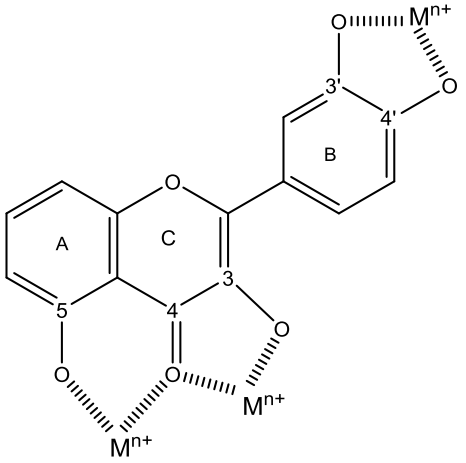



Figure 4.14 Potential phenolic binding sites for trace metals,

Optimum metal-binding occurs where structures contain hydroxy-keto group (a 3-OH or 5-OH plus a 4-C = O) (Pietta 2000), Figure 4.14, and a large number of catechol/gallol groups (Khokhar *et al.* 2003), producing an increased depth of colour.

Method

Ferric chloride reagent, (Ferric chloride, $FeCl_3$ solution, 60%w/v) was used in this test. The test had to be reduced in scale to reflect the small amount of sample available; positive reactions were obtained using the

following proportions: $FeCl_3$ reagent 40µl + 100µl pH2 water and 40µl freshly prepared filtered acidic digest, were placed in a white porcelain well plate. Where an initial negative result (no colour change) was seen further 20µl sample was added. The resultant colour was noted.

#### 4.6.2.3.3 Flavonoid content

Development of a yellow colour when a strong base e.g. NaOH is added to possible flavonoid in solution and which is reversed by addition of HCl is indicative of the presence of a flavonoid. The ionisation of the most acidic OH group in the presence of a strong base causes a bathochromic shift of both adsorption maxima. The ionisation is reversed by the addition of HCl. Any reduction in the intensity of the colour upon standing is indicative of 4'OH-3-OR substitution (Bohm 1999). Interaction with metal e.g. aluminium ions also produce a bathochromic shift due to formation of stable complexes with OH and keto groups (Figure 4.14) and acid labile complexes with ortho-dihyroxyl groups.

##### Method

500µl of freshly prepared filtered acidic digest was placed in a white porcelain well plate and a few drops, 0.1M solution of Sodium hydroxide (NaOH, Analar grade) in water added and the colour noted. The mixture was allowed to stand for 10 mins before addition of a few drops of conc. HCl acid and colour changes were noted.

##### Method

500µl of freshly prepared filtered acidic digest was placed in a white porcelain well plate and a few drops of a solution of 5% anhydrous aluminium chloride ($AlCl_3$, anhydrous) in methanol was added. Colour changes were noted.

#### 4.6.2.3.4 UV-induced Fluorescence

Flavonoids under 365 nm UV light, show dark yellow, green, or blue fluorescence, depending on the structural type, which is intensified and changed by the use of a variety of reagents e.g. ammonia vapour.

Green plants emit UV induced red fluorescence RF (630-700nm), far-red fluorescence FRF (700-800nm) both from chlorophyll, and blue-green fluorescence BGF (400-630nm) mainly from hydroxycinnamic acids, caffeic, ferulic and sinapic acids (Caerovic *et al.* 1999, Evangelista 2005). Other plant secondary metabolites which also contribute ultraviolet induced BGF, include: flavonols, flavones (except 5-hydroxyflavones), isoflavones, flavanones, chalcones, phenolic acids (salicylic, gentisic, ellagic), and folic acid (Caerovic *et al.* 1999).

Typical colours observed in 365nm UV light are: quercetin and its 3- and 7-O-glycosides: orange-yellow, luteolin and its 7-O-glycoside: orange; apigenin and its 7-O-glycoside: yellow-green (Andersen *et al.* 2010), chlorogenic acid and phenolic acids and phenylpropanoids bright blue (Ćetković *et al.* 2003).

##### Method

A drop from each freshly prepared aqueous digest was placed on filter paper. The paper was then

placed under a UV lamp (UVLS-28 230V 50Hz 8W 365nm / 254nm) and the colour noted at 254 and 365nm. The filter paper was then suspended in a chromatography tank containing a small volume of concentrated ammonia solution (35%$NH_3$ Solution) and any colour change noted.

Results for all phytochemical tests are presented in Sections 4.15.4-4.15.9 and summarised in Section 4.15.10, Tables 4.21-4.22.

## 4.6.3. UV characteristics *Phoradendron* digests

UV-VIS spectroscopy plays an important role in analytical chemistry and has widespread application in chemistry, physics and life sciences and pharmacokinetics (Perkampus *et al.* 2012, Takemoto *et al.* 2012). It is particularly important in the analysis of flavonoids (Andersen *et al.* 2010), procyanidins (Svedstrom *et al.* 2002) and has been used for mistletoes (Luczkiewicz *et al.* 2001). Single wavelength UV and multiple wavelength DAD-UV detection is widely used in HPLC analysis e.g. (Pan *et al.* 2002, de Rijke *et al.* 2006, Romani *et al.* 2006, Harnly *et al.* 2007, Lin *et al.* 2007, Diez *et al.* 2008, Takemoto *et al.* 2012).

In order to have a UV spectrum a molecule must contain functional groups which absorb light i.e. chromophores. Chromophores are commonly double or triple bonds e.g. conjugated double bond C=C absorbs between 200-400nm; the carbonyl groups, C=O of saturated aldehydes and ketones, have a weak absorption band between 270-300nm, examples of UV absorption maxima are shown in Table 4.10. However when a carbonyl group is connected to a conjugated system with a double bond, this shifts the absorption maxima to 300-350nm. Tryptophan, tyrosine, and phenylalanine, are the only amino acids that have a conjugated system enabling them to absorb UV light. Phenylalanine has a weak absorbance. The absorption maximum for these amino acids is ~ 280 nm. These are the amino acids detected in spectra of peptides and proteins.

Table 4.10 UV maxima for potential candidate compounds present in mistletoe leaf samples, (Harborne 1989a).

| **Class** | **Spectral maxima $\lambda_{max}$ (nm).** | | |
|---|---|---|---|
| amino acids, peptides | | 280 | |
| simple phenols | | 266-295 | |
| phenolic acid | | 235-305 | |
| hydroxycinnamic acids | 227-245 | | 310-332 |
| flavanones, dihydroflavonols | | 275-290** | 310-360* |
| flavones, flavonols | 245-270 | | 300-360** |
| hydroxycoumarins | 250-260 | 280-303 | 312-351 |

*May be small peak or just an inflection; ** most intense band

The UV spectra obtained for the digests are those of a mixture of the absorbance spectra of the components in the mixture. Absorbance is concentration dependent. Absorbance at any individual wavelength will be the sum of the absorbance contributed by each component at that wavelength. However a UV scan provides an indication of the ranges of potential constituents e.g. luteolin and its 7-O-glycoside have absorbance at 346/47nm whilst quercetin 362nm and its 7-O-rhamnoside at 372nm, these have been reported in different mistletoe species.

Both phoratoxins and lectins present in the digests will contribute to absorbance at 280nm. Phoratoxins B-F have either 41-46 amino acid residues and sequencing determined that each contained a single residue of tryptophan, tyrosine, and phenylalanine (Johansson *et al.* 2003). Mistletoe lectins A chain contains 254 amino acids, tryptophan (2), tyrosine (11), and phenylalanine (12) (Soler *et al.* 1996) and the B chain contains 246

amino acids, tryptophan (9), tyrosine (7), and phenylalanine (5) (Soler *et al.* 1998). Methanol was chosen as the solvent as it is the recommended solvent for the UV spectroscopic analysis of flavonoids, particularly for investigation of wavelength shift behaviour using $AlCl_3$ (Porter 1989a).

#### 4.6.3.1 Standardised UV Method

A standardised method was used to permit comparisons between individual samples and treatments. The polarity of a solvent, pH and temperature can alter the position of the adsorption maxima (Skoog *et al.* 2006). 10µl of freshly prepared acidic aqueous host tree leaf and mistletoe digests were diluted to 10ml in a volumetric flask with MeOH (HPLC grade) and analysed using scanning Perkin Elmer, LAMBDA 25 Scanning UV spectrophotometer with Winlab software. Results are presented in Section 4.16.

#### 4.6.3.2 UV spectra in the presence of shift reagents

Shift reagents are used to differentiate between different phenylpropanoids and flavonoids. The reagents interact with free-OH groups and carboxylic acid groups. The UV spectra of examples were determined using two standard shift reagents, to provide information on the classes of compounds contributing to the UV spectra. Two characteristic UV/Vis bands are present in flavonoids, Band I in the 300 to 550 nm range, arising from the B ring, and Band II in the 240 to 285 nm range, arising from the A ring (Figure 4.14). The effects of the shift reagent can be seen in Tables 4.11-4.12.

Table 4.11 Structural information from sodium acetate and sodium acetate/boric acid induced shift reagents (Markham 1989).

| **reagent** | **Shift observed** | **interpretation** |
|---|---|---|
| NaOAc | Band II +5 to 20nm, (reduced if 6- or 8-oxygenation present) | 7-OH |
| | Decreasing intensity with time | Alkali sensitive groups e.g. 5,6,7 or 5,7,8 or 3',4' - OH |
| $NaOAc/H_3BO_3$ | Band I +12 to 36nm, | B-ring o-dihydroxy |
| | Band I smaller shift than +12 to 36nm, | A-ring o-dihydroxy 6,7 or 7,8 |

NaOAc interacts with the most acidic OH group, commonly the 7-OH resulting in a bathochromic shift of Band II absorbance 250-285nm range. The addition of boric acid causes a shift in the Band I, 320-385nm due to interaction with o-dihydroxy groups.

Addition of $AlCl_3$ forms a complex with dihydroxy groups (3',4'-, 5,6- and 7,8-) and between OH at 3 or 5 and carbonyl at 4. The addition of the HCl degrades any dihydroxy complexes but not those involving the carbonyl group, e.g. Figure 4.14.

Table 4.12 Structural information from aluminium chloride and aluminium chloride/hydrochloric acid induced shift reagents (Markham 1989).

| **reagent** | **Shift observed in Band I, 300 to 550nm** | **interpretation** |
|---|---|---|
| $AlCl_3/HCl$ | + 35 to 55 | 5-OH |
| | + 17 to 20 | 5-OH with 6 oxygenation |
| | No change | No free 5-OH or possible 5-prenyl group |
| | + 50 to 60 | 3-OH possible with or without 5-OH |
| $AlCl_3$ | $AlCl_3/HCl$ plus 30-40nm | B-ring o-dihydroxy |
| | $AlCl_3/HCl$ plus 20-25nm | A-ring o-dihydroxy (additive to B-ring o-dihydroxy shift) |

Method - Sodium acetate (NaOAc)/boric acid ($H_3BO_3$)

10µl of freshly prepared acidic aqueous host tree leaf and mistletoe digests were diluted to 10ml in a volumetric flask with MeOH (HPLC grade, Fisher Scientific). A few crystals of NaOAc were added to a cuvette containing the methanolic solution of digest, shaken to mix and UV spectra obtained. This was followed by addition of a similar amount of $H_3BO_3$ crystals the cuvette shaken to mix and a second UV spectrum obtained. Typical shifts with which results were compared are presented in Table 4.11. Results are presented in Section 4.16.1

Method- Aluminium chloride ($AlCl_3$)/hydrochloric acid (HCl)

Two drops of 5% anhydrous $AlCl_3$ in methanol solution was added to a fresh cuvette of digest, (10µl in 10ml MeOH) shaken to mix and UV spectra determined. This was followed by the addition of two drops of 3M HCl acid, shaken to mix and spectra determined. Typical shifts with which results were compared are presented in Table 4.12. Results are presented in Section 4.16.1

## 4.7 Determination of biological activity

### 4.7.1 Assay methods Introduction

During the period 1966-2007 there were more than 420 publications investigating medicinal products in plants (Ríos *et al.* 2005). A review of over 500 publications cites anti-microbial testing as a precursor for investigations into work such as anti-inflammatory, anti-oxidant and antispasmodic properties of plant material (van Vuuren 2008). The majority of the publications have focused on identification of a single active compound however any activity may be due to interactions of more than a single compound with synergistic effects (van Vuuren 2008). The extraction method had significant effects on measured activities however it is more appropriate to attempt an extraction method which relates to the method of use such as liquid decoction (Ríos *et al.* 2005).

Brine shrimp *(Artemia salinas*) have been extensively used for toxicity testing. There are a number of critical factors with regard to the reproducibility of the results. Factors which need to be controlled are the origin of the strain, the temperature during incubation and hatching, time of harvesting of the larvae, the period of time between the harvest and the start of the bioassay, and the temperature and salinity of the medium during the test (Sorgeloos *et al.* 1978). Brine shrimp testing was advocated for plant extracts (Meyer *et al.* 1982) and has recently been used to determine the bioactivity and cytoxicity of aqueous extracts of widely used medicinal species in eastern Nicaragua (Coe *et al.* 2012). The literature review undertaken by the author showed that lectin binding sites in gut of *Artemia* are mannose specific (Vasconcelos *et al.* 1991, Arruda *et al.* 2013). Brine shrimp assay would not detect toxicity due to the presence of mistletoe lectins (Lee *et al.* 1992, Wacker *et al.* 2005). *Caenorhabditis elegans* (a nematode) is an alternative organism (Leung *et al.* 2008) however the required facilities to undertake its use were not available.

Lectins are widely used in the identification of bacteria (Cole *et al.* 1984, Costa *et al.* 2010) and the prevention of infection (Pistole 1981, Firon *et al.* 1983, Llovo *et al.* 1993, Pusztai *et al.* 1995, Lee *et al.* 1998). It was therefore decided to assess biological activity using bacterial screening as a precursor to any subsequent work on other potential activities. This was also appropriate in that many of the other potential PSM classes

also had antibacterial/mixed properties e.g. (Didry *et al.* 1999, Cushnie *et al.* 2005, Hatano *et al.* 2005, Ozçelik *et al.* 2006, Wang *et al.* 2010, Chew *et al.* 2011).

## 4.7.2 Microbiological Activity Testing Method selection

Most methods are based on using known concentrations of an antibacterial agent e.g. the Broth dilution test. This requires relatively large amount of reagents for each test. This was not practicable given the amounts of digest potentially available and the uncertainty as to constituents and the concentrations. An alternative was micro dilution method using 96 well trays, each containing 0.1 ml extract (Reller *et al.* 2009). This uses an automated plate reader to measure bacterial growth. This method was attempted however during incubation the *Phoradendron* digests changed colour becoming very dark brown. This prevented changes in absorbance/transmission measurements relating to organism growth being obtained. Following consultation with the technical staff, (P. Burgess and Dr. E. Gaskell) it was decided to trial the Agar Diffusion Assay method.

The Agar Diffusion Assay for microbial sensitivity is a standard method of The European Committee on Antimicrobial Susceptibility Testing – EUCAST. This method has been evaluated for use and employed to test antimicrobial activity in plant extracts e.g. (Wilkinson 2006, Valgas *et al.* 2007, Klancnik *et al.* 2010, Gautam *et al.* 2012). The test relies on the diffusion of the agent through the agar to inhibit the growth of the organism growing in it. The Zones of Inhibition (ZI) are taken to be representative of the susceptibility of the organism to the digest constituents.

### 4.7.2.1 Preparation of Seeded Agar plates.

To enable comparisons of the digests it was necessary to ensure that the agar plates contained standard number of organisms and that this could be replicated for all the tests. It was therefore necessary to seed each plate with the same number or organisms. This was accomplished using the McFarland standard method.

### 4.7.2.2 McFarland Standard

The turbidity and absorbance of a liquid culture increases as the number of organism increase. The McFarland standard has been used since 1907 and is still used by many regulatory bodies worldwide (American Society of Microbiologists/ ASM 5.14.1 Technical Bulletin). Each McFarland Standard equates to a particular Absorbance value relating to number of Colony Forming Units (CFU), for specific bacteria. The McFarland 0.5 Standard has a UV absorbance 0.08-0.10, at 600nm, and equates to the broth culture $<3 \times 10^8$ CFU. The number of CFU is confirmed by direct measurement of organisms using serial dilution and plating (Figure 4.15).

The McFarland Standard 0.5 was prepared as follows: Two stock solutions were prepared 0.18M Sulphuric acid ($H_2SO_4$) and 0.048M barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$). The barium solution was wrapped in foil and stored in the refrigerator. When the standard was required 50µl of $BaCl_2$ solution was added to 9.95ml $H_2SO_4$ acid. The solution was vortexed to mix and turbidity measured as absorbance at 600nm.

#### 4.7.2.3 Preparation of culture for standard agar plates

Overnight cultures in Oxoid Nutrient Broth (Code: CM0001) were grown to exponential growth phase. The turbidity (absorbance at 600nm) of the culture was measured and sufficient added to 9ml nutrient broth to produce an absorbance 0.08-0.10 i.e. standard culture equivalent to a McFarland 0.5 standard <300 x $10^8$ Colony Forming Units/ml (CFU/ml). The number of CFU in the McFarland 0.5 dilution was confirmed using standard serial dilution and plating methods (Figure 4.15).

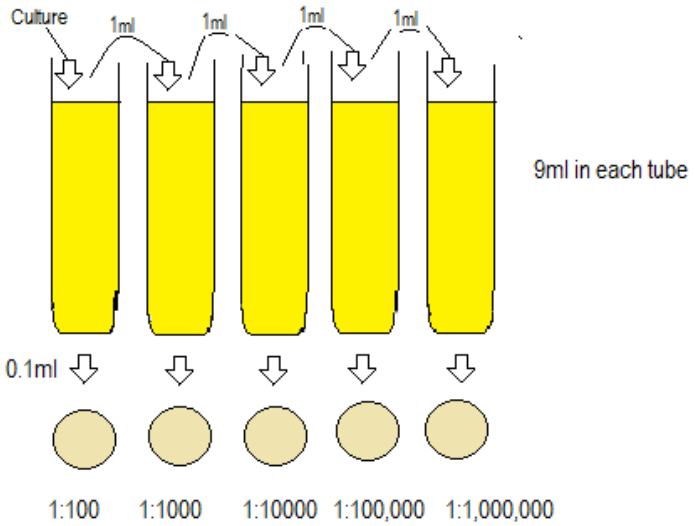



Figure 4.15 Serial dilution and plating. Number of bacteria/ml = number of colonies x dilution Drawn in Paint®.

The McFarland 0.5 culture was further diluted 1:100 and 1ml added to 19ml molten Oxoid Nutrient Agar (Code: CM0003) and the agar poured into sterile Petrie dish and allowed to set. This produced plates containing $10^5$ CFU/ml. Once cooled and set a sterilised No.2 cork-borer was used to create wells in the agar. Each well accommodated 50µl liquid.

#### Confirmation of number of organisms.

McFarland 0.5 broth culture <3 x $10^8$ CFU, was diluted according to the procedure (Figure 4.15) and agar plates (triplicate) were prepared for $10^5$, $10^6$ and $10^7$ dilutions. The plates were incubated overnight and the number of colonies counted and used to calculate the number of viable Colony Forming Units, CFU/ml in the broth, confirming the McFarland 0.5 standard number of organisms, ensuring that all digests were tested against equivalent numbers of viable microorganisms.

### 4.7.3 Method for preparation of *Phoradendron* and geophagy digests

8ml freshly prepared high purity deionised water adjusted to pH2 with HCl, warmed to $37^0$C, was added to 500 ±10mg *Phoradendron* leaf samples in polypropylene centrifuge tubes which were then incubated on an orbital mixer in an incubator at $37^0$C for 60 mins. The tubes were then placed in a fridge to cool and particles to sediment before being centrifuged in a Sigma 2-6 Compact Centrifuge, 12,500 rpm (15G) for 20 mins. 750µl of the supernatant were decanted into sterile Bijou bottles, frozen at -$80^0$C then freeze-dried; once dry the bottles were capped and stored in the dark in airtight containers. Digests were concentrated by reconstituting freeze-dried samples with 250µl pH2 water (sterile) immediately before use. 50µl of reconstituted supernatant was added to wells in agar plates seeded with a range of organisms.

#### 4.7.3.1 Preliminary Screening organisms selected

ATCC microbial strains available for testing were ones quoted in the United States and European Pharmacopeias' for QC testing.

Table 4.13 Micro-organisms organisms used in testing *Phoradendron* and geophagy digests

| Organism | strain/type number | Gram stain |
|---|---|---|
| *Bacillus cereus* | NCTC 6474 | +ve |
| *Bacillus megaterium* | NCTC 5635 | +ve |
| *Bacillus subtillis* | NCIMB 8054 | +ve |
| *Escherichia coli* | ATCC 8739™ | -ve |
| *Micrococcus luteus* | ATCC 10240 | +ve |
| *Pseudomonas aeruginosa* | ATCC 9027 | -ve |
| *Staphylococcus aureus* | NCTC 6571 | +ve |
| *Serratia marcescens* | ATCC 14756 | +ve |
| *Proteus vulgaris* | ATCC 6380 | -ve |
| *Candida albicans* | ATCC 10231 | yeast |

ATCC organisms obtained from LGC Standards. Other bacteria/organisms obtained from National Collection of Type Cultures (NCTC), The National Collection of Industrial, Food and Marine Bacteria (NCIMB) (Table 4.13). Organisms were grown in appropriate media and conditions to yield a culture suitable for seeding agar plates as previously described. Results are presented in Section 4.17.1, Table 4.29.

#### 4.7.3.2 Measurement of Zone of Inhibition (ZI).

Figure 4.16 is an illustration of a plate seeded with *Bacillus subtilis* 8054 and the wells treated with a known concentration of antibiotic, minocycline.

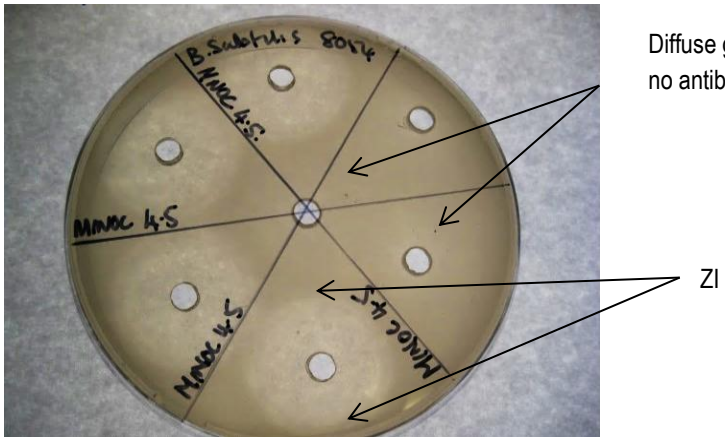



Figure 4.16 Well plate assay results for minocycline control antibiotic, ZI >20 mm, Author's photograph.

The agar plates were incubated for 24 h at 36ºC ± 1ºC, under aerobic conditions. After incubation, diffuse bacterial growth was observed in untreated areas in the agar. Inhibition of the bacterial growth, Zone of Inhibition (ZI) seen as clear zones around the wells was measured in mm. Plates were placed on a light box and ZI were measured using digital callipers (Figure 4.17). The ZI was measured across three axes and the mean value recorded.

Following the screening tests, two organisms were selected for subsequent testing, following the results of preliminary testing, Results Section4.17.1 Tables 4.29(a-b): *B. subtilis* and *S. aureus. P. quadrangulare (*60 Samples) and *P. robustissimum (*26 samples) were tested. Results are presented in Section 4.17.2, Tables 4.30a-c.

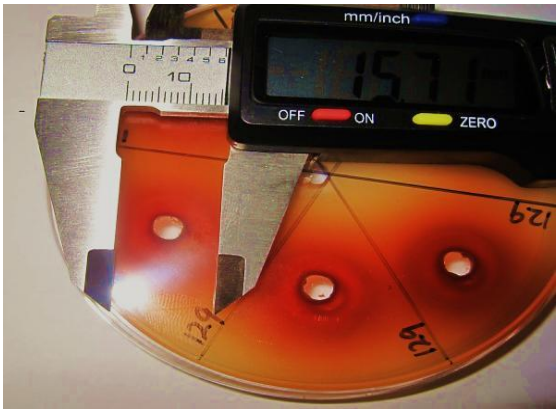



Figure 4.17 Example of the use of digital callipers to measure a *Phoradendron* plate ZI.

#### 4.7.3.3 Antimicrobial testing of geophagy digests

Interest in the influence of metal ions on growth of microorganisms and parasites has developed over the last 20 years e.g. (Bullen *et al.* 1978, Silver 1996, Tatara *et al.* 1998, Zhao *et al.* 1998, Glanfield *et al.* 2007, Cunningham *et al.* 2010, Otto *et al.* 2010, Otto *et al.* 2013). Geophagy samples were incubated in the absence of any leaf material in order to determine if there was any activity due to mineral ions potentially released during extraction. A leachate prepared from 10% suspension of an iron rich clay mineral had been tested against *E. coli* (Cunningham *et al.* 2010). The aqueous digests of geophagy samples tested by ICP-MS were deficient in iron content (Chapter 3). Samples from Sites 2, 6, 8, 9 and 10 were tested against the trial organisms. *Phoradendron* leaf samples were then incubated with geophagy material and digests similarly tested against the trial organisms to determine if there was any interaction e.g. loss of leaf activity or synergy.

##### Method

1.00g±50mg geophagic material and 8ml of warmed freshly prepared pH2 adjusted deionised water was added to each tube and mixed. The tubes were then placed on an orbital shaker in an incubator at $37^0$C for 60 mins. Following incubation samples were placed in a fridge to sediment, digest was decanted then centrifuged Sigma 2-6 Compact Centrifuge, 12,500 rpm (15G) for 20 mins and filtered through 0.45µm filter, the filtrate was freeze-dried and reconstituted as previously described. Reconstituted leachate was tested against *B. subtilis, S. aureus* and *E. coli*. Following the first determinations, the geophagy sample was increased to 2g ±50mg/8ml. Results are presented Section 4.17.3.

Direct application of 100mg geophagy sample (remaining from Loss on Ignition treatment) to pre-seeded plates was also undertaken, as there were literature reports of direct antibacterial activity from topical application of clays (Williams *et al.* 2010). Results are presented in Section 4.17.3.

#### 4.7.3.4 Antimicrobial testing of combined *Phoradendron* leaf and geophagy digest

##### Method

500±10mg portions *Phoradendron* leaves were placed in 15ml conical centrifuge tubes, with 1.000±50mg geophagic material from known eating site. The samples were treated as Section 4.7.3.3. Organisms were tested with both types of digests from the sample leaf sample in a single plate, to permit direct comparisons of effect of geophagy digestion. Results are presented in Section 4.17.3.

### 4.7.4 Parasitology - Faecal Sample collection and identification

One of the potential functions initially hypothesised for the *Phoradendron* consumption was that its use may be related to elimination/controlling a parasite load. It was intended to test gastric digests against examples of parasites identified in *Ateles* faecal samples.

*Ateles* faecal samples were collected opportunistically from identified monkeys between July 2007-October 2008. Samples were collected and stored in 10% formaldehyde/phosphate buffered saline solutions using standard protocols. Examples were subsequently examined by specialist staff Chesley Walsh, Dept. of Anthropology and McGill School of Environment, Montreal, Quebec, Canada. Further samples were analysed by technical staff under Dr W Bailey, Senior Principal Experimental Officer, in charge of Clinical Diagnostic Parasitology Laboratory at Liverpool School of Tropical Medicine, Liverpool, L3 5QA. The author was not able to test the aqueous digests against example parasites, due to the limited amounts of *Phoradendron* sample available. The parasites identified are presented in Section 4.22.

## 4.8 HPLC Analysis *Phoradendron* digests

### 4.8.1 Instrument and conditions

Following the initial method development Section 4.3.2-4.3.4 the remaining analyses used a gradient method using MeOH/water/acetic acid (Figure 4.18).

Gradient conditions:

After development work, the following gradient conditions provided the optimal conditions for analysis of the *Phoradendron* digests. Flow rate 1ml/min; column oven temp. $35^0$C. Gradient conditions (Figure 4.18).

$T_0$ – $T_5$ - Hold 5% MeOH: 95% HPLC water + 2% acetic acid; $T_5$ 5% MeOH: 95% HPLC water + 2% acetic acid gradient to $T_{60}$ –95% MeOH: 5% HPLC water + 2% acetic acid, $T_{60}$-$T_{75}$ Hold at 95% MeOH: 5% HPLC water + 2% acetic acid.

Column:

Optimal ODS-L 25cm x 4.6 cm id. 5µm particle size, pore size, details Section 4.3.4.

HPLC Instrument: Agilent 1200 Series HPLC,

UV Diode Array Detector (UV-DAD).UV-DAD settings: 280, 325 and 350nm UV detection. UV spectra recording at 215-560nm. 1200 series (FL) Fluorescence Detector: FL detector settings: Multiple scan excitation and emission during method development indicated that combinations of the following wavelengths produced FL chromatograms of varying signal intensities Excitation: 226, 265, 300, 330, 370nm, Emission 350,430,440, 452, and 540nm. Agilent Chemstation® Software Package was used to control the instrument and generate chromatograms.

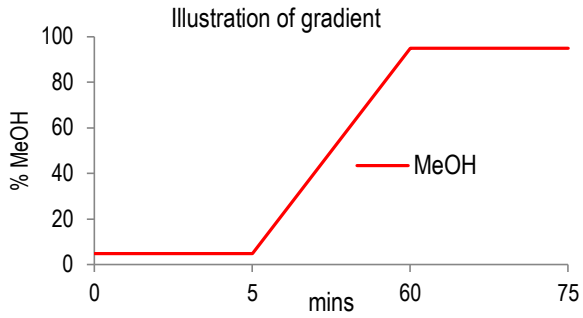



Figure 4.18 Graphical illustration of HPLC gradient conditions, MeOH proportion increasing

Solvents and Mobile Phases (Obtained from Fisher Scientific):

Methanol 99.8%, for HPLC gradient analysis; Acetic Acid, Glacial, HPLC grade; High purity 18Ω deionised water. All mobile phases were filtered through 0.2µm nylon filter and degassed with helium, and maintained in amber containers. Results are presented in Section 4.18.

## 4.9 Identification of constituent of major HPLC peaks

### 4.9.1 HPLC Standard Compounds selection

In an attempt to identify the components eluting in the 3 major peaks which were present in *P. quadrangulare* samples and absent in *P. robustissimum* samples particularly, potential reference standards were researched. Of particular interest was the peak which had highest concentration, which eluted at ~ 19 mins.

Table 4.14 The reported flavonoids for *Phoradendron* spp.(Dossaji *et al.* 1983, Varela *et al.* 2004, Rivero-Cruz *et al.* 2005, López-Martínez *et al.* 2012).

| **Species** | **Compound** | **UV data ±2nm** |
|---|---|---|
| *P. robinsonii* | apigenin-6-C-glucoside (isovitexin)* | 272, 337 in EtOH |
| | apigenin-6-C-galactoside | |
| *P. liga* | apigenin-8-C-galactoside | |
| | 4',5,7-trihydroxyflavone (apigenin)* | 268, 300sh, 338nm in EtOH |
| | apigenin-4'-O-glucoside | |
| | luteolin-6-C-xyloside-8-C-glucoside | |
| *P. tomentosum* | apigenin-8-C-glucoside (vitexin)* | 270, 296sh 334nm in EtOH |
| | 6-C-glucosyl-8-C-arabinosylapigenin (schaftoside)* | 273, 300sh, 333nm in MeOH |
| | 6-C-arabinosyl-8-C-glucosylapigenin (isoschaftoside)* | 273, 300sh, 333nm in MeOH |
| *P. brachystachyum* | 5,7-Dihydroxy-4'-methoxyflavone (acacetin ) | 271, 328 |
| | acacetin 7-methyl ether. | |
| | 3-desoxyprocyanadins | |
| Viscaceae | apigenin-7-O-glucoside* | 268, 332 in MeOH |
| | luteolin-6-C-glucoside* | 257, 270, 350nm in MeOH |
| | luteolin-7-O-glucoside* | 255, 267sh, 350nm in MeOH |
| | 3',4',5,7-tetrahydroxyflavone (luteolin)* | 254, 266, 292sh, 347 in MeOH |
| | 3-(3,4-dihydroxycinnamoyl)quinic acid (chlorogenic acid)** | 236, 244, 300sh, 329 in MeOH |
| | 4-Hydroxy-3-methoxycinnamic acid (ferulic acid)** | 235, 297sh, 324nm in EtOH |
| | 3,4-Dihydroxycinnamic acid (caffeic acid)** | 219, 236sh, 244, 299, 327 EtOH |
| | 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (quercetin) | 255,269sh, 301sh,370nm in MeOH |

The UV spectral data for the above potential candidate compounds (Table 4.14) were obtained from the

literature where available or from the Product Data sheets from the suppliers of the reference standards (Sigma Aldrich UK and Extrasynthèse S.A. France). These included compounds known to be present in the Viscaceae and those previously reported in *Phoradendron*. This data was compared with the UV spectral data of the *Phoradendron* digests.

The compounds listed in Table 4.14. Compounds marked with * had UV induced fluorescence brown, purple, orange or yellow (Markham 1989) and whose UV spectra data did not match that of the UV-DAD spectra for the major peaks present in the HPLC analysis of the *P. quadrangulare* digests. The three compounds listed in Table 4.14 marked with ** matched the *Phoradendron* digests fluorescence in response to UV light and the UV spectra. Fresh samples of these three standards were then purchased from Sigma Aldrich UK:

3-(3,4-dihydroxycinnamoyl)quinic acid (chlorogenic acid) - C3878 ≥95% titration

3,4-Dihydroxycinnamic acid (caffeic acid) - C0625 ≥98% HPLC

4-Hydroxy-3-methoxycinnamic acid (ferulic acid) – 99% HPLC.

## 4.10 HPLC-MS determination of accurate mass

### 4.10.1 Introduction

The mobile phase exiting from the HPLC-DAD detector is diverted to the mass spectrometer, where it undergoes Electro Spray Ionisation generating ions. Ions are generated by inducing either the loss or gain of a charge from a neutral species. The ions pass through a magnetic field where the ions are electrostatically deflected according to their mass-charge ratio (m/z), and then pass to the detector. The mass of a molecule is determined by measuring the mass-to-charge ratio (m/z) of its ion. The result is a spectrum that can provide molecular mass and even structural information. The mass spectrum provides information on the molecular weight and the fragmentation pattern is highly sensitive and can be used to determine the identity of compounds eluting from the HPLC. The use of tandem MS-MS generates more fragmentation ions through collision-induced dissociation of the molecular ions, thus providing much more information allowing structure elucidation (Sarker *et al.* 2005).

Analysis and identification of phenolic acids using a combination of HPLC-MS and Tandem Mass Spectrometry (HPLC-MS-MS) is widely used (Clifford *et al.* 2003, Simonovska *et al.* 2003, Jander *et al.* 2004, Frank *et al.* 2006, Bystrom *et al.* 2008, Orčić *et al.* 2014). The use of negative ion mode using 60V or lower cone voltage is preferred for some classes of phenolic compounds (Gioacchini *et al.* 1996, Pérez-Magariño *et al.* 1999, Robbins 2003, Waksmundzka-Hajnos *et al.* 2010). Negative ion mode produces a higher abundance of the molecular ion $[M-H]^-$ as deprotonation is high. There is little other fragmentation making the interpretation simpler. At higher voltages there is more fragmentation and a base peak due to higher abundance of low molecular mass ion (Pérez-Magariño *et al.* 1999).

Determination of the mass ion, comparison of fragmentation patterns of reference compounds and data bases and comparison of RT of a standard are used in identification.

### 4.10.2 Equipment and Conditions

#### Instrument

Waters Alliance 2695 HPLC system attached to MassLynx® 4 Mass Electrospray Ionisation Time of Flight Mass Spectrometer, (EI-TOF-MS).

The Optimal L column, previously used HPLC solvents and gradient conditions were used for the LC-MS analysis. Negative ionisation mode was used. Sample Cone voltage 50V; Solvation temperature $200^0$C: Acceleration 200. Research Technician: Dr N Dempster operated the instrument on behalf of the author. Results are presented in Section 4.19.

Determination of the Mass ion for the major peaks of interest in the digests produced from *P. quadrangulare* growing on host MC was undertaken. Data was also obtained for chlorogenic acid, 3-(3,4-dihydroxycinnamoyl)quinic acid (Sigma Aldrich). Results are presented in Section 4.19.

## 4.11 Antimicrobial activity of chlorogenic acid

Following the identification of chlorogenic acid in peak RT ~19 mins the following investigations were undertaken: Response of chlorogenic acid standard to shift reagents and antibacterial activity against *B. subtilis*, *S. aureu*s and *E. coli* bacteria as there were reports of activity against these organisms in several papers (Chirumbolo 2011, Hemaiswarya *et al.* 2011, Lou *et al.* 2011). Methods were those previously detailed Sections 4.7.2.1- 4.7.2.2.

50µl of chlorogenic acid solutions equivalent to adding 12.5, 25 and 37.5µg of chlorogenic acid were added to seeded well plates, prepared as described in Section 4.7.2.3. The results are presented in Section 4.20.1-4.20.2.

## 4.12 Estimation of chlorogenic acid content of *P. quadrangulare* digests

### 4.12.1 Calibration Data

An aqueous solution of chlorogenic acid was prepared and the diluted using volumetric glassware to provide solutions suitable for HPLC analysis, using the standard gradient conditions already detailed.

Calibration solutions ranged from 0.5-20µg/10µl. The mean peak areas from five replicate determinations for each sample were obtained. The calibration data are presented in Results Section 4.21.1

### 4.12.2 Chlorogenic acid Recovery, SPE elution process

This was undertaken in order to determine the effect of the PA SPE sample clean-up procedure on the amount of chlorogenic acid collected and hence its effect on chlorogenic acid in the SPE eluents obtained from the digests, Results are presented in Section 4.21.2.

#### Method

150mg/200ml (7.5µg/10µl) chlorogenic acid in deionised water was prepared. This concentration was chosen following determination of the calibration plot. 4ml was added to a 500mg PA SPE cartridge and the liquid allowed to drip through, and fractions collected as previously described Section 4.2.2.1 Final Procedure.

10µl of each fraction was analysed by HPLC. Results are presented in Section 4.21.3.

# 4.13 Results

## 4.13.1 Drying Method development

On examination after ~72 hours the silica gel in the samples marked ** were obviously green. The weights were recorded and all silica gel was exchanged. After ~96 hours there were a few green crystals in the samples marked *. The samples were reweighed and silica gel replaced (Table 4.15). There were no changes in dried leaf mass between 72-96 hours. The results suggested that a ratio of 2g fresh leaf to 50g silica gel, in the glass container, Method 2 produced the most consistent drying within 72 hours, in laboratory conditions. The mean weight loss was 65.47% SD 1.16.

Table 4.15 Comparison of *Viscum album* Drying method trial

| Drying method | 15.2.07 weight (g). | 16.2.07 weight (g). | 19.2.07 weight (g). | Cumulative % weight loss | 20.2.07 weight (g). | Cumulative % weight loss | Mean Total % Weight loss | SD |
|---|---|---|---|---|---|---|---|---|
| 1 | 4.29 | 2.42 | 1.60** | 62.61 | 1.60* | 62.71 | 63.19 | 1.33 |
| 1 | 2.18 | 0.94 | 0.77 | 64.69 | 0.77 | 64.69 | | |
| 1 | 2.59 | 1.16 | 0.98 | 62.15 | 0.98* | 62.15 | | |
| 2 | 2.70 | 1.29 | 0.93 | 65.62 | 0.93 | 65.63 | 65.47 | 1.16 |
| 2 | 3.69 | 1.99 | 1.32* | 64.15 | 1.32 | 64.23 | | |
| 2 | 1.52 | 0.65 | 0.51 | 66.40 | 0.51 | 66.54 | | |
| 3 | 5.14 | 3.14 | 1.88** | 62.46 | 1.88* | 63.36 | 64.56 | 2.24 |
| 3 | 2.59 | 1.47 | 0.83** | 67.90 | 0.83* | 67.91 | | |
| 3 | 1.23 | 0.73 | 0.46 | 62.42 | 0.46 | 62.42 | | |
| 4 | 3.46 | 1.94 | 1.24** | 64.07 | 1.24* | 64.12 | 65.76 | 1.97 |
| 4 | 2.23 | 1.25 | 0.72** | 67.97 | 0.72* | 67.95 | | |
| 4 | 1.75 | 0.85 | 0.61* | 65.22 | 0.61 | 65.21 | | |
| 5 | 3.21 | 1.91 | 1.04** | 67.72 | 1.03* | 67.84 | 65.86 | 1.85 |
| 5 | 2.63 | 1.17 | 0.90** | 65.57 | 0.90* | 65.55 | | |
| 5 | 1.20 | 0.57 | 0.43* | 64.21 | 0.43 | 64.18 | | |

** dark green silica gel. * orange silica gel with some green crystals, gel changed.

Further fresh samples of *Viscum album* leaf were purchased and dried using these conditions mean weight loss was 62.68% SD 2.57. The samples were stored in airtight boxes in a cool dark cupboard. Dried *Viscum album* leaf material was subsequently used to develop the HPLC analysis methods. The four samples of this batch remaining were examined after 16 months, before being used in further method development. There was no obvious visual deterioration in the samples.

## 4.13.2 Field Drying Trial

The results of the field drying trial using a leaf/gel ratio of approx. 1:10 indicated that a max of 14g/150g should be used for subsequent sample drying. This permitted rapid drying with the least number of gel changes (Table 4.16).

Following consultation with the National Park Authorities, it was agreed that approx. 5g dried sample material be permitted for export. The mean weight loss was then used to determine the sample size necessary to obtain approx. 5g dried material for the subsequent analyses. This however was not always achieved due to the amount and accessibility of suitable leaf available. An illustration of the differences required for sample

collection between the species can be seen as approx. 16g fresh weight *Phoradendron* leaves required 45 leaves from host *Manilkara chicle*; 18 leaves from host *Luehea speciosa* and 8 leaves from host *Tabebuia ochracea.* The more fleshy leaves of *P. robustissimum* required longer (7-8 days) to achieve dried to constant weight than *P. quadrangulare* (3-5 days).

Table 4.16 Drying results for *Phoradendron* samples

| **Host Tree Species** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MC** | | | | **LS** | | | | **TO** | | | |
| **Sample No.** | **Initial wt (g).** | **Dried Wt (g).** | **% Loss** | **Sample No.** | **Initial wt (g).** | **Dried Wt (g).** | **% Loss** | **Sample No.** | **Initial wt (g).** | **Dried Wt (g).** | **% Loss** |
| 125 | 4.8 | **1.8** | 62.5 | 119 | NA | **0.8** | NA | 123 | 43.7 | **12.9** | 70.5 |
| 127 | 2.7 | **1.1** | 59.3 | 134 | 15.2 | **5.5** | 63.8 | 131 | 41.2 | **14.9** | 63.8 |
| 128 | 10.2 | **4.6** | 54.9 | 135 | 17.9 | **7.1** | 60.3 | 136 | 14.7 | **4.8** | 67.3 |
| 132 | 24.4 | **7.1** | 70.9 | 138 | 14.0 | **5.5** | 60.7 | 137 | 14.2 | **4.9** | 65.5 |
| 133 | 13.5 | **4.5** | 66.7 | 158 | 18.2 | **6.7** | 63.2 | 161 | 26.7 | **9.3** | 65.2 |
| 139 | 10.8 | **3.6** | 66.7 | 159 | 20.1 | **6.9** | 65.7 | 162 | 22.9 | **7.9** | 65.5 |
| 155 | 23.2 | **6.2** | 73.3 | 160 | 23.0 | **8.5** | 63.0 | 171 | 20.5 | **7.2** | 64.9 |
| 156 | 16.5 | **5.2** | 68.5 | 168 | 18.4 | **6.8** | 63.0 | 172 | 19.6 | **6.2** | 68.4 |
| 157 | 19.5 | **5.3** | 72.8 | 169 | 20.8 | **7.6** | 63.5 | 189 | 12.9 | **4.8** | 62.8 |
| 165 | 17.0 | **4.7** | 72.4 | 170 | 18.8 | **7.9** | 58.0 | 196 | 10.7 | **3.9** | 63.6 |
| 166 | 16.8 | **5.6** | 66.7 | 190 | 9.3 | **4.8** | 48.4 | 200 | 19.5 | **8.0** | 59.0 |
| 167 | 16.6 | **4.3** | 74.1 | 194 | 12.5 | **6.6** | 47.2 | 205 | 10.0 | **3.9** | 61.0 |
| 191 | 7.2 | **2.4** | 66.7 | 199 | 8.8 | **4.1** | 53.4 | 209 | 12.4 | **4.7** | 62.1 |
| 195 | 8.9 | **3.1** | 65.2 | 203 | 10.3 | **4.4** | 57.3 | 214 | 13.3 | **4.8** | 63.9 |
| 201 | 9.7 | **3.3** | 66.0 | 204 | 9.6 | **6.1** | 36.5 | | | | |
| 202 | 8.9 | **3.4** | 61.8 | 208 | 9.0 | **3.4** | 62.2 | | | | |
| 207 | 8.6 | **2.9** | 66.3 | 212 | 9.0 | **3.3** | 63.3 | | | | |
| 210 | 9.8 | **3.3** | 66.3 | 213 | 10.7 | **4.7** | 56.1 | | | | |
| 211 | 8.0 | **3.0** | 62.5 | | | | | | | | |

## 4.13.3 SPE method Development

Preliminary fraction collecting of several *Phoradendron* samples was undertaken. The fractions were subjected to UV and a limited phytochemical and antibacterial screening. Sample 165 is presented as typical of the digests obtained from *P. quadrangulare*/MC host tree. The UV profile of each fraction illustrates a range of potential types of compound present. It is accepted that each fraction may contain multiple components. The spectra shown in Figures 4.19-4.20, illustrate the differences in character of the fractions particularly the change from F7- to F 8 and F9.

The UV, solution colour, $FeCl_3$ test and fluorescence results are variable due to the relative strength in response contributed by all the compounds present. These changes are also reflected in the colours of the eluents which change from (F2) yellow→(F4,5,6,7) colourless →(F8,9) deep yellow. There are also changes in the $FeCl_3$ and gelatine test results and the fluorescence colour observed (Table 4.17). Yellow coloured solutions are associated with both luteolin and apigenin (Favaro *et al.* 2007, Amat *et al.* 2009). The change in colour observed with the $FeCl_3$ test suggests a change from predominantly condensed tannins (green-brown colour) to hydrolysable tannins which produce a blue/black colour. This is supported by the position of the UV maxima. The maximum absorbance for F3-7 was 319-329nm whilst in F8 and F9 maximum absorbance occurs at 282 and 280nm respectively, with a shoulder around 325nm.

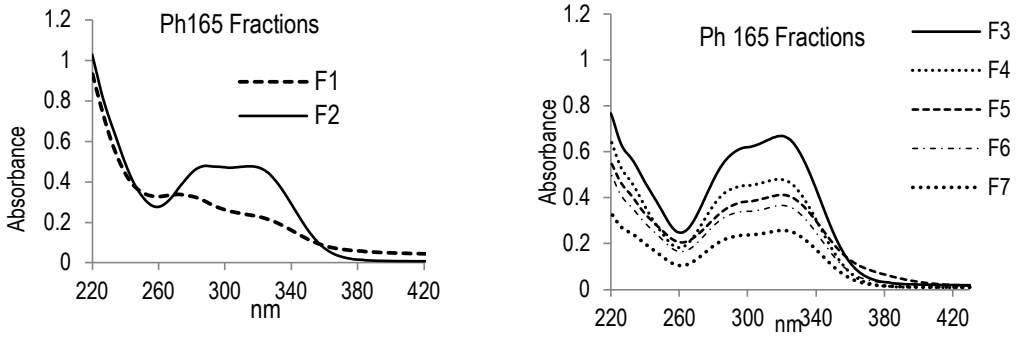



Figure 4.19 Method Development UV spectra Fractions 1-7, Sample 165

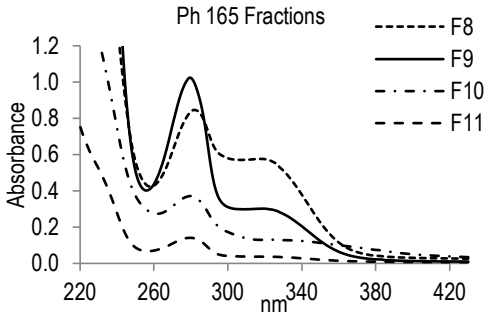



Figure 4.20 Method development UV spectra for Fractions F8-F11, sample Ph165,

Table 4.17 Colour description and observed test results for SPE produced Ph165 fractions.

| Fraction | solution colour | gelatine test | $FeCl_3$ test | Fluorescence at 366nm |
|---|---|---|---|---|
| F1 | colourless | + | neg | VioletBlue |
| F2 | yellow | +++ | +++ green | VioletBlue |
| F3 | yellow | +++ | +++green | VioletBlue |
| F4 | colourless | + | + green | VioletBlue |
| F5 | colourless | + | + green | VioletBlue |
| F6 | colourless | slight haze | + green | VioletBlue |
| F7 | colourless | slight haze | + green | bright Blue |
| F8 | deep yellow | +++ | blue black→dk green | Blue/yellow green outer ring |
| F9 | deep yellow | +++ | blue black→dk green | Blue/yellow green outer ring |
| F10 | pale yellow | | blue black | dark red |
| F11 | v pale yellow | | blue black | yellow |

The gelatine response is indicative of the presence of procyanidins (CT). Gelatines precipitate polymeric tannins with the precipitation of tannins by gelatine increasing with the degree of polymerization and the number of gallic units bound to the tannins (Maury *et al.*2001). Ferric chloride results suggest the presence of simple phenolics, HT, and CT in the different fractions. Violet blue fluorescence is associated with hydroxycoumarins, bright blue fluorescence with hydroxycoumarins and caffeoyl phenylpropanoids. Flavones and flavonols - dark red/purple, yellow - various different flavonols and flavonol aglycones and hydroxycoumarins and yellow-green fluorescence is seen with apigenin-7-O-glycosides (Markham 1989, Harborne 1998a). These preliminary results suggest complex mixtures of phenolic constituents are present in the *P. quadrangulare* digests.

The spectra (Figures 4.21a-b) and data (Table 4.18) are also representative of those obtained for *P. robustissimum* from (a) LS and (b) TO host trees.

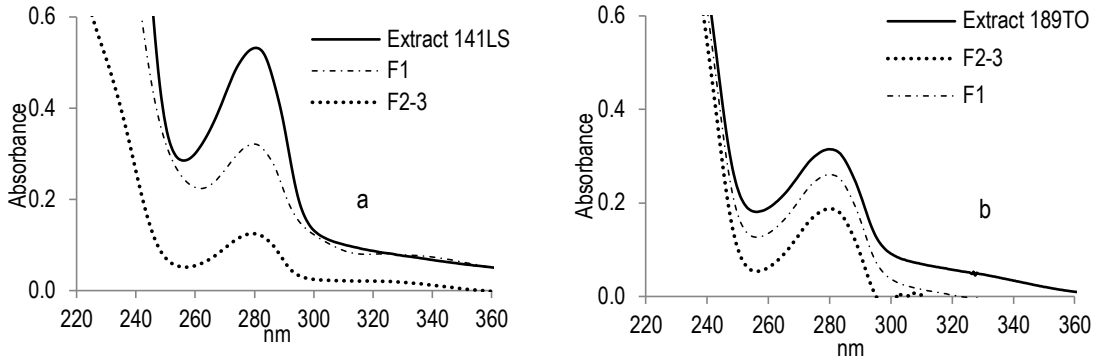



Figure 4.21 Method development UV spectra for *P. robustissimum* Fractions F1-3, Sample141LS (a) and 189TO (b).

The maximum absorbance for the digest and the SPE treated fractions 141LS and 189TO *(P. robustissimum)* were at 280nm.The peak was broader and less intense in the *(P. robustissimum)* TO samples (Figure 4.21b).

Table 4.18 Colour description and test results for SPE produced *P. robustissimum* 141LS and 189TO digest and fractions.

| | Fraction | solution colour | gelatine test | $FeCl_3$ test | Fluorescence at 366nm |
|---|---|---|---|---|---|
| Sample 141LS | digest | 'tea coloured' | neg | neg | dull yel/Bright blue ring |
| | F1 | pale brown | neg | neg | dull yel/Bright blue ring |
| | F2-3 combined | slight brown tint | neg | neg | dull yel/Bright blue ring |
| Sample 189TO | digest | pale brown | neg | neg | dull yel/Bright blue ring |
| | F1 | slight brown tint | neg | neg | dull yel/Bright blue ring |
| | F2-3 combined | V slight brown tint | neg | neg | dull yel/Bright blue ring |

Absorbance for Fractions 4-5 were < 0.1 and therefore data are not included. There was no visible shoulder in the 319-325nm region. The lack of reaction with both ferric chloride and gelatine may be a concentration effect and or constituents. The gelatine may be due to differences in constituents as the precipitation is greatest with CT. In the *P. quadrangulare* sample the absorbance were >0.3 for fraction 5 compared to <0.2 for F2-3 in *P. robustissimum* fractions. The fluorescence and UV spectra are suggestive of hydroxycoumarins in *P. robustissimum.*

## 4.13.4 Preliminary Antibacterial testing SPE elution fractions

### 4.13.4.1 Antibacterial Results *P. quadrangulare*

Zones of inhibition (ZI) for *B. subtilis* seen for fractions from the three *P. quadrangulare*/(MC) samples were very similar. ZI were seen for fractions F2-F9, but not F1, 10 and 11. No activity was seen against *S. aureus*. The constituents in F1, 10 and 11 may have had no antibacterial activity or were at such low concentrations that antibacterial activity was not detected against either organism at the used inoculum levels. F3, 8 and 9 had the greatest activity. Both HT and CT are reported to have antibacterial activity with CT being assayed using their ability to bind to bacteria and inhibit growth (Schofield *et al.* 2001). HTs have been reported as being more potent than CT in ability to bind to bacteria and inhibit growth. The binding/inhibition activity of HT is related to number of galloyl groups. For inhibition of bacterial growth to be due to tannins, sufficient levels must be present to overcome potential competitive binding to protein constituents of the agar (Schofield *et al.* 2001).

#### 4.13.4.2 Antibacterial Results *P. robustissimum*

No ZI were seen for the digest or fractions from *P .robustissimum*/LS, for either organism. The digest from *P. robustissimum*/TO had a less opaque area around the well with lower density of culture with *B. subtilis*, i.e. there was no clear ZI and after a further incubation no clear ZI were seen. The concentrations in the digest may have been sufficient to inhibit growth to some degree but this was not complete inhibition. Levels of CT or HT present in the fractions were below the concentration range of the current $FeCl_3$ test protocol.

**In Summary**:

- The fraction development work illustrated differences between the *Phoradendron* /host combinations.
- The analysis of the SPE fractions suggests that there are differences in the classes of PSM in the *Phoradendron* species (Figures 4.19-4.21).
- There may also be differences in the biological activity as indicated by the antibacterial results.

## 4.14 EDXRF analysis of *Phoradendron* leaf

The instrumental output for this method reports values as ppm. Instrumental values were converted to mcg or mg/g dry leaf weight. All the elements present in vertebrate tissues and minerals and considered essential nutrients that were detected were then examined in detail. Following this examination the data is presented for **all** the elements that were **quantifiable** at levels >1ppm using the FP method. The values were then plotted according to the sample identity. The values plotted for the *P. quadrangulare* samples are for *ad hoc* collected examples of leaf known to be eaten. Where available, samples of host tree leaf were analysed.

Statistical analysis of **host vs mistletoe** concentrations was not possible as host leaves were collected on a single occasion. Kruskal-Wallis Non-Parametric 1-Way Anova was performed on the samples of *P. robustissimum* as the samples came from an individual host tree in each instance.

Statistical analysis was **not undertaken** on the *Phoradendron*/MC samples as many of the samples were collected from multiple host trees at the time of consumption. There were insufficient samples from a single host tree to permit statistical comparisons.

### 4.14.1 Silicon

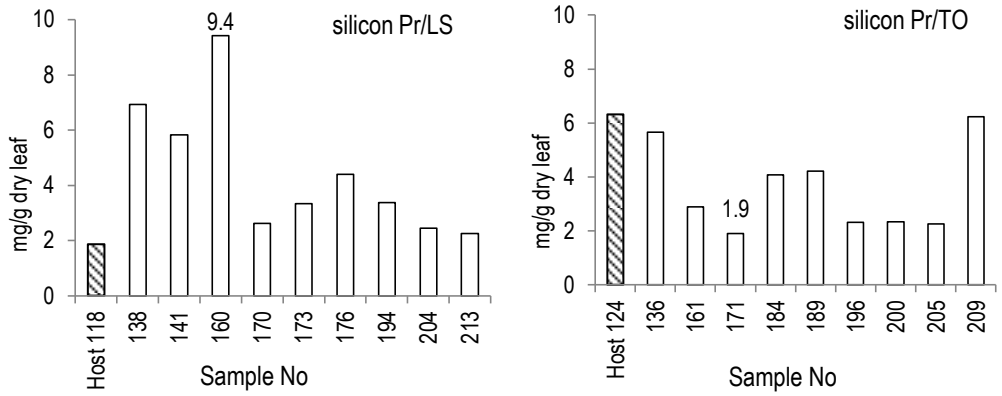



Figure 4.22 Silicon levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

Silicon levels detected in *P. robustissimum* ranged from 0.7-5.0mg/g dry leaf. Mistletoe levels were higher in

the case of host LS samples but this was not the case with all the *P. robustissimum/TO* samples (Figure 4.22). Kruskal-Wallis 1-Way Anova showed there was a significant difference between the silicon content of the mistletoe leaves of: *P. robustissimum*/LS $p<0.05$, K-W statistic 21.35 n = 9 and *P. robustissimum*/TO $p<0.05$, K-W statistic 23.86 n = 9.

Silicon levels detected in *P. quadrangulare*/MC ranged from 1.9-9.4mg/g dry leaf and were much lower than the examples single host tree leaves (Figure 4.23). There were insufficient *Phoradendron*/MC samples from a single host tree to permit statistical comparisons.

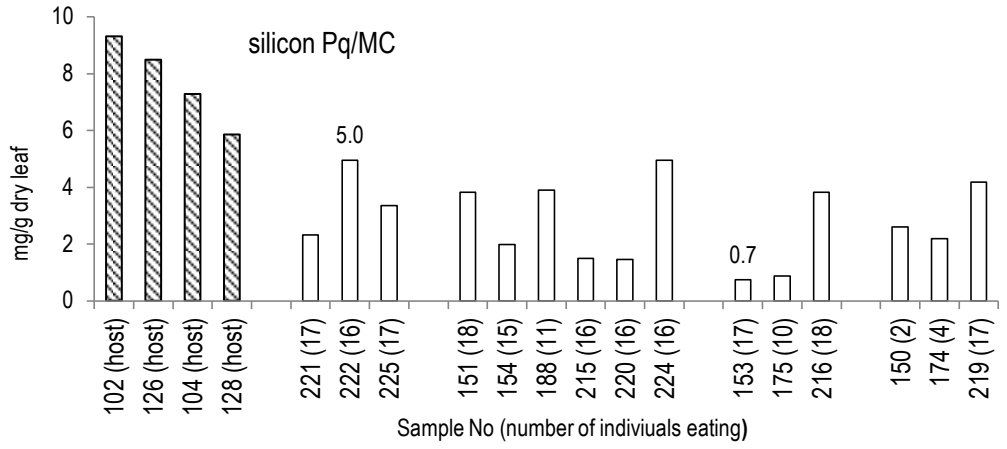



Figure 4.23 Silicon levels *P. quadrangulare*/MC eaten examples.

## 4.14.2 Potassium

Potassium levels were higher in the mistletoe than the host leaf sample in both sets of *P. robustissimum* samples. The range for *P. robustissimum* were 7.7-27.1 mg/g dry leaf (Figure 4.24) with the two host samples 6mg/g (LS) and 13mg/g dry leaf (TO). Kruskal-Wallis 1-Way Anova showed a significant difference between the potassium content of the mistletoe leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic 21.05 n=9 and *P. robustissimum*/TO $p<0.05$, K-W statistic 23.34 n = 9.

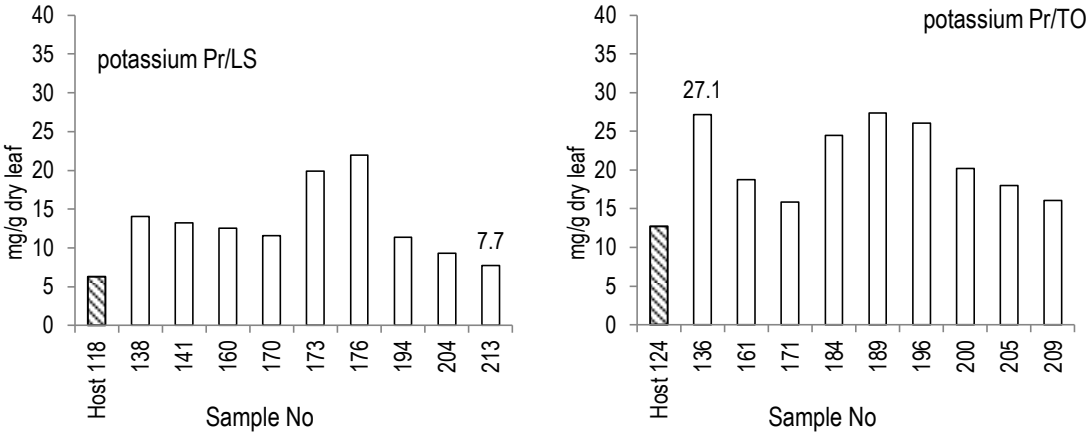



Figure 4.24 Potassium levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

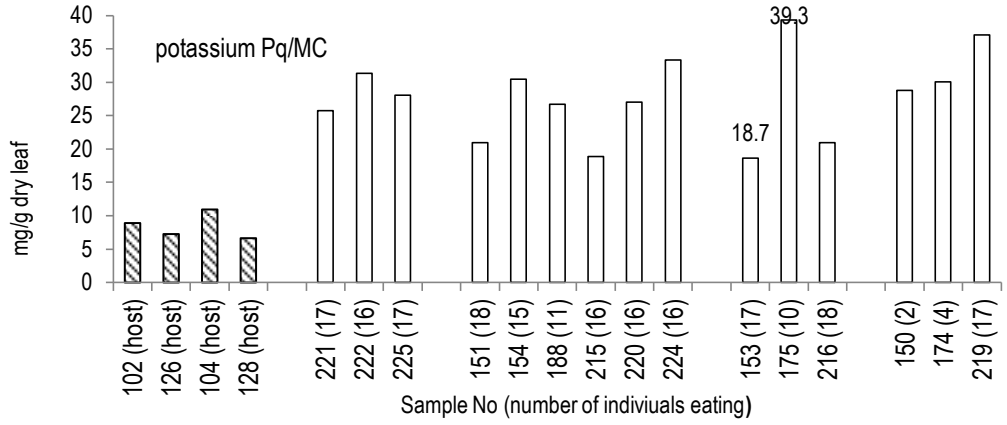



Figure 4.25 Potassium levels *P. quadrangulare*/MC eaten examples

Potassium levels in *P. quadrangulare* samples ranged from 18.7-39.3 mg/g dry leaf (Figure 4.25) and would therefore provide more K than *P. robustissimum* (Figure 4.24). These levels were also much higher than the MC host leaf levels of 6.6-10.9mg/g dry leaf. There were insufficient *Phoradendron*/MC samples from a single host tree to permit statistical comparisons.

## 4.14.3 Sodium

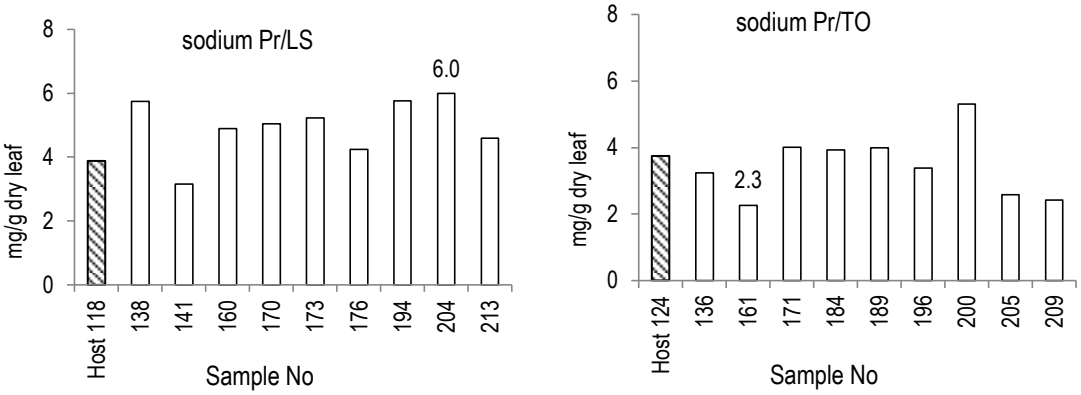



Figure 4.26 Sodium levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

Kruskal-Wallis 1-Way Anova showed a significant difference between the sodium content of the mistletoe leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic18.62 n = 9 and *P. robustissimum*/TO $p<0.05$, K-W statistic 20.68 n = 9, (Figure 4.26).

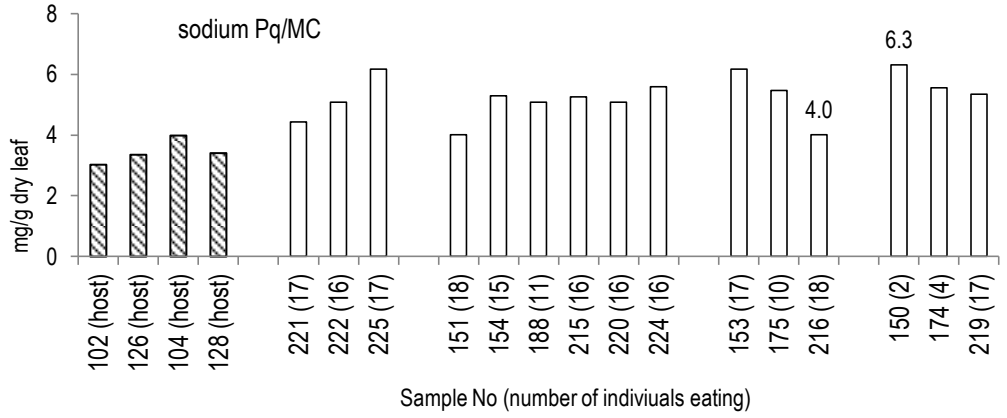



Figure 4.27 Sodium levels *P. quadrangulare*/MC eaten examples

The levels of Na in *P. quadrangulare* were marginally higher than the MC host leaf range 4.0-6.3mg/g dry leaf

(Figure 4.27) and P. robustissimum sample range 2.3-6.0mg/g dry leaf (Figure 4.26). There were insufficient *Phoradendron*/MC samples from a single host tree to permit statistical comparisons.

## 4.14.4 Calcium

Calcium levels in both host leaf examples were very different. *P. robustissimum* ranged from 2.5-7.7mg/g dry leaf (Figure 4.28). Kruskal-Wallis 1-Way Anova showed a significant difference between the calcium content of the both leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic 22.63 n = 9 and *P. robustissimum*/TO $p<0.05$, K-W statistic 22.66 n = 9.

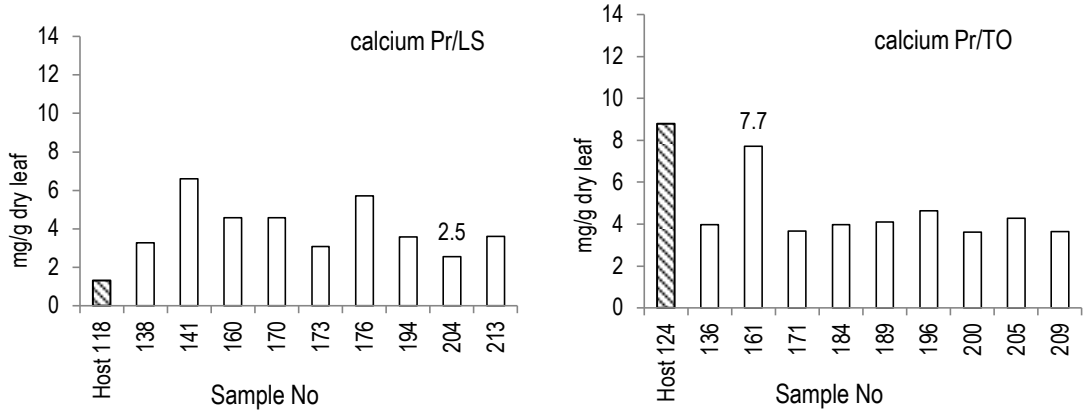



Figure 4.28 Calcium levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

In the case of host tree pairs *P. robustissimum*/TO and *P. quadrangulare*/MC both *Phoradendron* species had lower calcium levels than the host leaves collected (Figures 4.28-4.29).

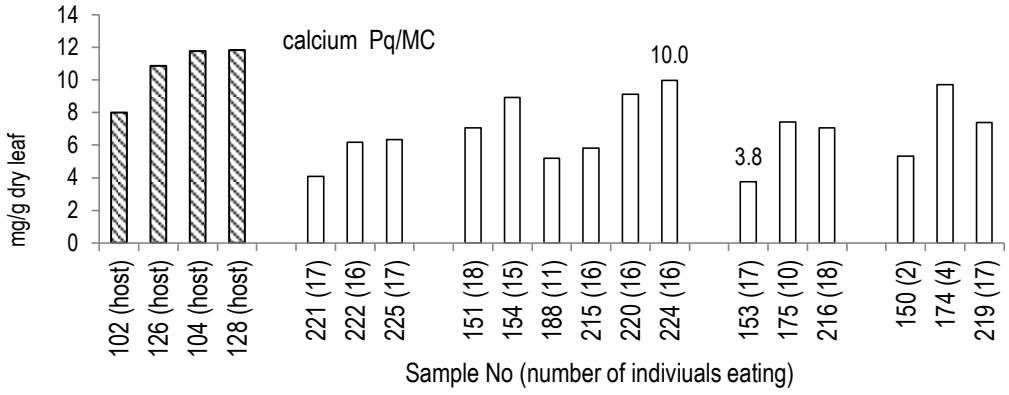



Figure 4.29 Calcium levels *P. quadrangulare*/MC eaten examples

There were insufficient *P. quadrangulare* samples from a single host tree to permit statistical comparisons.

## 4.14.5 Sulphur

Sulphur levels in *P. robustissimum* were slightly higher than host leaf LS for all samples but were much higher in the case of host TO (Figure 4.30). All the sulphur levels in *P. robustissimum* were < 1.1mg/g dry leaf. Kruskal-Wallis 1-Way Anova showed no significant difference between the sulphur content of the leaves: *P. robustissimum*/LS $p>0.05$, K-W statistic 14.62 n = 9 and *P. robustissimum*/TO $p>0.05$, K-W statistic 19.58 n = 9.

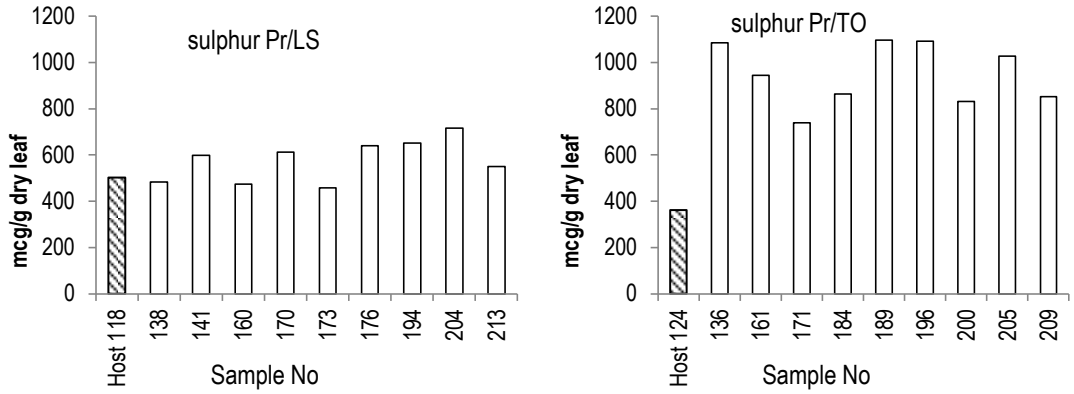



Figure 4.30 Sulphur levels *P. robustissimum*/LS and *P. robustissimum*/TO – non-eaten species

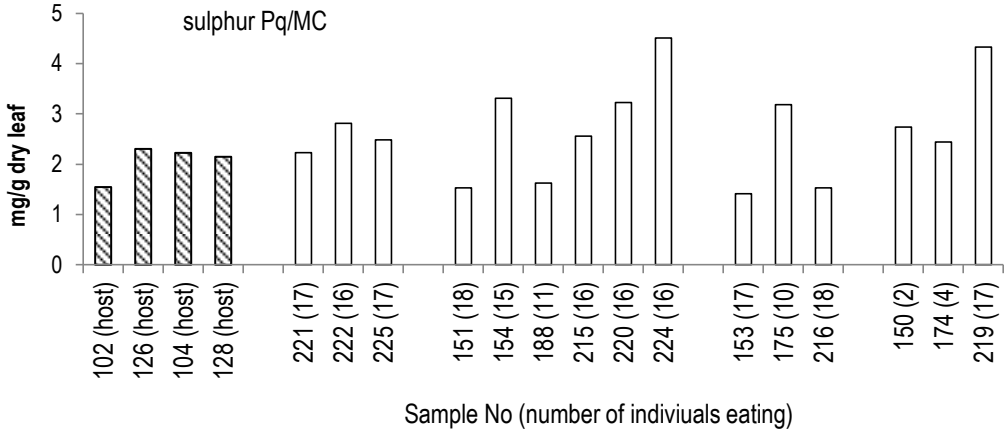



Figure 4.31 Sulphur levels *P. quadrangulare*/MC eaten examples

The sulphur levels in *P. quadrangulare*/MC ranged from 1.4-4.5**mg/g** dry leaf (Figure 4.31) higher than those of *P. robustissimum* on either TO or LS hosts which were **mcg/g** levels (Figure 4.30). There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

## 4.14.6 Phosphorus

Phosphorus levels in *P. robustissimum*/LS ranged from ~168-414mcg/g and *P. robustissimum*/TO from ~101-370mcg/g, in latter case markedly different from the sole host leaf (Figure 4.32). Kruskal-Wallis 1-Way Anova showed a significant difference between the phosphorus content of the mistletoe leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic 19.42 n=8 and no significant difference in *P. robustissimum*/TO $p>0.05$, K-W statistic 10.56 n = 9.

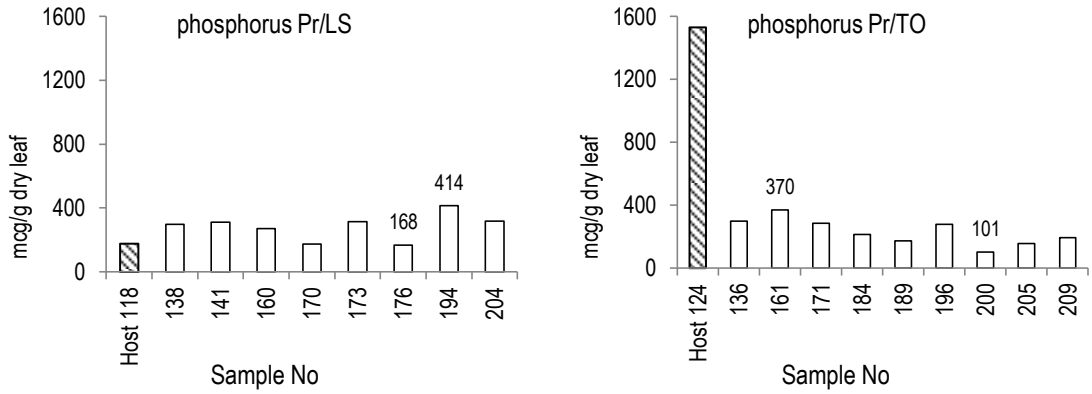



Figure 4.32 Phosphorus levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

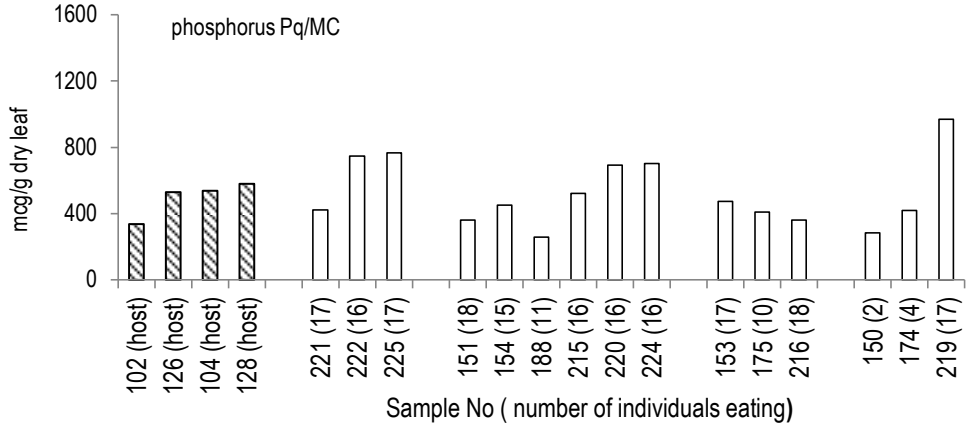



Figure 4.33 Phosphorus levels *P. quadrangulare*/MC eaten examples

Phosphorus levels in *P. quadrangulare*/MC samples ranged from 257-968mcg/g dry leaf (Figure 4.33) and so were higher than the majority of samples of *P. robustissimum* (101-414 mcg/g dry leaf). There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

## 4.14.7 Magnesium

Magnesium levels in *P. robustissimum* growing on host LS were higher than the single value for the host leaf (Figure 4.34). Kruskal-Wallis 1-Way Anova showed a no significant difference between the magnesium content of the mistletoe leaves: *P. robustissimum*/LS $p>0.05$, K-W statistic 7.81 n = 8 and a significant difference *P. robustissimum*/TO detected levels $p<0.05$, K-W statistic 17.86 n=7. As stated previously there may be large analytical errors with the lighter elements (Stephens *et al.* 2004).

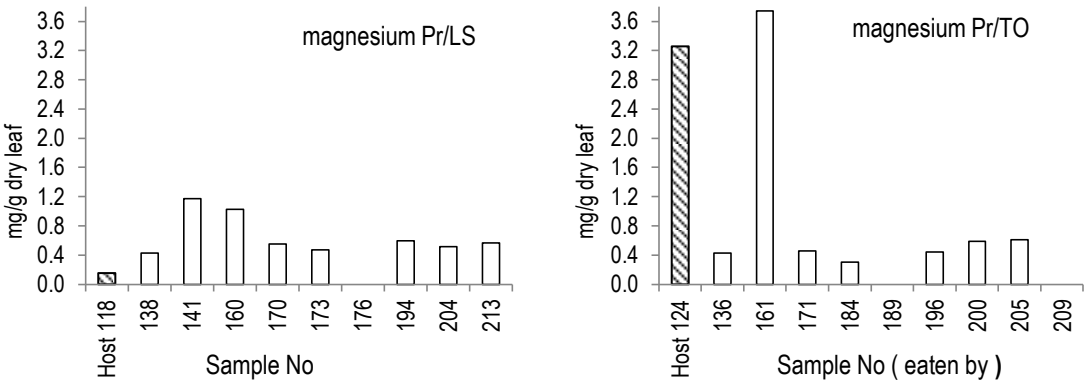



Figure 4.34 Magnesium levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

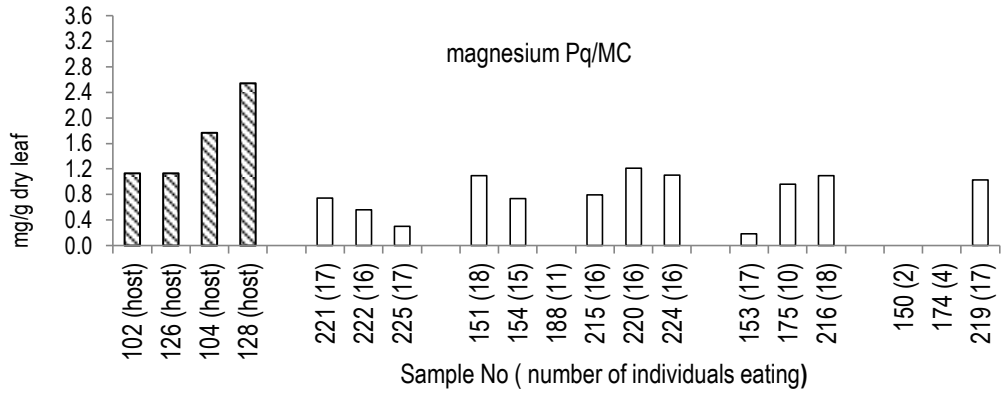



Figure 4.35 Magnesium levels *P. quadrangulare*/MC eaten examples

Magnesium was not detected at levels > 1ppm in three of the opportunistically eaten *P. quadrangulare* samples (Figure 4.35) and the remaining samples had levels much lower than the host leaf samples. There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

## 4.14.8 Manganese

Manganese content of the *P. robustissimum* samples on both host trees, whilst low were higher than that of the single host leaf samples (Figure 4.36). The values ranged from 0.3–1.6**mg**/g dry leaf on host LS and 97-260**mcg**/g dry leaf on host TO. Kruskal-Wallis 1-Way Anova showed a significant difference between the manganese content of both mistletoe leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic 19.84 and *P. robustissimum*/TO $p<0.05$, K-W statistic 14.72.

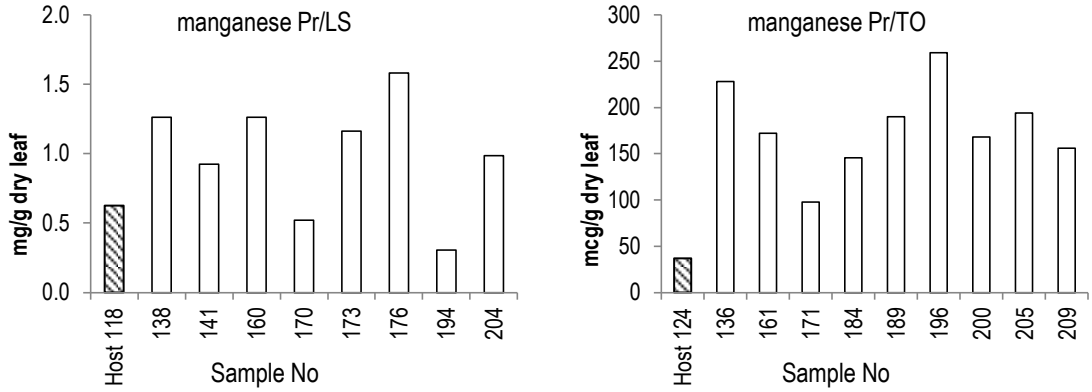



Figure 4.36 Manganese levels *P. robustissimum*/LS and *P. robustissimum*/TO - non eaten species

Manganese was not detected at levels > 1ppm in any of the *P. quadrangulare* samples.

## 4.14.9 Zinc

There were a range of Zn values in *P. robustissimum* samples 5.1-13.1mcg/g dry leaf these were higher than the single host leaf determination 2.8mcg/g dry leaf (Figure 4.37). Kruskal-Wallis 1-Way Anova showed a significant difference between the zinc content of mistletoe leaves: *P. robustissimum*/TO $p<0.05$, K-W statistic 20.79 n = 9. Zinc was not detected at levels > 1ppm in any of the *P. robustissimum*/LS samples.

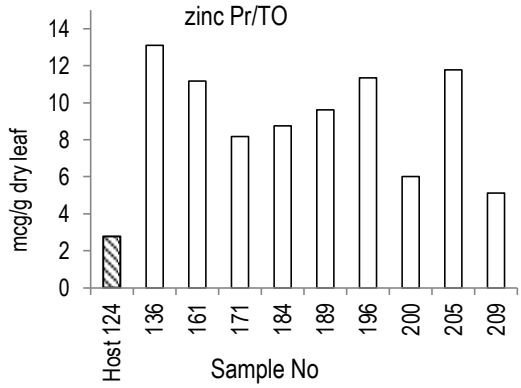



Figure 4.37 Zinc levels *P. robustissimum*/TO – non eaten species

The zinc levels detected in the *P. quadrangulare* eaten samples (Figure 4.38) ranged from 3.7- 9.5mcg/g dry leaf these were 2-4 times higher than the host tree leaf examples. The *P. quadrangulare* results were lower

than the values for *P. robustissimum* (5.1-13.1mcg/g dry leaf).

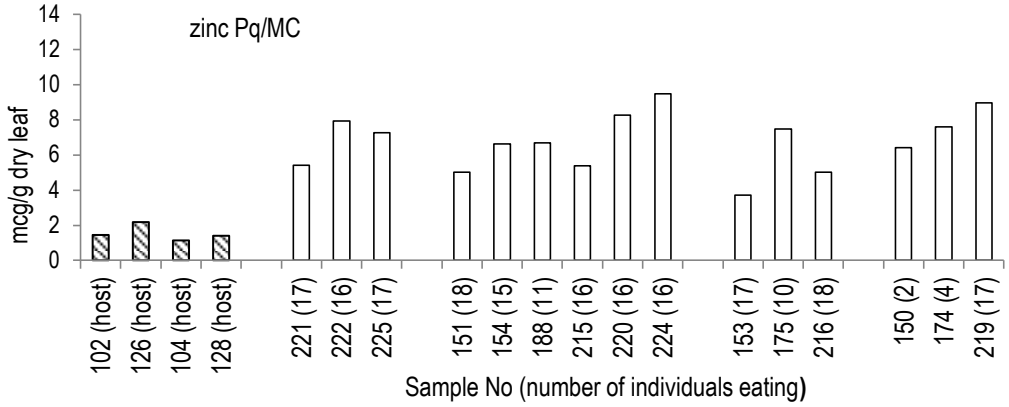



Figure 4.38 Zinc levels *P. quadrangulare*/MC eaten examples

There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

## 4.14.10 Copper

Copper was not detected at levels > 1ppm in host leaves LS or *P. robustissimum*/LS samples.

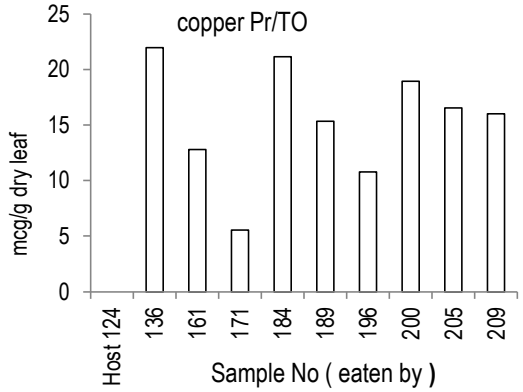



Figure 4.39 Copper levels *P. robustissimum*/TO – non eaten species

Copper levels in *P. robustissimum*/TO range between 6-22mcg/g dry leaf and was not detected at levels > 1ppm in the sole example of host leaf. Kruskal-Wallis 1-Way Anova showed a significant difference between the copper content of the mistletoe leaves: *P. robustissimum*/TO $p<0.05$, K-W statistic 19.38 n = 9.

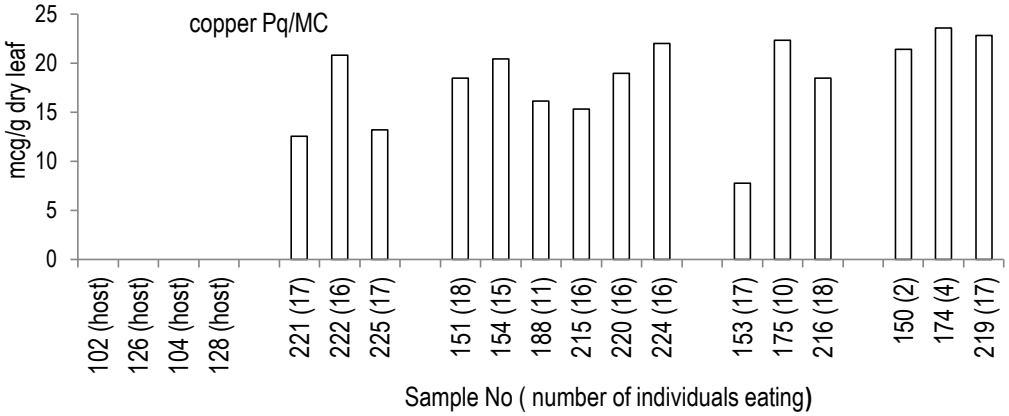



Figure 4.40 Copper levels *P. quadrangulare*/MC eaten examples

Copper was not detected MC host leaves (Figure 4.40). Copper levels in the eaten samples of *P.*

*quadrangulare* varied from 7.7-23.6mcg/g dry leaf (Figure 4.40) which was a similar range to that of the *P. robustissimum* samples. There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

## 4.14.11 Iron

In both cases *P. robustissimum* levels were in the mcg/g range but both were higher than the host leaf examples (Figure 4.41). Kruskal-Wallis 1-Way Anova showed a significant difference between the detected iron content of the mistletoe leaves: *P. robustissimum*/LS $p<0.05$, K-W statistic 15.97 n=7 and no significant difference in *P. robustissimum*/TO $p>0.05$, K-W statistic 15.97 n=9.

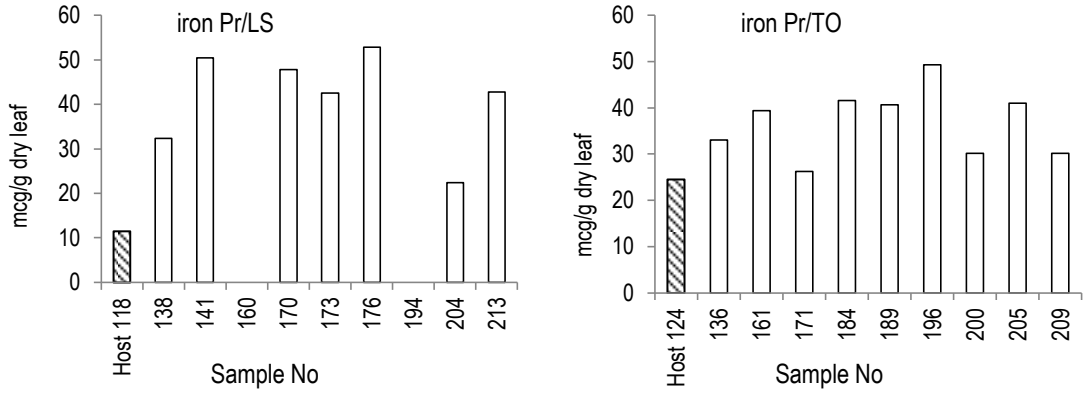



Figure 4.41 Iron levels *P. robustissimum*/LS and *P. robustissimum*/TO – non eaten species

*P. quadrangulare* samples had iron levels similar to or slightly higher than the host leaf examples (Figure 4.42). The levels were low in all cases being only in the mcg/g range. There were insufficient *P. quadrangulare/MC* samples from a single host tree to permit statistical comparisons.

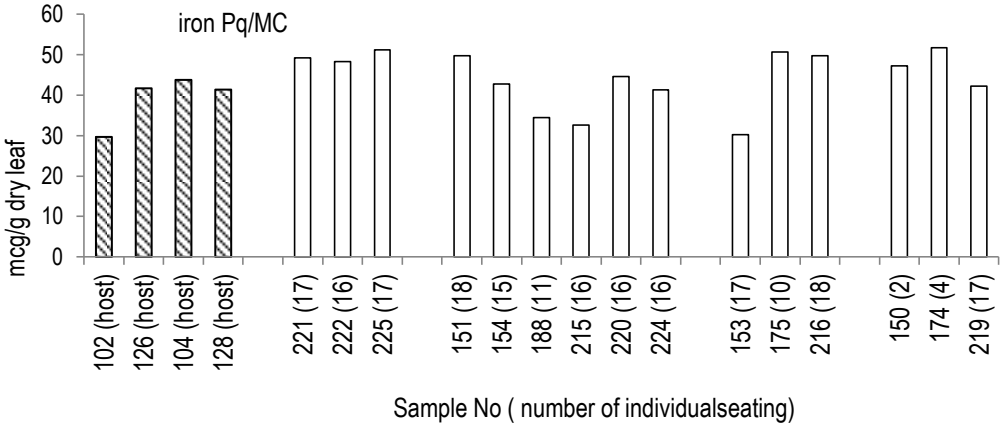



Figure 4.42 Iron levels *P. quadrangulare*/MC eaten examples

In Summary:

- Higher Si was observed in the physically more robust *P. robustissimum* samples.
- K, Na, and Ca were detected at mg/g levels and were higher in the *P. quadrangulare* samples
- S was higher at mg/g dried leaf level in *P. quadrangulare* samples and at mcg/g dried leaf.in *P. robustissimum* samples.
- P, Zn, Cu and Fe, when detected in both *Phoradendron* species were at mcg/g dried leaf levels.

## 4.15 Phytochemical Results *Phoradendron* digests

### 4.15.1 Preliminary observation- Colour

The colours of the freshly prepared mistletoe digests were distinctly different. *P. quadrangulare*/host MC digests were pale green. *P. robustissimum* digests had two differing colours according to the host tree, those from host LS being a much darker tea-like colour, Figure 4.43.

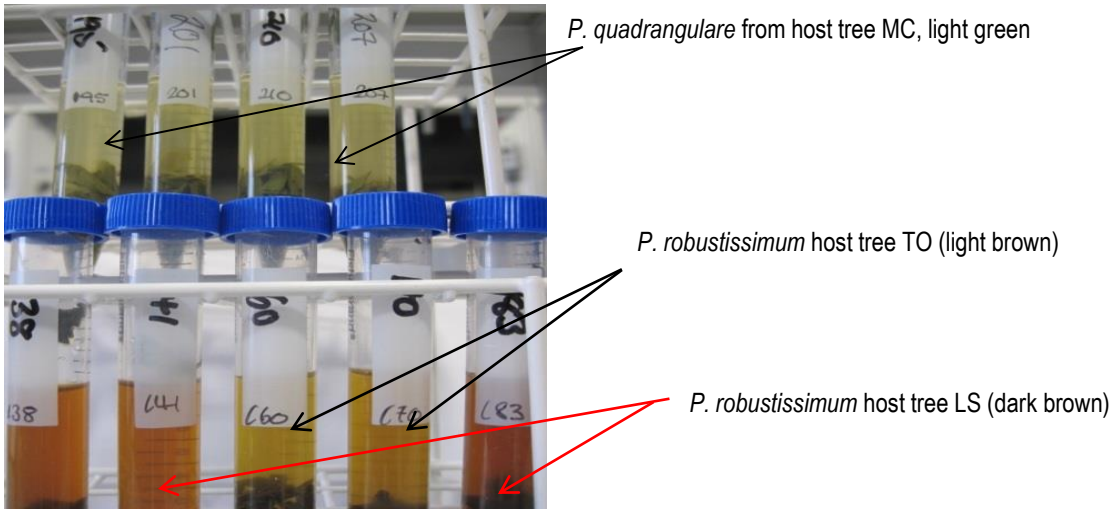



Figure 4.43 Examples of typical colours of fresh digests, before filtration.

### 4.15.2 Lectin agglutination

Clear or coloured solutions greenish (gr), brown (br) pale yellow/pale green were seen in wells 1-3 (Table 4.19).

Table 4.19 Well plate schedule for lectin agglutination testing and results for tested leaf digest

| | **Positive control** | | | **Negative control** | | | **Sample** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| plant digest | 50µl | 50µl | 50µl | | | | 50µl | 50µl | 50µl |
| PBS | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | | | |
| rbc in PBS | | | | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |
| | **Results** | | | | | | | | |
| *Tabebuia ochracea leaf* | gr | gr | gr | ● | ● | ● | ● | ● | ● |
| *Luehea speciosa leaf* | gr | gr | gr | ● | ● | ● | ● | ● | ● |
| *Manilkara chicle leaf* | gr | gr | gr | ● | ● | ● | ● | ● | ● |
| *P. robustissimum* (131) | br | br | br | ● | ● | ● | + | + | + |
| *P. robustissimum* (137) | br | br | br | ● | ● | ● | + | + | + |
| *P. robustissimum* (134) | br | br | br | ● | ● | ● | + | + | + |
| *P. robustissimum* (141) | br | br | br | ● | ● | ● | + | + | + |
| *P. quadrangulare* (125) | pale yell/gr | pale | pale | ● | ● | ● | + | + | + |
| *P. quadrangulare* (152) | pale yell/gr | pale | pale | ● | ● | ● | + | + | + |

Key - **+**agglutination is seen as a haze throughout the well, negative agglutination ● a spot in centre of well.

A red spot which dispersed on gentle agitation was indicative of no agglutination. No evidence of agglutination was seen in wells 4-6 (no plant digest) nor in the wells 7-9 (digests of the host tree leaf). The red spot was more obvious in the samples from *P. quadrangulare,* because of the paler colour of the incubated solution.

## 4.15.3 Inhibition of lectin activity

No agglutination or turbidity was observed in the absence of plant digest (wells 1-6). When plant digest was present agglutination was not inhibited by d-mannose (wells 11-12), but was inhibited by d-galactose (wells 7-8), (Table 4.20). The mixture of spot and turbidity was interpreted as partial inhibition by GalNAc (wells 9-10). This partial inhibition seen may also be due to an insufficient concentration of GalNAc for complete inhibition. The concentration of inhibitory sugars was determined using *V. album.* Levels of ML are known to vary with mistletoe species, host species and mistletoe phenology (Urech *et al.* 2006, Urech *et al.* 2009).

As with previous results interpretation was easier in the *P. quadrangulare* samples as the digests exhibited much less darkening during the incubation period than did the *P. robustissimum* samples. The pattern of reactivity is similar to that reported for *V. album* (Eifler *et al.* 1993). A d-galactose specific lectin has been isolated from the mistletoes *Phoradendron californicum* (Endo *et al.* 1989), *Phoradendron liga* (Varela *et al.* 2004) and the Argentinian mistletoe *Ligaria cuneifolia (Fernandez et al. 2003)*. All three report that these lectins share epitopes with *V. album* ML1 which is inhibited by d-galactose. The results suggest the presence of ML1and 3. ML2 is inhibited by GalNAc and ML3 is inhibited by both d-galactose and GalNAc, therefore the partial inhibition seen may be due to the presence of either ML2 or ML3.

Table 4.20 Well plate schedule results for inhibition of lectin agglutination testing

| | **Control** | | | | | | **Sample** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
| plant digest | | | | | | | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |
| d-galactose in PBS | 50µl | 50µl | | | | | 50µl | 50µl | | | | |
| GalNAc in PBS | | | 50µl | 50µl | | | | | 50µl | 50µl | | |
| D-mannose in PBS | | | | | 50µl | 50µl | | | | | 50µl | 50µl |
| PBS | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | | | | | | |
| rbc in PBS | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |
| | **Results** | | | | | | | | | | | |
| *P. robustissimum* (131) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |
| *P. robustissimum* (137) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |
| *P. robustissimum* (134) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |
| *P. robustissimum* (141) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |
| *P. quadrangulare* (125) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |
| *P. quadrangulare* (152) | *N* | *N* | *N* | *N* | *N* | *N* | *N* | *N* | ●+ | ●+ | + | + |

Key- N – no agglutination; **+** agglutination **;** ●**+** mixture of red spot and haze throughout the solution, partial inhibition

## 4.15.4 Alkaloid screening

All the aqueous digests produced were tested with both reagents. No positive tests were observed. These results suggest that the major classes of alkaloid compounds were absent in the aqueous acidic digests. The results are summarised in Section 4.15.10, Tables 4.21-4.22.

## 4.15.5. Saponin Screening

All the aqueous digests produced were tested. The duration of the foams ranged from <8 mins to > 15 mins suggesting the presence of saponins. Differing levels of saponins may have been responsible for the variable duration of the foams. The variability was seen in digests from both mistletoe species. This may therefore be due to seasonal/phenological differences in leaf composition or leaf age. The results are summarised in Section 4.15.10, Tables 4.21-4.22.

## 4.15.6 Gelatine precipitation test

The gelatine precipitation method can be used for digests containing phenolic and non-phenolic components and is capable of detecting tannins at levels >30µg/ml in distilled water (Hagerman *et al.* 1978). The results are summarised in Section 4.15.10, Tables 4.21-4.22.

The results ranged from clear no reaction, slight haze, opalescent and precipitate formed, Figure 4.44 shows (+++med haze). Condensed tannins (CT) proanthocyanidins have a high affinity for proteins with high content of proline. Gelatine contains 18mol% proline and has a highly specific structurally related affinity for CTs (Hagerman *et al.* 1981). Results suggest variable amounts of CT in *P. quadrangulare* samples and the results for *P. robustissimum* suggest much lower levels CT.

Figure 4.44 Example of a +++ positive gelatine precipitation test. Author's photo.

## 4.15.7 Ferric chloride test

The mistletoe samples growing on two host trees produced varying degrees of brown or green colours in response to the ferric chloride test, as can be seen in Figure 4.45.

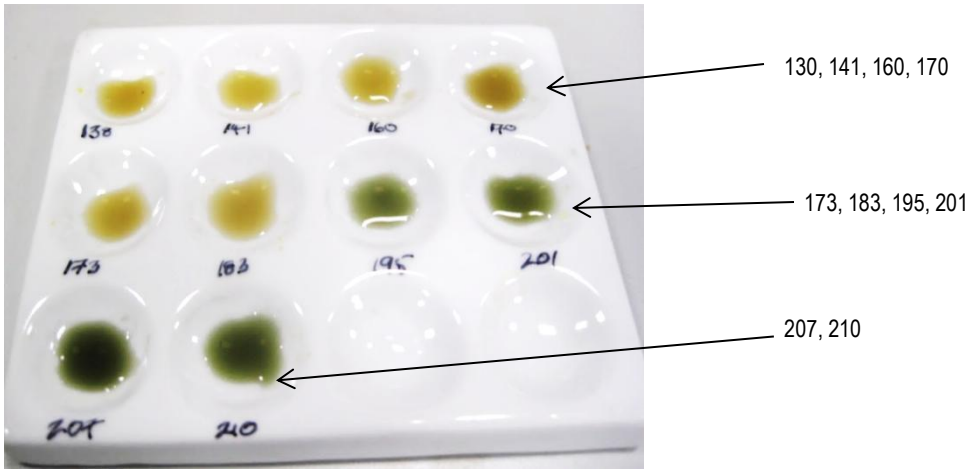



Figure 4.45 Spot plate showing examples of ferric chloride test results author's photo. Sample 138, 141, 160, 170, 173 and 183 growing on host LS. Samples 195, 201, 207 and 210 growing on host MC.

The mistletoe samples growing on two host trees produced varying degrees of brown or green colours in response to ferric chloride test, as can be seen in Figure 4.45. MC and GU host leaf digest developed a pale green colour, LS host leaf dark brown and TO host leaf brownish, very little different from the colour of

mistletoe digests. *P. robustissimum* growing on LS produced digests that resulted in a transient green colour which became dull green/brown and TO a dull brown/green colour. The digests from *P. quadrangulare* growing on MC upon adding the first drop of reagent produced a deep blue colour which after all the reagent had been added and the solution agitated produced varying depth of colour but all strongly green, Figure 4.45. *P. quadrangulare* growing on GU produced med/dark green colour similar to sample195 and 210. Hydrolysable tannins (gallitannins and elagitannins) produce a blue/black colour and condensed tannins a green-brown colour. In a digest mixture the variable proportions of individual constituents will impact upon the colour formation e.g. where both types of tannin are present the initial colour may be blue which then changes to dark green (Evans 2009b).

These results suggest that there are differences in the types of tannins and concentrations in the mistletoe samples. *P. robustissimum* digests showing differences in result depending on host, appear to contain mainly condensed tannins. *P. quadrangulare* results suggest a mixture of both types of tannin, possibly with variable hydrolysable tannin levels contributing to the intensity of the green colour and higher concentrations of hydrolysable tannin than in *P. robustissimum.* Chlorogenic acid has also been shown to have a high binding capacity for iron > gallic acid and protocatechuic acid and compounds with a galloyl group being a stronger chelators than those bearing a catechol group Andjelković *et al.*( 2006). The results are summarised in Section 4.15.10, Tables 4.21-4.22.

## 4.15.8 Flavonoid screening

The brown of the digests produced from *P. robustissimum* on both host trees made colour determination problematic. The tests were repeated on the combined SPE eluents from *P. robustissimum* to overcome this issue.

### Results - Addition of NAOH

The *P. quadrangulare* samples growing on the MC produced a deep yellow colour which was reversed upon the addition of the HCl, whilst those on the SPE eluents from *P. robustissimum* produced a very faint yellow colour reaction also reversed by addition of HCl. This test detects flavonols, flavones, xanthones and hydroxycinnamic acids. These compounds also give a positive phenolic results for the ferric chloride test (Harborne 1989a), yielding blue, green, brown or red colours. The results indicate possible differences in flavonoid content/concentration in the two mistletoe species.

### Results - Addition of methanolic $AlCl_3$

A variable yellow colour was produced in eluents from *P. quadrangulare* samples growing on the MC and the SPE eluents from *P. robustissimum* produced a no yellow colour. The method using aluminium chloride is based on the formation of a complex between the aluminium ion, Al (III), and the carbonyl and hydroxyl groups of flavones and flavonols that produce a yellow colour (Popova *et al.* 2004). The results for both tests are summarised in Section 4.15.10, Tables 4.21-4.22.

## 4.15.9 UV Induced Fluorescence

A bright blue/ violet blue coloured spot with dark outer ring was seen under 365nm wavelength for digests of *P. quadrangulare* growing on MC. Both the spot and much of the darker ring became yellow/green after ammonia exposure. This is characteristic of flavones/flavonols lacking a free 5-OH group, Figure 4.32, (Markham 1989). However chlorogenic acid solution when exposed to ammonia turns yellow (Trease and Evans' Pharmacognosy 2009, p 228).The yellow colour with ammonia may therefore be due to more than one component.

The mistletoe samples from LS and TO produced a central dull yellow/brown spot with a faint bright blue outer ring which did not change after ammonia fuming. This response is characteristic of flavonol aglycones (Markham 1989). Results are presented in Section 4.15.10, Tables 4.21-4.22.

## 4.15.10 Summary of phytochemical screening results

Key for test results

- gelatine test: +++++very heavy immediate ppt; ++++heavy immediate ppt; +++med haze with or without ppt ; ++ light haze; + slight haze with or without ppt.
- ferric chloride test – Pale BrG - Pale brown/green; + represent darkness of colour green
- fluorescence – BrB- bright blue; BVB- bright violet blue, dk- dark, Dyb – dull yellow/brown; YG- bright yellow green

Table 4.21 Summary of phytochemical screening, *P. robustissimum* samples – host TO/LS

| Sample no. | alkaloid | saponin | gelatine | $FeCl_3$ | flavonoid test | Fl spot at 365nm | Fl spot 365nm & NH3 fumed |
|---|---|---|---|---|---|---|---|
| 136 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 144 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 161 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 171(TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 184 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 189 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 196 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 200 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 205 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 209(TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 214 (TO) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 134 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 159 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 169 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 182 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 199 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 208 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 138 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 141 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 160 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 170 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 176 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 194 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 204 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |
| 213 (LS) | - | + | Clr/ trace | Pale BrG | weak | Dyb/Brb ring | No change |

Table 4.22(a) Summary of phytochemical screening, *P. quadrangulare* samples – host MC/GU

| Sample no. | alkaloid | saponin | gelatine | $FeCl_3$ | flavonoid test | Fl spot at 365nm | Fl spot 365nm & $NH_3$ fumed |
|---|---|---|---|---|---|---|---|
| **Host MC 1 Control** | | | | | | | |
| 125 | - | + | ++ ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 130 | - | + | ++ ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 132 | - | + | +++++ | +++++ v dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 147 | - | + | + lt ppt | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 155 | - | + | ++ ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 165 | - | + | ++++ | ++++ dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 178 | - | + | ++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 195 | - | + | ++ | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 201 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 210 | - | + | ++++ | +++ dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 221 | - | + | ++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 225 | - | + | ++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |

Table 4.22(b) Summary of phytochemical screening, *P. quadrangulare* samples – host MC/GU

| Sample no. | alkaloid | saponin | gelatine | $FeCl_3$ | flavonoid test | Fl spot at 365nm | Fl spot 365nm & $NH_3$ fumed |
|---|---|---|---|---|---|---|---|
| **Host MC21 Control** | | | | | | | |
| 127 | - | + | ++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 139 | - | + | + lt ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 146 | - | + | trace | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 156 | - | + | ++ ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 166 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 179 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 191 | - | + | ++ | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 198 | - | + | +++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 207 | - | + | ++ | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| **Host MC76 Control** | | | | | | | |
| 148 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 157 | - | + | trace | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 180 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 186 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 202 | - | + | + lt ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 211 | - | + | + lt ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 226 | - | + | + lt ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| **Host MC4 Eaten** | | | | | | | |
| 153 | - | + | +++ ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 175 | - | + | ++ | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 216 | - | + | +++ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 217 | - | + | +++ppt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| **Host MC2 Eaten** | | | | | | | |
| 151 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 154 | - | + | +++ ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 187 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 215 | - | + | + lt ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 220 | - | + | + lt ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 224 | - | + | + lt ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |

Table 4.22(c) Summary of phytochemical screening, *P. quadrangulare* samples – host MC/GU

| Sample no. | alkaloid | saponin | gelatine | $FeCl_3$ | flavonoid test | Fl spot at 365nm | Fl spot 365nm & NH3 fumed |
|---|---|---|---|---|---|---|---|
| 115 | - | + | ++++ ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 116 | - | + | + | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 150 | - | + | + lt ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 174 | - | + | +++ ppt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 219 | - | + | ++ | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| **Miscellaneous MC hosts Eaten** | | | | | | | |
| 113 | - | + | ++ | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 114 | - | + | ++ | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 121 | - | + | +lt | +++ dk green | + both strong | BVB/dk ring | BrB/YG ring |
| 122 | - | + | +lt | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 152 | - | + | ++ ppt | ++ green | + both strong | BVB/dk ring | BrB/YG ring |
| 163 | - | + | ++ | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 177 | - | + | ++ | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 188 | - | + | ++ | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| 206 | - | + | ++ | + lt green | + both strong | BVB/dk ring | BrB/YG ring |
| 223 | - | + | ++ | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |
| **Host tree GU** | | | | | | | |
| Host 140 | - | + | clear | + lt green | V pale yellow | Pale blue | No change |
| 164 | - | + | clear | ++ med green | Pale yellow | Pale blue | No change |
| 197 | - | + | clear | ++ med green | Pale yellow | Pale blue | No change |
| 115 | - | + | ++++ ppt | ++++dk gr | + both strong | BVB/dk ring | BrB/YG ring |
| 116 | - | + | + | ++ med green | + both strong | BVB/dk ring | BrB/YG ring |

The fluorescence results for *P. quadrangulare* are suggestive of phenylpropanoids with caffeoyl groups, flavones and glycosyl flavones.

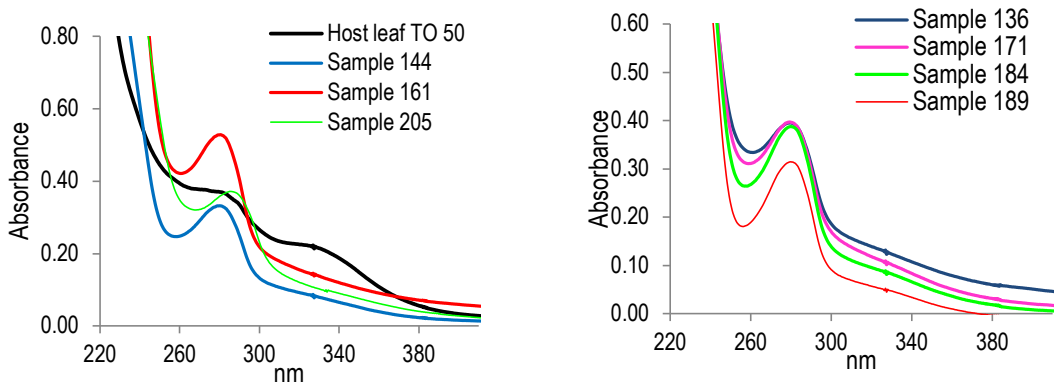
Caerovic *et al.* (1999) and Tattini *et al.* (2004) report that several factors including species, growth conditions, leaf temperature, water status and nutrients status can strongly influence the UV-induced fluorescence emission as all these factors influence the accumulation of flavonoids and hydroxycinnamates in leaves. Thus both host and mistletoe status will determine content and quantities of such compounds. Hydroxycinnamic acids, which exhibit blue fluorescence (BF) are shown to accumulate in leaves with age. High levels of BF are associated with nitrogen (N) deficiencies in plants. Johnson *et al* (1997), working in Costa Rican tropical dry forest, showed that it was potentially a N deficient habitat. Nitrogen was concentrated in leaf litter, which was only very slowly decomposed and so was easily leached in the high rainfall of the wet season, leading to low nitrogen availability. There are a range of potential candidate compounds which exhibit these patterns of fluorescence, including 5- OH flavones/flavonols and 7-glycosides e.g. apigenin and its glycosides (Harborne 1989b) and the phenylpropanoids 3-caffeoylquinic acid, 3-feruloylquinic acid, caffeoylglucose and feruloylglucose. Phenolic acids and derivatives have been isolated from other species of mistletoes (Luczkiewicz *et al.* 2001), the major component being chlorogenic acid and its glycosides.

In Summary:

- Lectin activity suggests MI, II and III present.
- Absence of alkaloids and presence of saponins and phenolic compounds
- Mistletoe species differences in hydrolysable and condensed tannins and host tree induced differences in types present
- Potential presence of 5-OH flavones/flavonols, 7-glycosides and phenylpropanoids

## 4.16. Comparison of UV Characteristics aqueous digests

The initial UV data is presented for examples from each mistletoe species and host tree combination. Where host leaf samples were obtained the spectra have been included for comparison. The values for the wavelength of the UV spectral maxima ($\lambda_{max}$) are included to illustrate the variation in the samples.




UV spectra for *P. robustissimum* /Host Tree TO

Figure 4.46 UV spectra for host leaf and examples of *P. robustissimum*/host TO

Table 4.23 Spectral maxima, *P. robustissimum* digests, host TO 50.

| Sample Id | nm. | Abs. |
|---|---|---|
| Host TO 50 | Shoulder 276-277; shoulder 327 | |
| 144 | 279 | 0.332 |
| 161 | 280 | 0.5282 |
| 205 | 279 | 0.3719 |
| 136 | 279 | 0.3952 |
| 171 | 280 | 0.3971 |
| 184 | 280 | 0.3881 |
| 189 | 280 | 0.3148 |

*P. robustissimum* samples differ from host leaf TO in that the spectra have no shoulder in the 327nm region, and have distinct spectral maxima at 279-280nm (Figure 4.46, Table 4.23).

UV spectra for *P robustissimum*/Host LS

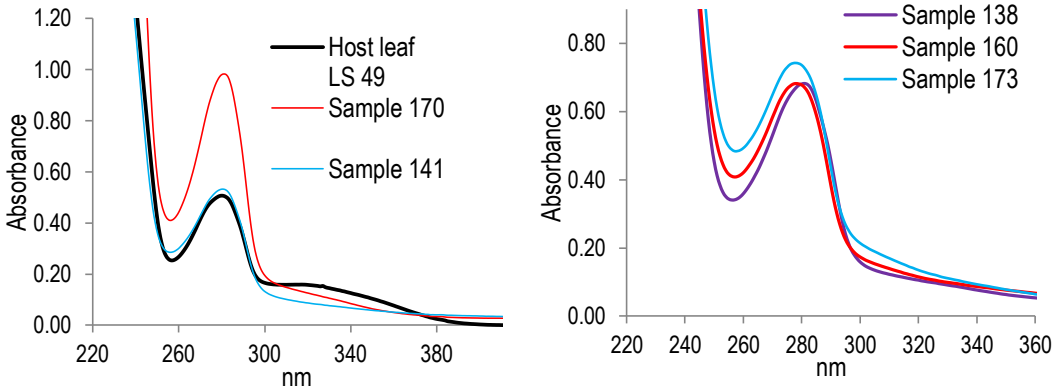



Figure 4.47 UV spectra for host leaf and examples of *P. robustissimum*/host LS

Figure 4.47 and Table 4.24 illustrate the similarities within the *P. robustissimum*/LS samples of the spectral maxima. The absorbance varied with sample 160 (0.6822) the lowest and 194 (0.7843) the highest. *P.*

*robustissimum* samples differ from host leaf LS having no shoulder in the 327nm region, Figure 4.47.

Table 4.24 Spectral maxima, *P. robustissimum* digests, host LS

| Sample Id | nm. | Abs |
|---|---|---|
| Host LS 49 | 280nm and shoulder327 | 0.5066 |
| 141 | 280 | 0.5319 |
| 170 | 281 | 0.9824 |
| 138 | 278 | 0.6695 |
| 160 | 278 | 0.6822 |
| 173 | 278 | 0.7426 |

The digests were all prepared using 500mg ± 10mg of dried leaf and digested using the same volume of acidified water. Samples were all diluted in the same manner using volumetric flasks before obtaining the UV. The absorbance values can therefore be used to make comparisons. The data suggests that LS samples contained higher concentration of components absorbing in the 278-281nm region. *P. robustissimum*/LS digests were a darker brown colour than those of *P. robustissimum*/TO, Figure 4.43. Table 4.10, Section 4.6.3 lists the types of compounds with significant absorbance in this region.

During phenol oxidation, aqueous acidic solutions become a dark brown colour and the compounds responsible for the colour change (catechol, resorcinol, hydroquinone, quinhydrones and benzoquinones) are associated with increased toxicity (Mijangos *et al.* 2006). Non-enzymatic browning may be due to the formation of B-type procyanidins from (+)-catechin and (-)-epicatechin molecules (Sun *et al.* 2003).

## UV spectra for *P. quadrangulare*/Host tree GU

The mistletoe growing on GU was identified as *P. quadrangulare*; the spider monkeys have been seen to eat mistletoe 2-3 times from this host. Both host tree and mistletoe had very little leaf material and so were not included in the regular collection. The results are included to illustrate the effect of host on UV spectra of *P. quadrangulare (*Figure 4.48 Table 4.25).

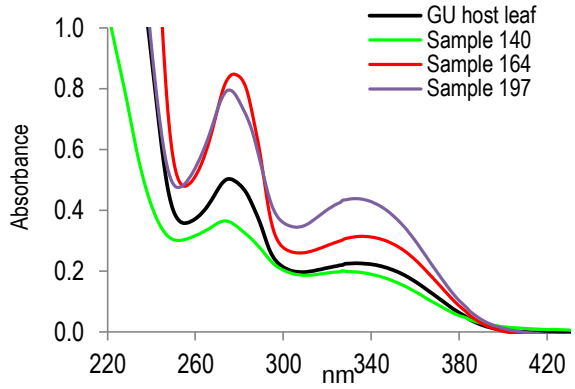



Figure 4.48 UV spectra for host leaf and examples of *P. quadrangulare/*host GU

Table 4.25 Spectral maxima, *P. quadrangulare* digests, host GU

| Sample Id | nm. | nm. |
|---|---|---|
| GU host leaf | 275 | 333/4 |
| 140 | 273 | 327 |
| 164 | 277 | 336 |
| 197 | 275 | 333/4 |

Figure 4.48 and Table 4.25 illustrate the similarities between the GU host and the *P. quadrangulare* samples. The UV spectral maxima for these samples encompass several classes of phenolic compounds Table 4.26.

Table 4.26 Spectral maxima, phenolic compounds (Harborne 1989a)

| Class/ compound | Spectral maxima | | Class/ compound | Spectral maxima | |
|---|---|---|---|---|---|
| Simple phenolics | 266-295 | | flavones | 250-270 | 330-350 |
| Phenolic acids | 235-305 | | flavonols | 250-285 | 320-385 |
| Hydroxycinnamic acids | 227-245 | 310-332 | apigenin-8-C-glucoside | 270 | 336 |
| flavanones | 275-290 | 310-330 | apigenin-7-O-glucoside | 268 | 333 |

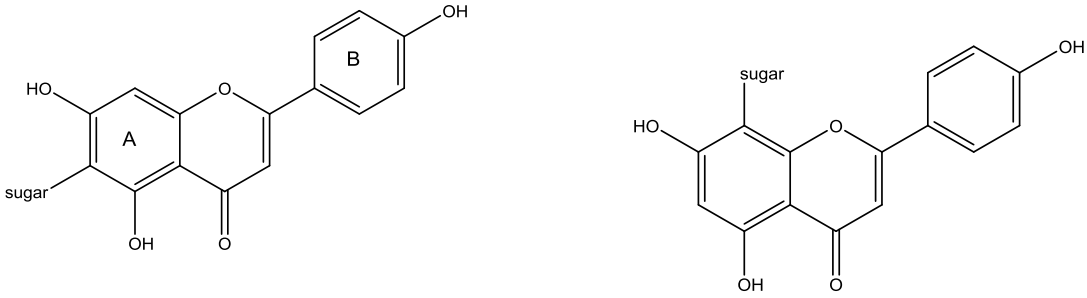



Figure 4.49(a) Structure apigenin–6-C-glycoside and Structure apigenin-8-C-glycoside

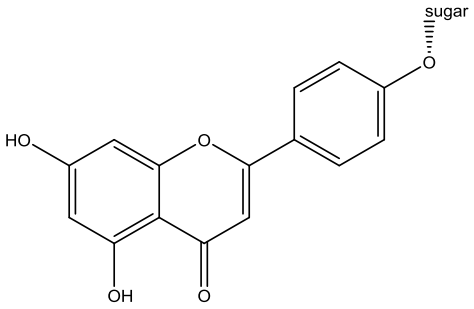



Figure 4.49(b) Structure apigenin–4'-O-glucoside

Spectral maxima at 270 and 333nm are reported for two glycosides, apigenin-8-C-β-glucopyranoside and apigenin-6-C-β-glucopyranoside (Mohammed *et al.* 2014). Several apigenin C and O glycosides (Figures 4.49a-b) have been identified in *Phoradendron spp.* (Dossaji *et al.* 1983, Harvala *et al.* 1984, Varela *et al.* 2004).

UV spectra for *P. quadrangulare*/Host MC1 and MC21- Regular collection

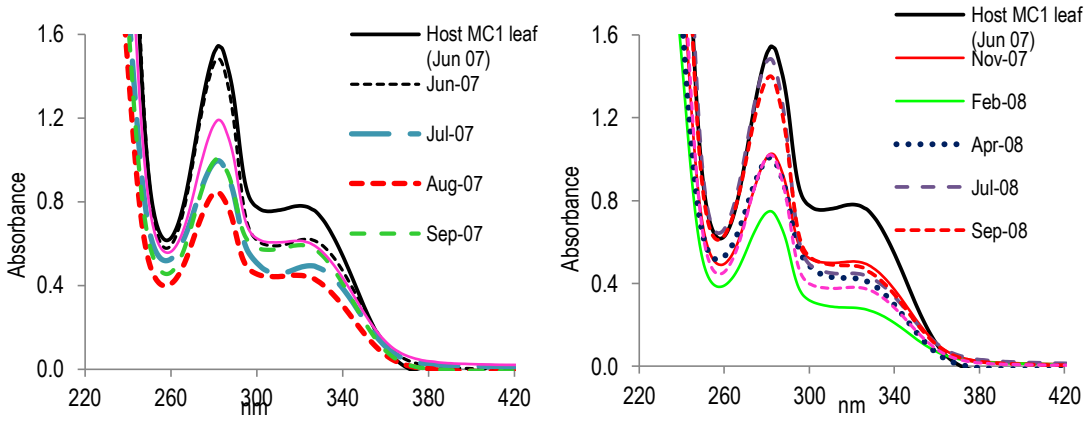



Figure 4.50(a) UV spectra for examples *P. quadrangulare* and host leaf MC1

Figure 4.50(a) shows the UV spectra for regular collections of *P. quadrangulare*/ MC1, including an

example from host tree leaf. The spectra illustrate the spectral similarities between the *Phoradendron* and the host leaf. Absorbance of the host leaf was higher than that of the *Phoradendron* digests. Table 4.27 lists the spectral maxima. It is accepted that the UV spectra obtained are as the result of a mixture of constituents, but the spectra do show consistency in absorbing wavelengths but with variable levels of absorbance. This variation may be due to mistletoe/host seasonal variations or mistletoe leaf age.

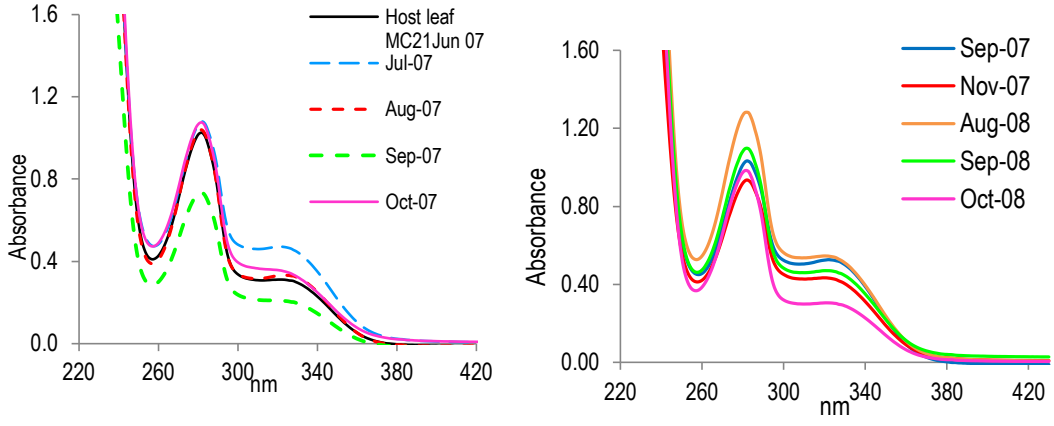



Figure 4.50(b) UV spectra for examples of *P. quadrangulare* and host leaf MC21

Figure 4.50(b) show the UV spectra for regular collections of *P. quadrangulare*/MC21, including spectra for host tree leaf. Samples collected in Jan, Feb and May 08 show greater variations in the components absorbing in 318-325nm range (Table 4.27). These spectra have less pronounced maxima in this region, classed as a shoulder. The maximum absorbance values were in some samples greater than the host tree leaf; this is different from Host tree MC1 data.

Table 4.27 Spectral maxima Host and *P. quadrangulare* from MC1/MC22

| Id/date | nm | Abs | nm | Abs | Id/date | nm | Abs. | nm | Abs |
|---|---|---|---|---|---|---|---|---|---|
| Host leaf MC1 | 282 | 1.5441 | 320 | 0.7808 | Host leaf MC21 | 282 | 1.0248 | 322 | 0.3109 |
| Jun-07 | 282 | 1.4820 | 324 | 0.6224 | Jul-07 | 282 | 1.0798 | 321 | 0.4714 |
| Jul-07 | 282 | 0.9966 | 325 | 0.4948 | Aug-07 | 281 | 1.0380 | 323 | 0.3325 |
| Aug-07 | 282 | 0.8463 | 320 | 0.4487 | Sep-07 | 282 | 1.0248 | 321 | 0.3109 |
| Sep-07 | 282 | 1.0058 | 321 | 0.5934 | Oct-07 | 282 | 1.0746 | 319 | 0.3582sh |
| Oct-07 | 282 | 1.1907 | 320 | 0.6137 | Nov-07 | 281 | 1.3054 | 320 | 0.3703 |
| Nov-07 | 282 | 1.0256 | 321 | 0.5062 | Jan-08 | 281 | 0.8217 | 318 | 0.1619sh |
| Feb-08 | 282 | 0.7843 | 322 | 0.2818 | Feb-08 | 281 | 0.8651 | 318 | 0.1811sh |
| Apr-08 | 282 | 1.0069 | 317 | 0.4269 | May-08 | 281 | 1.4692 | 318 | 0.4463sh |
| Jul-08 | 282 | 1.4835 | 321 | 0.4487 | | | | | |
| Sep-08 | 282 | 1.4000 | 318 | 0.4867 | | | | | |
| Oct-08 | 282 | 1.0202 | 321 | 0.3815 | | | | | |

Many classes of components may contribute to the absorbance between 254-290nm (Tables 4.10 and 4.26). The variation in the wavelength and the absorbance values in the 317-325nm ranges suggest variation in the components within these samples, which contribute to the absorbance at these wavelengths. A comparison of the spectra for mistletoe growing on host TO/LS (Tables 4.23-4.24) with that from host MC (Table 4.27) highlights that there is a major phytochemical difference between both the hosts and the *Phoradendron* species. There is also an apparent difference between *P. quadrangulare* from host GU (Table 4.25) and MC (Table 4.27). In the latter case the Band I (320-385nm) spectral maxima has increased by ~16-20nm. This suggests a higher component of flavonoid type compounds in *P. quadrangulare*/GU samples (Table 4.26).

UV spectra for *P. quadrangulare* collected at time of eating

There is a high level of consistency in the UV signature for all the *P. quadrangulare* samples consumed by the monkeys Figure 4.51. However the concentrations of the constituents contributing to the UV signature are variable. Absorbance in the 278-282nm range shows the greatest variation, this absorbance may also result from the presence of either the phoratoxins or mistletoe lectins, in *P. quadrangulare*/MC samples. The phenology of the host and mistletoe plants was not recorded and it was not possible from the dried sample to determine the age of the mistletoe leaf.

Mistletoe leaf senescence and abscission occur in the summer, when the metabolic activity of the host has reached its maximum (Schrader-Fischer *et al.* 1993). The sulphur-rich viscotoxin/phoratoxins are the only leaf proteins recycled by the mistletoe plants and consequently levels will decrease prior to leaf loss (Calder 1983).

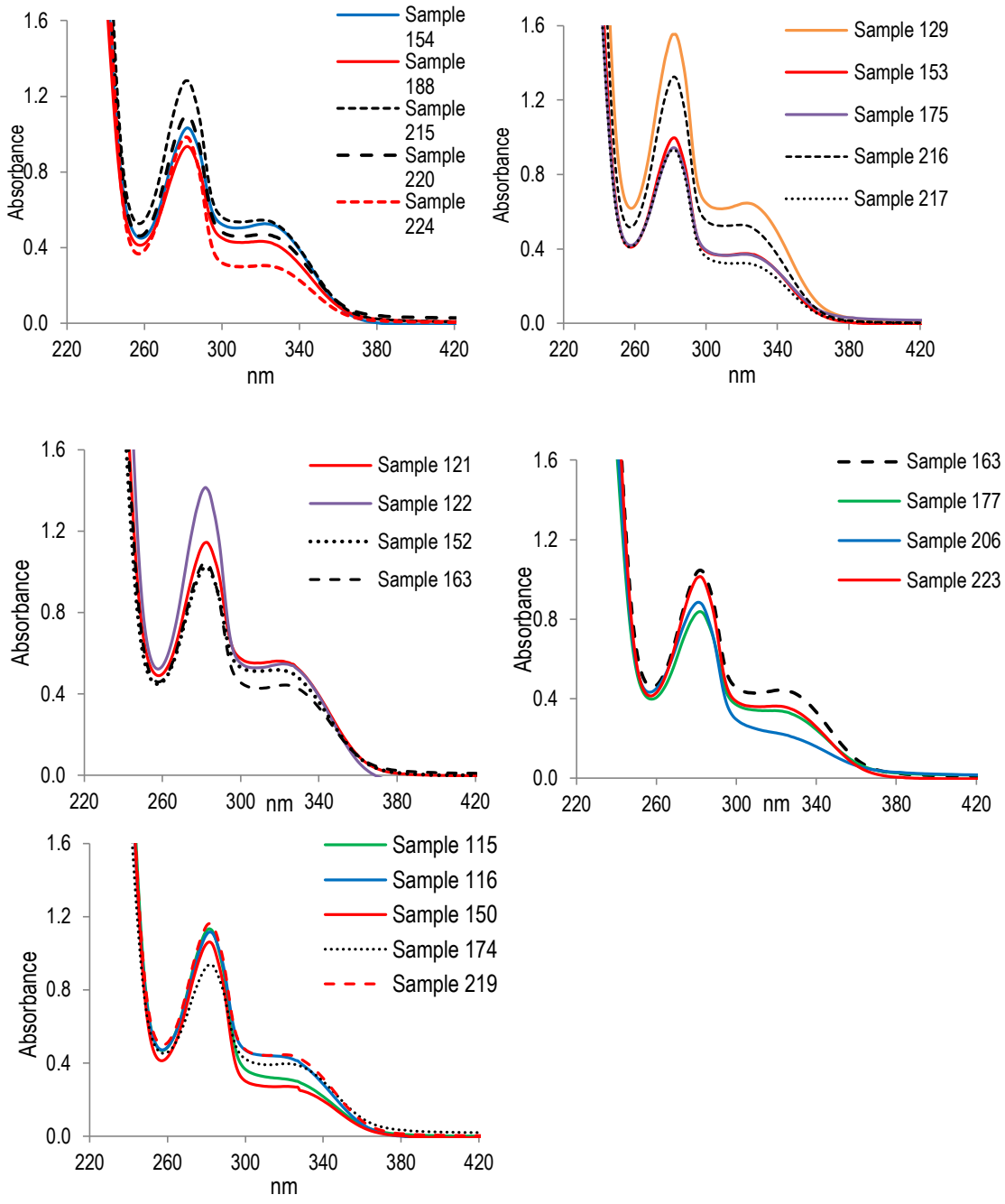



Figure 4.51 UV spectra for *P. quadrangulare ad hoc* samples collected as eaten.

In Summary:

- There were detectible differences in the UV signatures of the two *Phoradendron* species
- There was a difference in the signature of the *P. quadrangulare* growing on GU and MC host trees
- All of the samples growing on the host MC trees appear to have similar constituents but there may be variation in individual components each month or between MC hosts.

## 4.16.1 UV spectra in presence of shift reagents

Shift reagents were used to attempt to differentiate between different phenylpropanoids and flavonoids potentially present in the digests, Section 4.6.3.1. The interpretation of the shift data in the literature relates to individual compounds, whilst the data presented relates to a mixture of compounds.

Response of *P. robustissimum* digests to Na acetate (NaOAc)/boric acid ($H_3BO_3$)

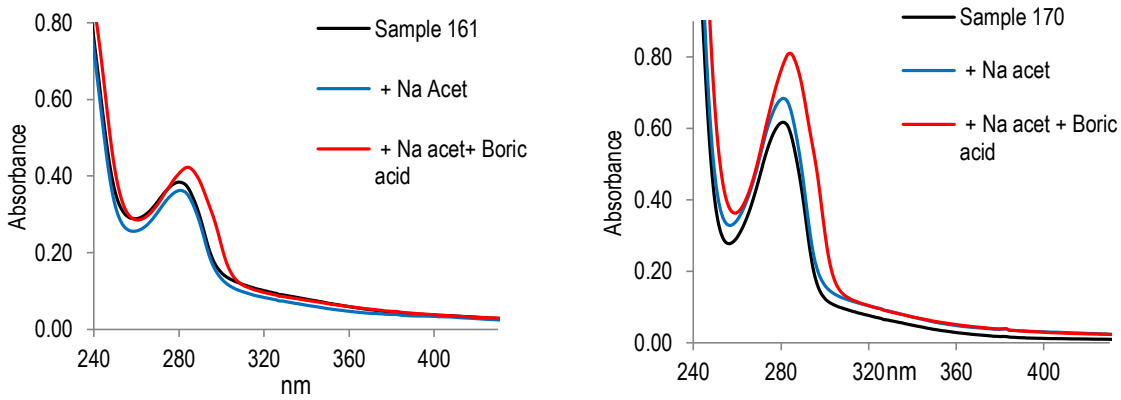



Figure 4.52 UV spectra NaOAc/boric acid*: P. robustissimum* host tree TO and *P. robustissimum* host tree LS

*P. robustissimum* digests show no shift in Band II (250-285nm) in response to the NaOAc reagent but an increase in intensity and a small bathochromic 3nm shift (i.e. the shift of a spectral band to lower frequencies, longer wavelengths) with the addition of $H_3BO_3$., Figure 4.52. The shifts may give some indication of functional groups present in the major components contributing to the UV spectra.

Response of *P. quadrangulare* digests to Na acetate (NaOAc)/boric acid ($H_3BO_3$).

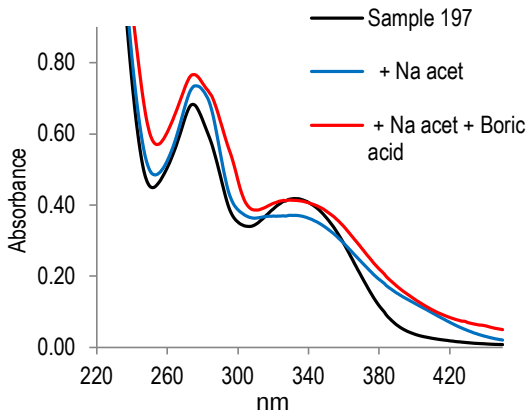



Figure 4.53 UV spectra NaOAc/boric acid*: P. quadrangulare* host/GU, Sample 197

*P. quadrangulare*/GU digest, Figure 4.53 shows no shift of Band II with NaOAc but a small hypsochromic shift from 332-329nm with the addition of $H_3BO_3$. (i.e. the shift of a spectral band to higher frequency or shorter

wavelength). There is also the possible appearance of a shoulder at ~285nm. The lack of shift of Band II with NaOAC suggests there is no free 7-OH group, potentially a 7-glycoside flavonoid compound.

The effects of the shift reagent are more obvious in the digests from *P. quadrangulare*/MC host tree Figure 4.54.

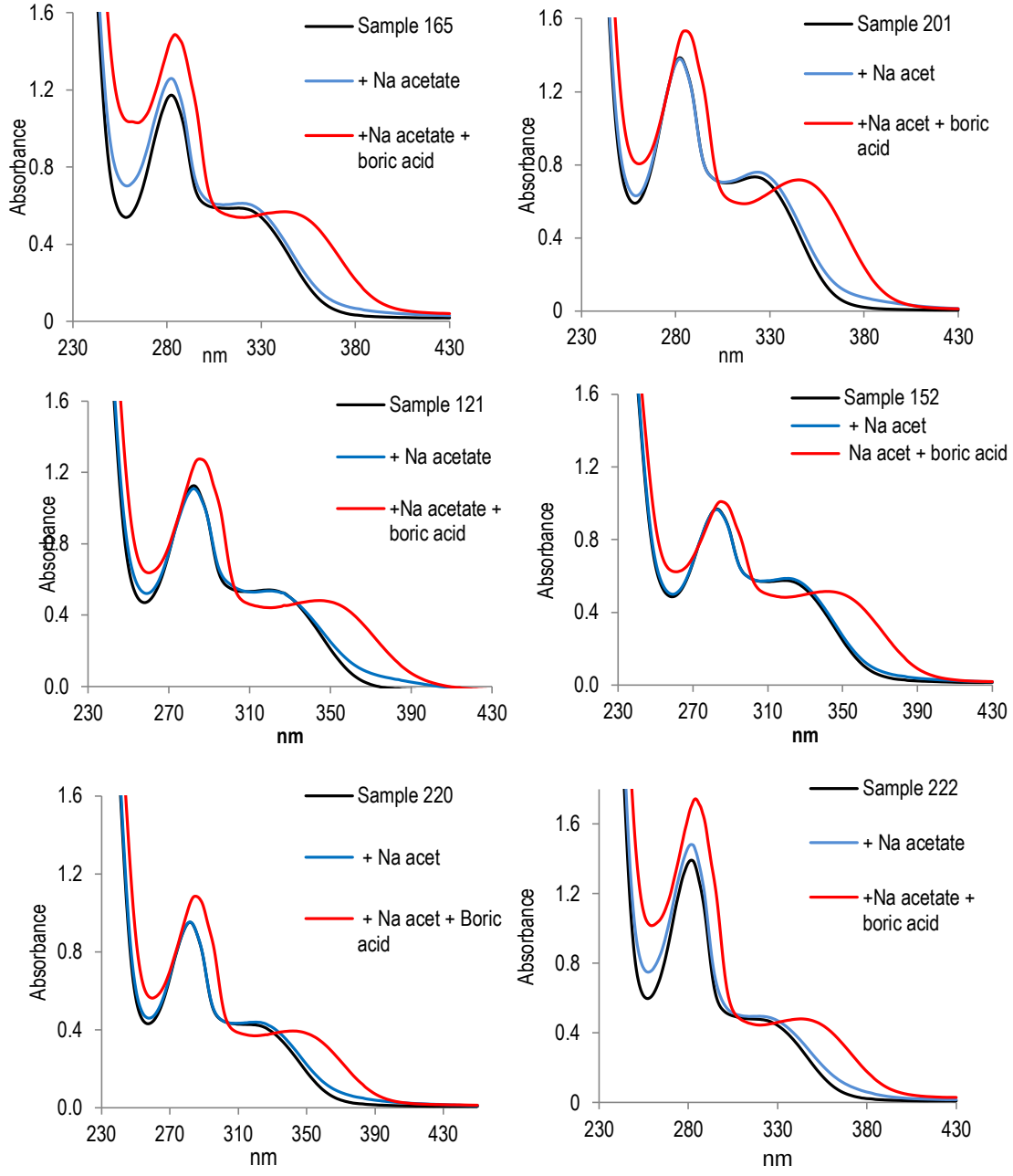



Figure 4.54 UV spectra NaOAc/boric acid: *P. quadrangulare* eaten leaves

Addition of boric acid produces a small bathochromic shift with increased intensity of Band II and a bathochromic shift of + 18-36nm Band I (320-385nm). This is suggestive of B ring *o*-diOH groups, (Figure 4.53-4.54(a-c), Table 4.28). However o-dihydroxycoumarins also give a bathochromic shift with NaOAc/$H_3BO_3$ (Ibrahim *et al.* 1989).

Table 4.28 Response of *P. quadrangulare* digests to Na acetate (NaOAc)/boric acid ($H_3BO_3$) shift reagent, (* bathochromic shifts)

| ID | reagent | Shift Band II | Shift Band I | ID | reagent | Shift Band II | Shift Band I |
|---|---|---|---|---|---|---|---|
| 165 | NaOAc | 282 n/c | 320→321* | 152 | NaOAc | 282 n/c | 320→321* |
| | + boric acid | 282→286* | 321→344* | | + boric acid | 282→284* | 321→344* |
| 201 | NaOAc | 282 n/c | 318→321* | 220 | NaOAc | 282 n/c | 318→321* |
| | + boric acid | 282→285* | 321→342* | | + boric acid | 282→285* | 321→344* |
| 121 | NaOAc | 282 n/c | 320→321* | 222 | NaOAc | 282 n/c | 320→321* |
| | + boric acid | 282→285* | 321→345* | | + boric acid | 282→286* | 321→343* |

## Response of *P. robustissimum* digests to $AlCl_3$/HCl shift reagent

Aluminium forms stable complexes with 4-keto-5-hydroxyl and 4 keto-3-hydroxyl groups with a bathochromic shift and an acid labile complex with any *ortho* dihydroxy groups present in flavonoids (Markham 1989). The presence of the latter is seen as a hypsochromic shift upon the addition of the HCl (Markham 1989).

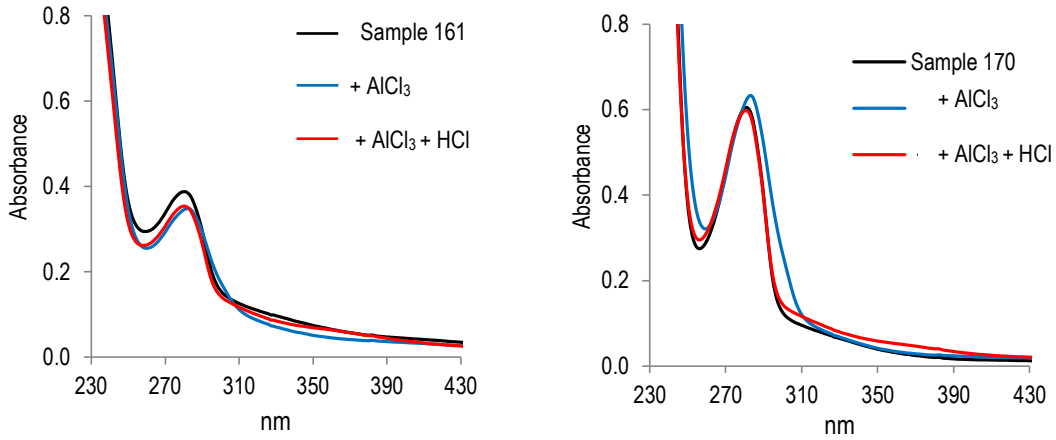



Figure 4.55 UV spectra $AlCl_3$/HCl: *P. robustissimum* host TO and LS.

The *Phoradendron* digests from TO and LS host differ (Figure 4.55) showing a 1-2nm shift Band II with $AlCl_3$ reversed by addition of HCl for Pr/LS. This indicates there was no free 5-OH or possibly a 5-OH and 6-prenyl group (Markham 1989), Figure 4.56. Also, o-dihydroxycoumarins exhibit a bathochromic shift with $AlCl_3$ (Ibrahim *et al.* 1989).

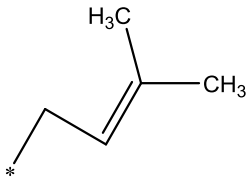



Figure 4.56 a prenyl group (* attachment point)

## Response of *P. quadrangulare* digests to $AlCl_3$/HCl shift reagent

The response for *P. quadrangulare*/host GU, Figure 4.57 shows a comple response to $AlCl_3$ with the appearance of shoulders at 303 and 388nm and bathochromic shift of Band 1 from 333 to 344 upon the addition of $AlCl_3$.

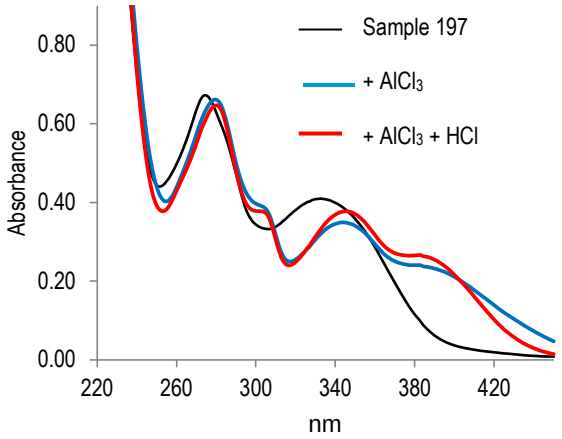



Figure 4.57 UV spectra $AlCl_3$/HCl *P. quadrangulare* host GU.

These changes are not reversed by the addition of HCl. The Band I shift of 333-344nm suggests that neither A or B ring contain *ortho*-diOH groups (Markham 1989).

The response to $AlCl_3$ was broadly similar in the majority of the samples tested, all weakly showing a shoulder suggestive of a bathochromic shift of Band I as shown by the examples Figures 4.58 a-c. There is not the complexity seen in the digest form *P. quadrangulare*/GU digest. Sample 152 (Figure 4.58b) illustrates a further possible difference in constituents in the region between240-220nm.

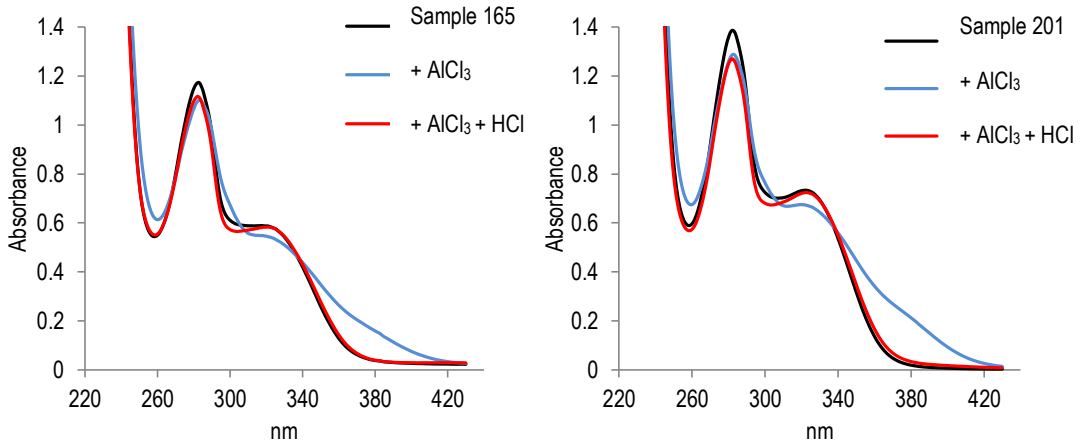



Figure 4.58a UV spectra $AlCl_3$/HCl *P. quadrangulare* Control tree.

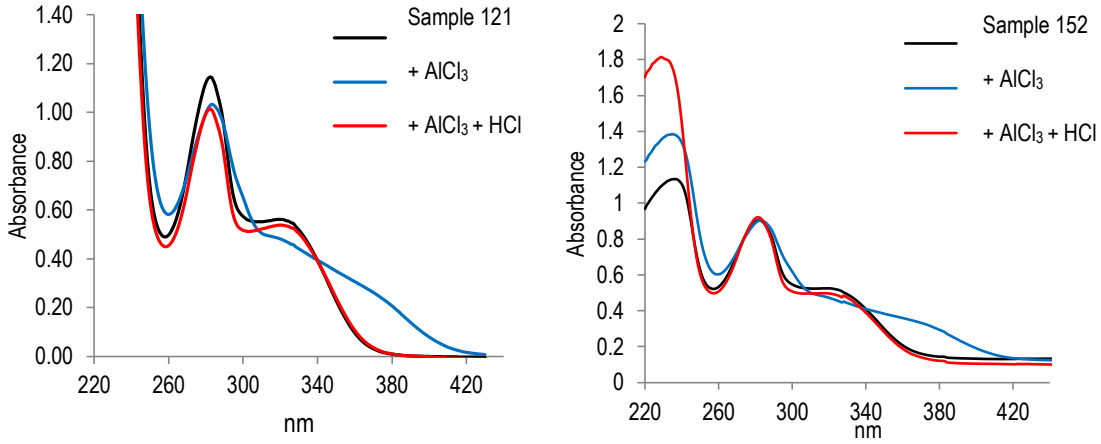



Figure 4.58b UV spectra $AlCl_3$/HCl *P. quadrangulare* eaten samples.

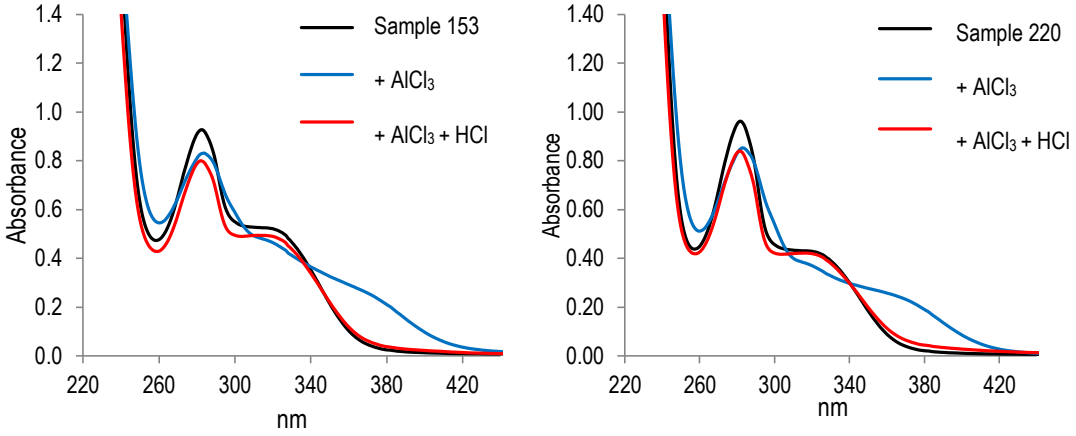



Figure 4.58c UV spectra $AlCl_3$/HCl *P. quadrangulare* eaten samples.

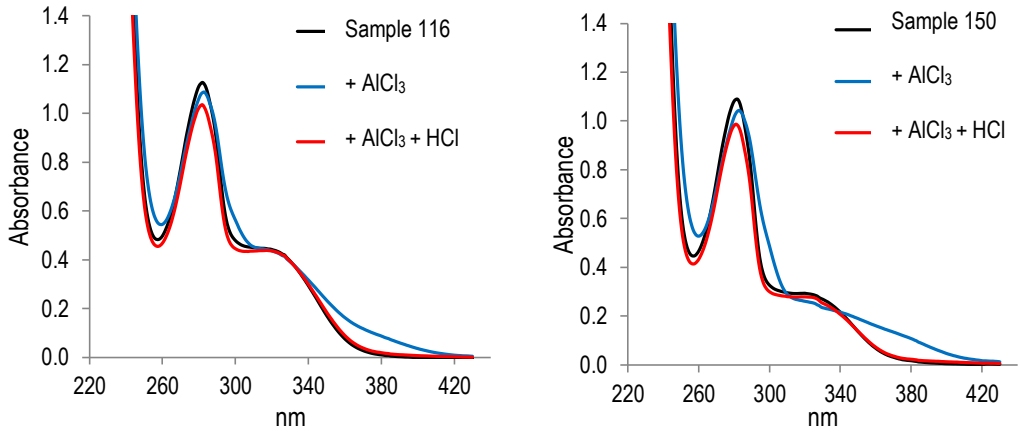



Figure 4.58d UV spectra $AlCl_3$/HCl Sample 116 *P. quadrangulare*/MC9 eaten (April) Sample 150 (August).

There is still a suggestion of a hypsochromic shift reversing the effect upon the addition of HCl. This when present is indicative of the presence of *o*-dihydroxy groups and the magnitude of the shift suggests the group is positioned on the B ring (Harborne 1989). This would be consistent with apigenin or its glycosides Figure 4.49(a-b). This is a compound which has been identified in other *Phoradendron* species (Dossaji *et al.* 1983, Varela *et al.* 2004).

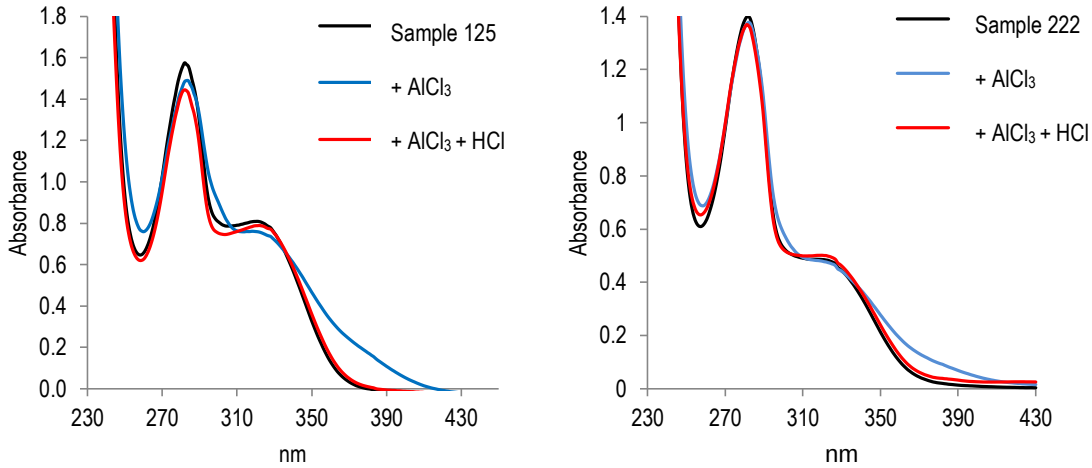



Figure 4.58e UV spectra $AlCl_3$/HCl *P. quadrangulare*/MC1 Sample 125 not eaten (May) Sample 222 eaten samples (Sept).

However there were samples which showed more limited response to $AlCl_3$, Figure 4.58(d-e). These included both eaten and non eaten samples from more than one host tree. The samples from MC9 are April and August and host MC1 are from May and September, showing that there are variations in constituents

within the samples eaten and it is not necessarily related to differences in host tree. Where seen the reaction to the $AlCl_3$ was a bathochromic shift of Band I from 319/320nm to a weak shoulder feature at 364-366nm. This shift is not well defined suggesting that there are multiple components within the mixture.

## 4.16.2 UV comparison of effect of digestion with geophagy material

Figure 4.59 illustrates the effect on the UV spectra when digested with geophagy sample material. There is a reduction in absorbance at 280nm ~ 37% (Sample 161TO) and ~ 46% (Sample 170LS). These results suggest that there would be a reduction in any potential toxicity associated with compounds which contributed to the absorbance at 280nm in *P. robustissimum.*

The following UV spectra are representative of all those determined for *Phoradendron* digests

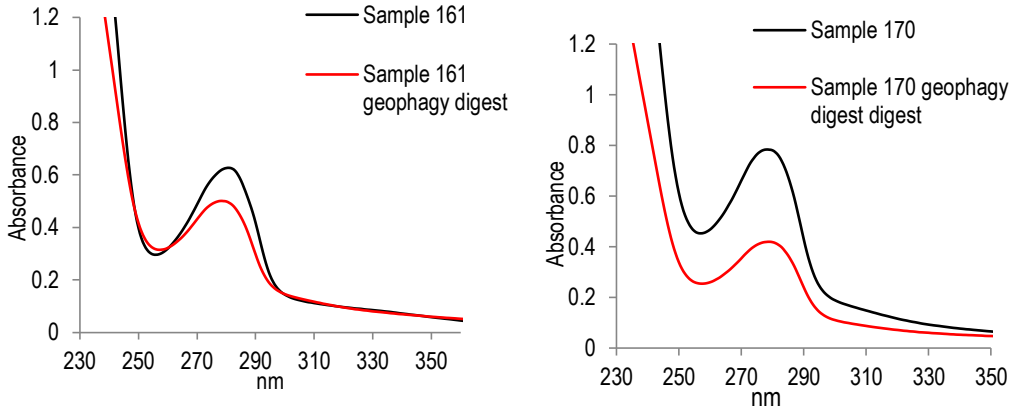



Figure 4.59 UV spectra *P. robustissimum*/rock digest /host LS and TO.

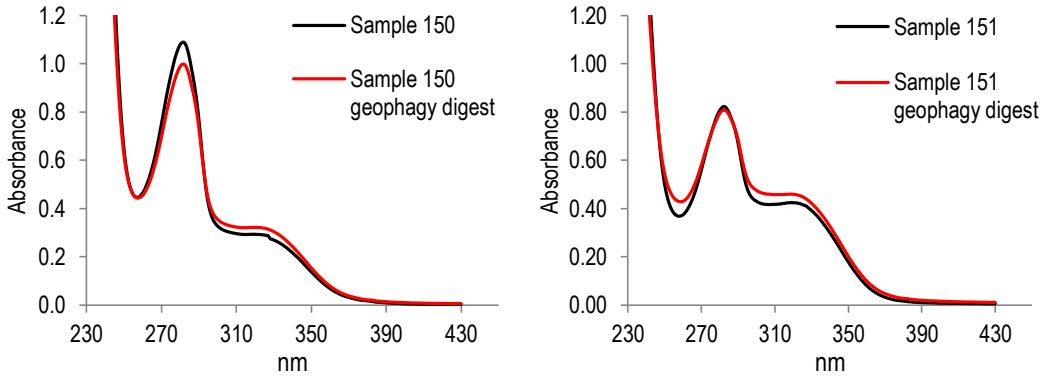



Figure 4.60(a) UV spectra *P. quadrangulare*/geophagy digests eaten leaves/host MC.

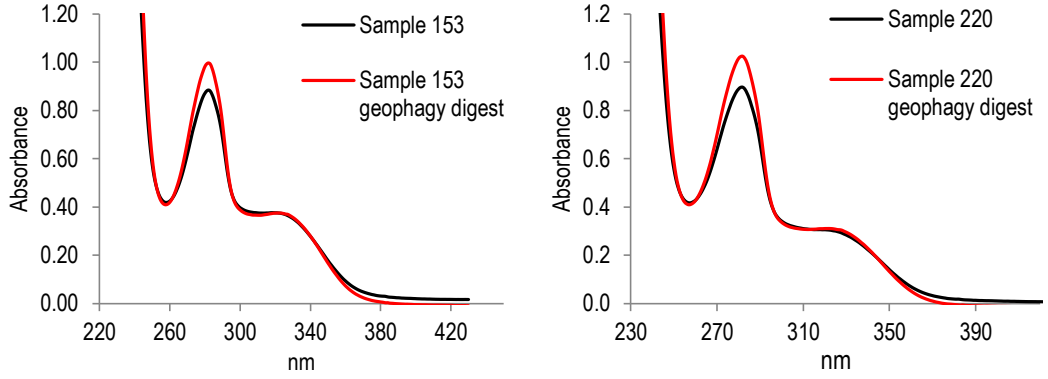



Figure 4.60(b) UV spectra *P. quadrangulare*/geophagy digests eaten leaves/host MC.

There is minimal reduction in the absorbance values when *P. quadrangulare* is digested in the presence of the geophagy material, Figure 4.60a. There were small increases in absorbance at 280nm in Samples

153/220 Figure 4.60(b) and absorbance 323/325nm in Samples 150/151 Figure 4.60(a), variable responses.

In Summary**:**

- The UV digest spectra indicate that there are differences in the principal constituents in the digests from the two *Phoradendron* species.
- The digest spectra suggest that *P. robustissimum* samples have negligible levels of aqueous soluble flavonoids or related compounds.
- The inferences are supported by the behaviour of the mistletoe digests to the shift reagents.
- There was no detectable adsorption of constituents following digestion of *P. quadrangulare* leaf with geophagy sample. The potential increases intensity at the $\lambda_{max}$ suggests there may be an increased in constituents released from the leaf.
- There was a reduction in absorbance following combined digestion in intensity at the $\lambda_{max}$ for the *P. robustissimum* digests, suggesting adsorption by the geophagy material.

# 4.17. Microbiological testing

## 4.17.1 Preliminary microbiological screening of mistletoe digests

Table 4.29 Results of preliminary antibacterial testing

| **Sample no.** | **Host tree** | ***B. cereus* NCTC 6474** | ***B. megaterium* NCTC 5635** | ***B. subtillis NCIMB 8954*** | ***S. aureus* NCTC 6571** | ***E. coli* ATCC 8739** |
|---|---|---|---|---|---|---|
| **host leaf samples** | | | | | | |
| 107 | LS | N | N | N | N | N |
| 118 | LS | N | N | N | N | N |
| 109 | TO | N | N | N | N | N |
| 124 | TO | N | N | N | N | N |
| 102 | MC | N | N | N | N | N |
| 104 | MC | N | N | N | N | N |
| 126 | MC | N | N | N | N | N |
| 128 | MC | N | N | N | N | N |
| ***Phoradendron* Samples** | | | | | | |
| 169 (Pr) | LS | N | N | N | N | N |
| 134 (Pr) | LS | N | N | N | N | N |
| 141 (Pr) | LS | N | N | N | N | N |
| 158 (Pr) | LS | N | N | N | N | N |
| 176 (Pr) | LS | N | N | N | N | N |
| 131 (Pr) | TO | N | N | N | N | N |
| 137 (Pr) | TO | N | N | N | N | N |
| 162 (Pr) | TO | N | N | N | N | N |
| 103 (Pq) | MC | ++A | A | ++A | +++A | A |
| 125 (Pq) | MC | ++A | A | ++A | +++A | A |
| 132 (Pq) | MC | ++A | A | ++A | +++A | A |
| 139 (Pq) | MC | ++A | A | ++A | +++A | A |
| 146 (Pq) | MC | ++A | A | ++A | +++A | A |
| 151 (Pq) | MC | ++A | A | ++A | +++A | A |
| 152 (Pq) | MC | ++A | A | ++A | +++A | A |
| 165 (Pq) | MC | ++A | A | ++A | +++A | A |
| 174 (Pq) | MC | ++A | A | ++A | +++A | A |
| 187 (Pq) | MC | ++A | A | ++A | +++A | A |

Key – *P. robustissimum* (Pr); *P. quadrangulare* (Pq); N – no measurable activity, A – activity < 2mm; ++A 2-4mm, +++A – activity > 4mm ZI.

A well of each diffusion plate was used as a control, it contained the appropriately treated and reconstituted digest fluid, no Zone of Inhibition (ZI) seen with gastric media or pH2 deionised water.

The results for *E. coli* were consistently less than 2mm ZI, this presented problems for accurate measurement, and therefore as this work was only intended as an indicator of biological activity and the limitations of sample. *E. coli* was not used in further investigations. There were three samples of *P. quadrangulare* which exhibited weak activity against *S. marcescens* and *P. vulgaris, Samples 139, 174 and 187.* The remaining results are summarised in Tables 4.29.

Figure 4.61 is an illustration of ZI and variation of colour and extent of the digest diffusion following incubation: ZI for MC host leaf Samples 102/128 (Left), *P. quadrangulare*/MC (Centre) and *P. robustissimum.*LS (Right).

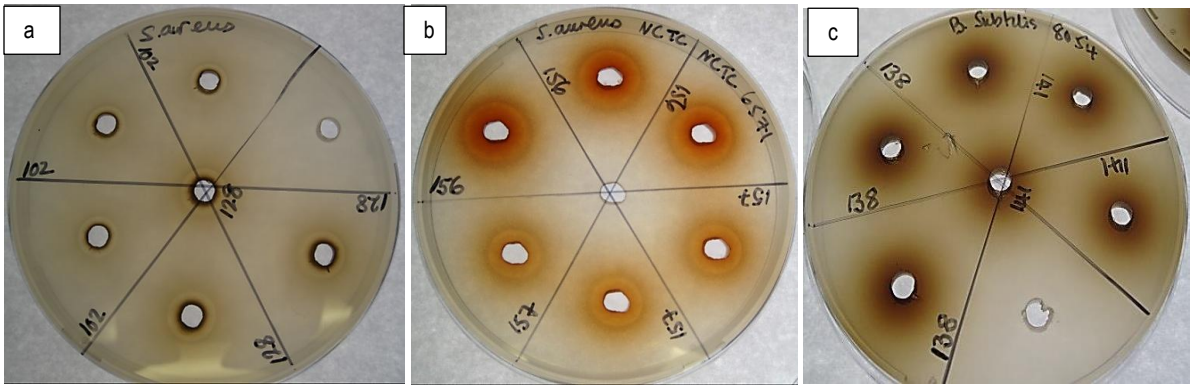



Figure 4.61 Examples of plates with ZI and colour variation Authors' photographs.

Figure 4.62 Plate (a) illustrates differences in ZI of two MC host leaf digests (102 and 128) and Plate (b) digests (156, 157) against *S. aureus.* Plate (c) has no visible ZI. These images also illustrate clearly the colour variations encountered with the different digests.

*B. cereus* under the conditions used did not produce a visually dense growth or a clear sharp edge to the ZI in the agar making measurement problematic. The remainder of the work used *B. subtilis*, NCIMB 8054 and *S. aureus* NCTC 6571 as these organisms appeared the most sensitive.

## 4.17.2 Antibacterial activity of *Phoradendron* digests

### 4.17.2.1 Activity against *B. subtilis*

There was a low level of activity in the *Manilkara chicle* (MC) host leaf. No activity was detected in the other host leaves (Table 4.29).

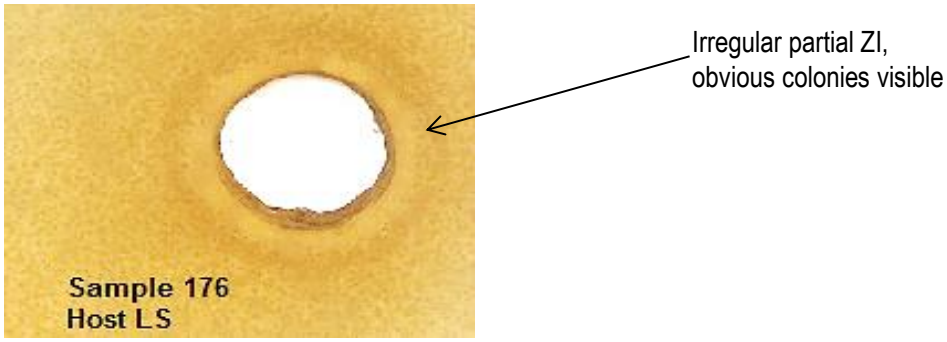



Figure 4.62 Illustration ZI - *B. subtilis* – digest Sample 176 *P. robustissimum*/host LS

*P. robustissimum*, on both host trees, showed lower amount of bacterial inhibition, ZI < 2mm, the zone edges were irregular/raggy and there was a small amount of clumps of growth within the 'ZI', Figure 4.62. The ZI generated by *P. quadrangulare*/MC host had sharp well-defined margins, clear ZI with no sign of growth, Figure 4.63. The zone was surrounded by an area where the agar was granular and this gradually became less obvious until it became the general culture in the plate. This is suggestive of bacteriostatic/bacteriocidal activity. There was no resumed growth if the plates were incubated for a further 36 hours, suggesting bactericidal activity.

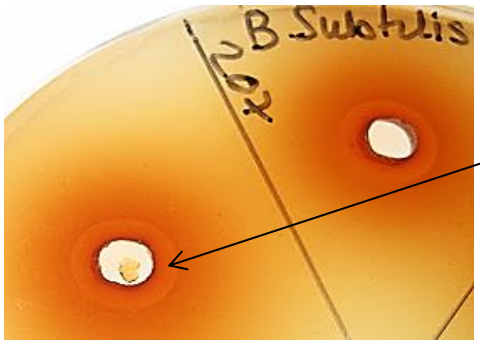



Figure 4.63 ZI *B. subtilis* digest *P. quadrangulare*/MC host

*P. quadrangulare* growing on host tree *Guazuma ulmifolia*, Figure 4.64 had an initial clear ZI but there was recolonisation. This recolonisation is usually considered to be due to colony formation by mutant organisms showing resistance to an agent. These differences suggest there is more than one agent responsible for inhibition of growth involved in the *P. quadrangulare* from hosts GU and MC leaf digests, as the bacteria show different responses to each type of digest. GU host leaf digest and *P. quadrangulare*/GU digests did not colour the agar green or brown as did the other digests, Figure 4.60 and 4.64. The results are summarised in Tables 4.30(a-c).

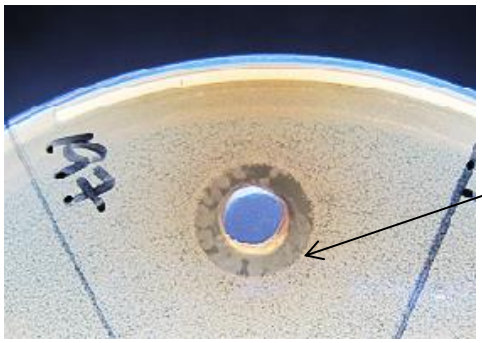



Figure 4.64 Illustrating ZI - *B. subtilis* – digest Sample 197, *P. quadrangulare*/host GU

Table 4.30(a) Summary Measured ZI, *P. robustissimum*– culture *B. subtilis*

| **Host TO** | | | | **Host LS 20** | | | |
|---|---|---|---|---|---|---|---|
| **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** |
| 136 | 1.44 | 189 | 1.82 | 134 | N/A | 141 | <1mm |
| 144 | 1.55 | 196 | 1.98 | 159 | N/A | 160 | N/A |
| 161 | 1.59 | 200 | 1.04 | 169 | N/A | 170 | N/A |
| 171 | 1.47 | 205 | 1.40 | 182 | N/A | 176 | 2.04 |
| 173 | 1.37 | 209 | 1.46 | 199 | N/A | 194 | 2.05 |
| 184 | 2.24 | 214 | 2.02 | 208 | N/A | 204 | 2.08 |
| | | | | 138 | <1mm | 213 | 2.02 |

Table 4.30(b) Summary Measured ZI, *P. quadrangulare*– culture *B. subtilis*

| **Host MC1** | | **Host MC21** | | **Host MC4** | | **Misc MC hosts** | |
|---|---|---|---|---|---|---|---|
| **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** |
| 125 | 3.95 | 127 | N/A | 129 | 4.29 | 113 | 3.89 |
| 130 | 5.05 | 139 | 3.89 | 153 | 3.26 | 114 | 4.51 |
| 132 | 4.21 | 146 | 2.56 | 175 | 2.71 | 121 | 3.98 |
| 147 | 3.32 | 156 | 3.35 | 188 | 2.63 | 122 | N/A |
| 155 | 3.04 | 166 | 3.18 | 216 | 4.65 | 152 | 2.98 |
| 165 | 3.16 | 179 | 3.08 | 217 | 3.08 | 163 | 3.84 |
| 178 | 3.52 | 191 | 1.57 | | | 177 | 2.66 |
| 195 | 3.19 | 198 | 2.89 | | | 188 | 3.27 |
| 201 | 2.64 | 207 | 4.31 | | | 206 | 2.74 |
| 210 | 4.50 | | | | | 223 | 3.04 |
| 221 | 3.40 | | | | | | |
| 222 | 3.95 | | | | | | |
| 225 | 3.82 | | | | | | |
| **Host MC76** | | **Host MC2** | | **Host MC 9** | | **Host GU** | |
| **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** |
| 148 | 2.32 | 151 | 3.06 | 115 | 4.46 | 140 | 0.71 |
| 157 | 1.95 | 154 | 2.84 | 116 | 3.19 | 164 | 1.34 |
| 167 | 0.94 | 187 | 2.66 | 150 | 3.08 | 197 | 1.49 |
| 180 | 2.29 | 215 | 2.28 | 174 | 3.25 | | |
| 186 | 2.62 | 220 | 2.62 | 219 | 4.23 | | |
| 202 | 3.05 | 224 | 2.87 | | | | |
| 211 | 3.16 | | | | | | |
| 226 | 2.86 | | | | | | |

Table 4.30 (c) Summary Measured ZI, *P. quadrangulare* digests – culture *S. aureus*

| **Host MC1** | | **Host MC21** | | **Host MC 4** | | **Misc MC hosts** | |
|---|---|---|---|---|---|---|---|
| **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** |
| 125 | 5.74 | 127 | N/A | 129 | 10.3 | 113 | 6.06 |
| 130 | 9.12 | 139 | 7.96 | 153 | 7.32 | 114 | 7.18 |
| 132 | 6.82 | 146 | 9.30 | 175 | 8.56 | 121 | 9.50 |
| 147 | 6.30 | 156 | 7.31 | 216 | 10.34 | 122 | N/A |
| 155 | 5.92 | 166 | 6.28 | 217 | 6.78 | 152 | 6.32 |
| 165 | 6.98 | 179 | 6.12 | | | 163 | 9.81 |
| 178 | 7.04 | 191 | 5.51 | | | 177 | 4.78 |
| 195 | 5.38 | 198 | 8.44 | | | 188 | 6.54 |
| 201 | 5.76 | 207 | 10.74 | | | 206 | 10.12 |
| 210 | 9.94 | | | | | 223 | 6.64 |
| 221 | 9.66 | | | | | | |
| 222 | 9.74 | | | | | | |
| 225 | 9.36 | | | | | | |
| **Host MC76** | | **Host MC2** | | **Host MC9** | | **Host GU** | |
| **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** | **ID** | **ZI mm** |
| 148 | 6.40 | 151 | 6.51 | 115 | 9.92 | 140 | 0.68 |
| 157 | 5.14 | 154 | 8.26 | 116 | 15.24 | 164 | N |
| 167 | 3.98 | 187 | 9.04 | 150 | 8.31 | 197 | 0.52 |
| 180 | 10.41 | 215 | 7.18 | 174 | 10.02 | | |
| 186 | 7.46 | 220 | 8.76 | 219 | 7.36 | | |
| 202 | 8.56 | 224 | 10.02 | | | | |
| 211 | 8.06 | | | | | | |
| 226 | 6.98 | | | | | | |
| | | | | | | | |

## 4.17.3 Antibacterial activity geophagy digests and combined digests.

### Direct application of geophagy sample to seeded agar plate:

The geophagy material was carefully removed from the plate to permit viewing using a light-box. There was an area with less density of *B. subtilis* and *S. aureus* under the geophagy sample, but it was not possible to quantify the possible change observed.

### Incubation with geophagy digests:

There was no measurable ZI seen with geophagy digests from Sites 2, 6, 8, 9 and 10 at either 1g/8ml or the higher 2g/8ml digests prepared with any of the three test organisms.

### Combined digests

In order to permit direct comparison of the effect of combined digestion the leaf and combined digests were prepared as a single batch and tested against the same sample of bacterial organisms. This ensured that the samples were tested against similar numbers of viable organisms in a single batch of prepared agar plates and were incubated as a single batch, using *B. subtilis* and *S. aureus* (Table 4.31). *B. subtilis* was selected as it had shown some response to direct application of the geophagy sample and *S. aureus* as it had the greater inhibition with *P. quadrangulare* alone. Sufficient material was available to utilise 14 plate wells for each sample. It was decided to use geophagy material from a single eating Site 10, as this was from a single collection and would have a greater degree of sample uniformity.

Table 4.31 Results *P. quadrangulare*/geophagy digests with *B. subtilis/S. aureus*

| **Host** | **ID** | **Mean ZI mm *P. quad.* digest** | **Mean ZI mm *P. quad.* digest + geophagy material** | **Host** | **ID** | **Mean ZI mm *P. quad.* digest** | **Mean ZI mm *P. quad.* digest + geophagy material** |
|---|---|---|---|---|---|---|---|
| ***S. aureus*** | | | | | | | |
| MC1 | 132 | 6.39 | 6.59 | MC9 | 150 | 5.89 | 6.17 |
| MC1 | 195 | 5.64 | 5.94 | MC9 | 219 | 6.23 | 6.76 |
| MC21 | 156 | 6.56 | 6.95 | MC2. | 187 | 8.76 | 5.37 |
| MC21 | 207 | 6.31 | 6.85 | Misc. | 206 | 5.74 | 6.01 |
| MC76 | 148 | 5.74 | 6.47 | | | | |
| MC76 | 180 | 8.05 | 8.35 | | | | |
| MC4 | 153 | 5.63 | 6.01 | | | | |
| MC4 | 217 | 5.92 | 6.17 | | | | |
| ***B. subtilis*** | | | | | | | |
| MC1 | 132 | 4.22 | 4.31 | MC9 | 150 | 2.98 | 3.09 |
| MC1 | 195 | 3.10 | 3.24 | MC9 | 219 | 4.17 | 4.26 |
| MC21 | 156 | 3.40 | 3.51 | MC2 | 187 | 2.59 | 2.69 |
| MC21 | 207 | 4.15 | 4.29 | Misc. | 206 | 2.61 | 2.72 |
| MC76 | 148 | 2.25 | 2.76 | | | | |
| MC76 | 180 | 2.20 | 2.69 | | | | |
| MC4 | 153 | 3.18 | 3.24 | | | | |
| MC4 | 217 | 2.99 | 3.14 | | | | |

A 2-tailed Mann-Whitney test was performed, in both cases the P value was >0.05. The difference between the treatments was not significant. It was not possible to detect a synergistic effect.

In Summary**:**

- *P. robustissimum*/TO samples had no activity in the preliminary testing and weak activity against *B. subtilis* < 2mm ZI in 10/12 digests: the remaining 2 digests had > 2mm ZI.
- *P. robustissimum* /LS had weak activity > 2mm ZI in 4/14 digests tested against *B. subtilis*
- None of the *P. robustissimum* digests exhibited activity against *S. aureus*.
- Preliminary activity of *P. quadrangulare*/MC digest against *S. aureus was* greater than *B. subtilis* >*B. cereus* > *B. megaterium.* Subsequent testing also showed greater activity against *S. aureus.*
- No activity was seen from the geophagy digests but there was a suggestion of potential activity from direct application of geophagy material against the two organisms tested.
- The increase in ZI seen from the combined digestion work was not statistically significant.

## 4.18 HPLC analysis *Phoradendron* digests

### 4.18.1 HPLC chromatogram of *Phoradendron* digests from method development

The following Figures 4.64(a-c) are examples of HPLC of acidic aqueous digests, obtained during method development, using an acetonitrile/water gradient. The chromatograms illustrate the complexity of the sample digests. These initial analyses suggested that there were clear differences in the components present in each digest.

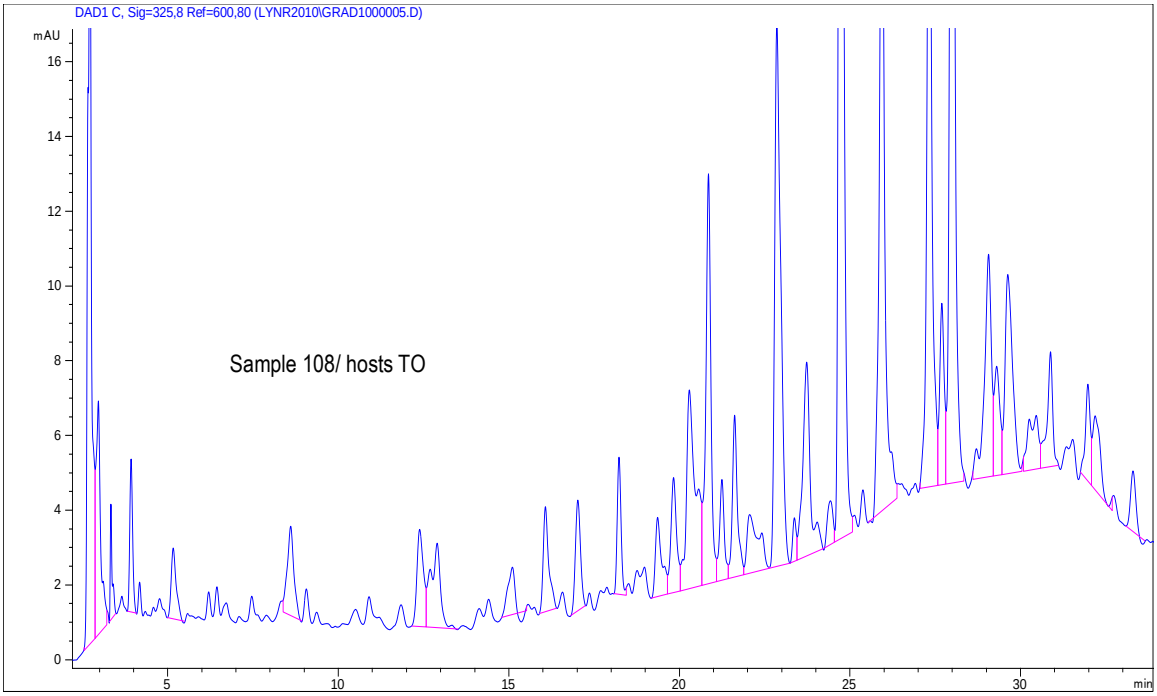



Figure 4.64(a) HPLC Chromatogram, 325nm detection, freshly prepared aqueous digest Sample 108/host TO

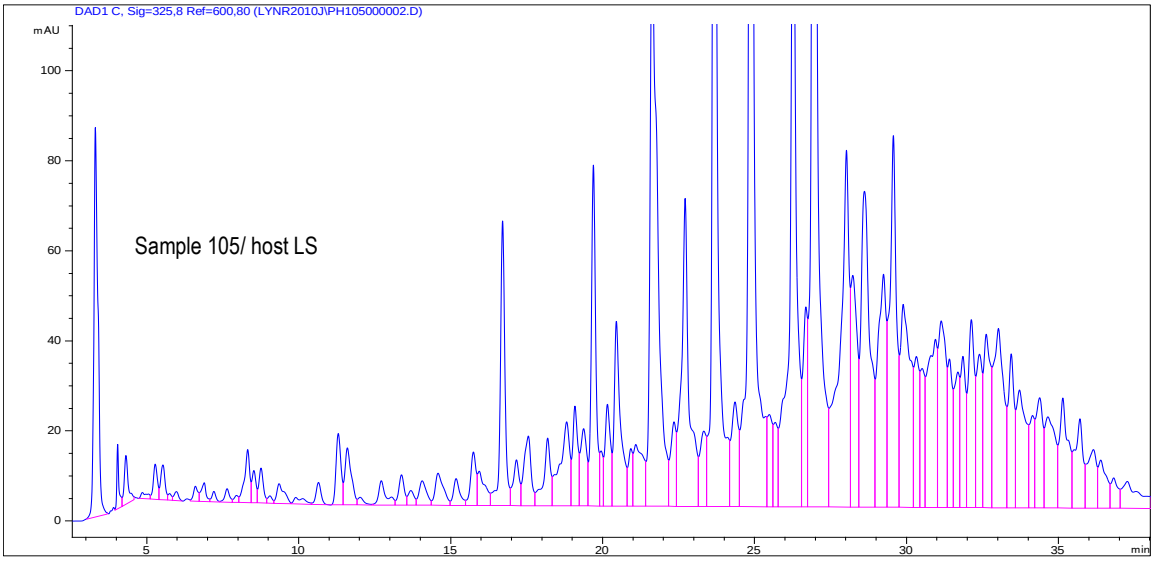



Figure 4.64(b) HPLC Chromatogram 325nm detection, freshly prepared aqueous digest Sample 105/host LS

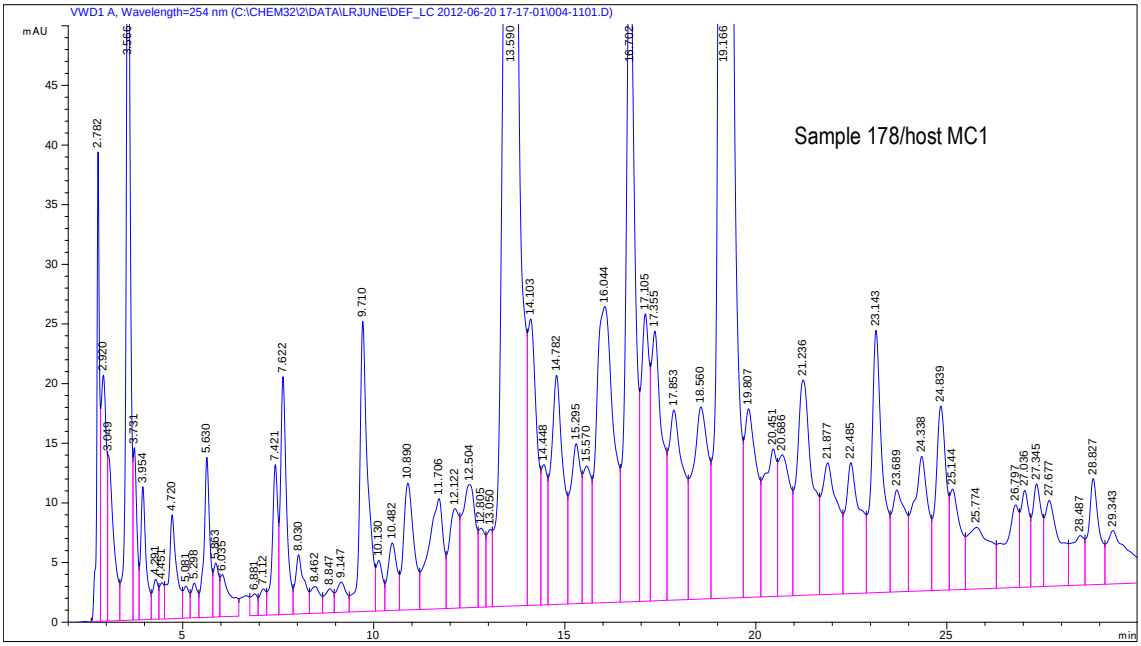



Figure 4.64(c) HPLC Chromatogram, 254nm detection, freshly prepared aqueous digest Sample 178/host MC1.

## 4.18.2 Comparison of *P. robustissimum* samples from host trees LS/TO.

Each of the digests was subjected to sample cleaning using the Chromabond 500mg PA SPE cartridges. The HPLC chromatograms of the eluents, Figures 4.65(a-b)-4.66(a-b) were obtained using the Optimal L column and the methanol/acetic acid/water gradient conditions detailed in Method section 4.8, Figure 4.18. Chromatograms are reported for the initial aqueous eluent (F1) and two methanolic eluents F2-F3 as detailed previously. UV analysis of methanolic fraction F4 had absorbance values < 0.1 therefore was not subjected to HPLC analysis. Chromatograms were obtained for eluents of the following samples 105,138,170,204 and 213 (Host LS) and Samples 108,136,161,205 and 214 (Host TO). DAD-UV detector wavelengths selected included 254 and 340nm. However the peak intensity and separation were clearer at the detection wavelengths of 280 and 325nm. Samples 161 (Pr/LS) and 170 (Pr/TO) contain all the peaks found in the individual samples and are therefore used as representative (determined at 280 and 325nm) of all those obtained.

## Aqueous eluent F1

Comparing the chromatograms obtained Figure 4.65(a-b) the contents of the two *P. robustissimum* eluents were broadly similar in constituents but varied in concentration. The differences are illustrated in comparison of the peak areas for specific peaks (Table 4.32).

Table 4.32 Peak area values principal peaks in F1 aqueous eluents, *P. robustissimum* Samples 161/170

| | **peak area mAu** | | | | |
|---|---|---|---|---|---|
| **Sample/RT mins** | **4.06** | **5.11** | **5.24** | **8.49** | **16.68** |
| Sample 161 280nm | 2194 | | 2386 | | |
| Sample 170 280nm | 830 | 5040 | | | |
| Sample 161 325nm | | | | 207 | 34 |
| Sample 170 325nm | | | | 209 | 148 |

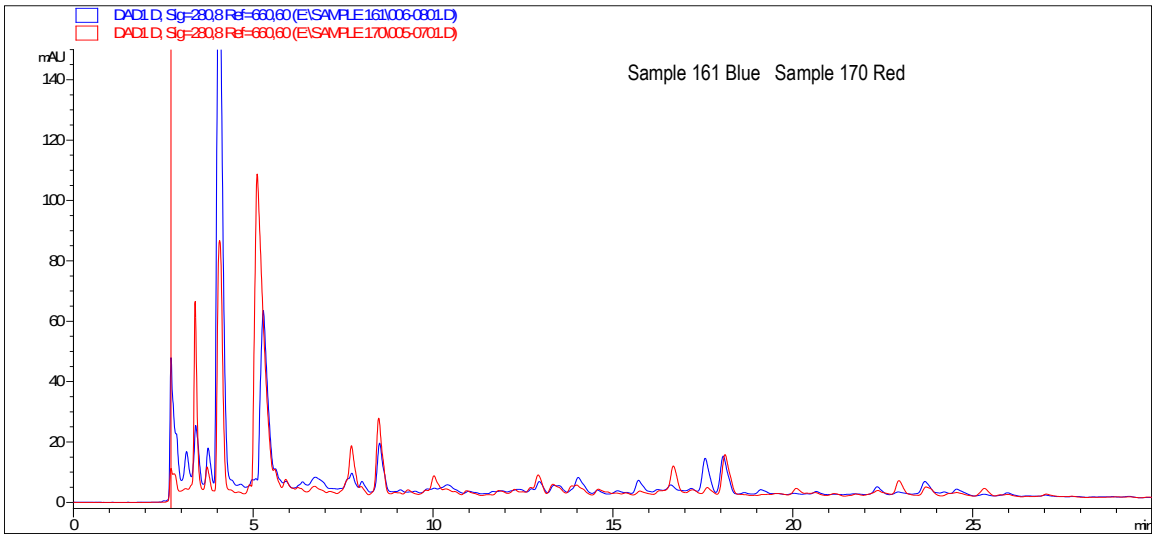



Figure 4.65(a) Chromatogram 280nm detection, (F1) *P. robustissimum* Samples 161/170.

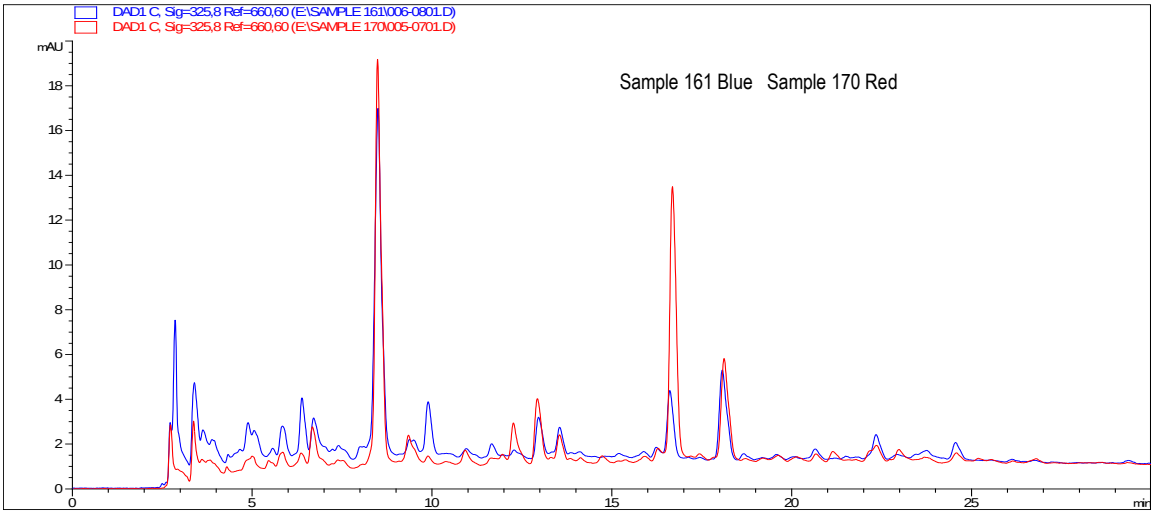



Figure 4.65(b) Chromatogram 325nm detection, (F1) *P. robustissimum* Samples 161/170.

## First methanolic eluent F2

The contents of the F2 eluents have some similar in constituents but theses varied in concentration, Figure 4.66(a-b).

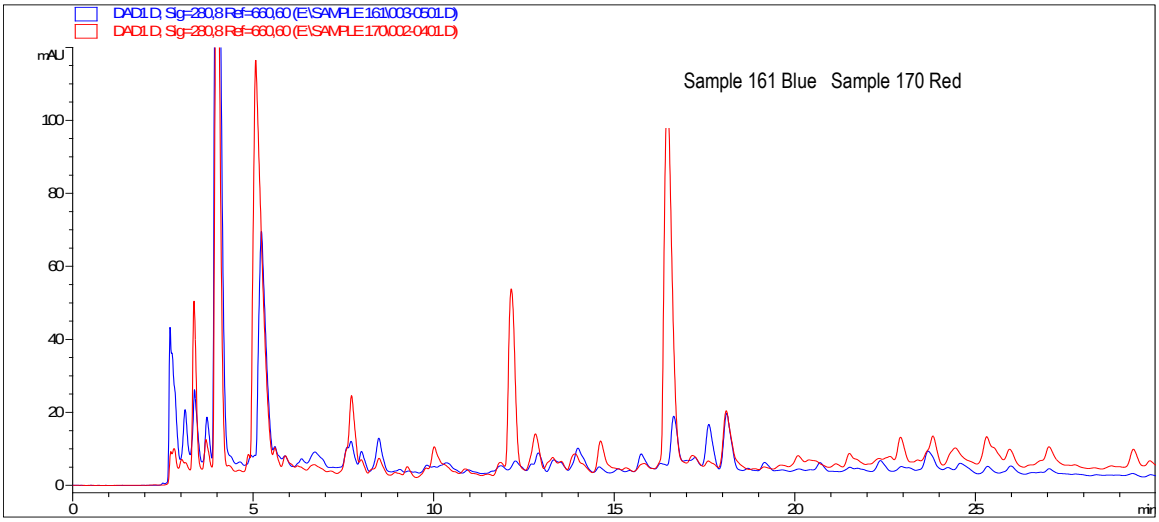



Figure 4.66(a) Chromatogram 280nm detection, (F2) *P. robustissimum* Samples 161/170.

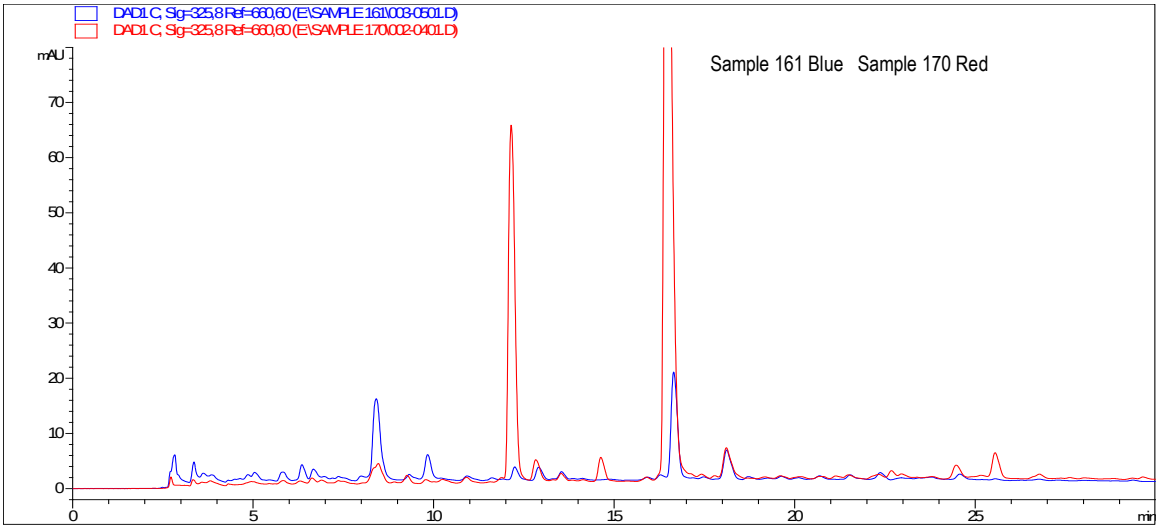



Figure 4.66(b) Chromatogram 325nm detection (F2) *P. robustissimum* Samples 161/170

The use of the PDA detector enables UV spectra to be obtained for the major peaks within the chromatograms. The quality of the spectra depends on the peak being well separated and containing a single component. Comparison of the spectra provides a further comparison between the components present in the mistletoe eluents.

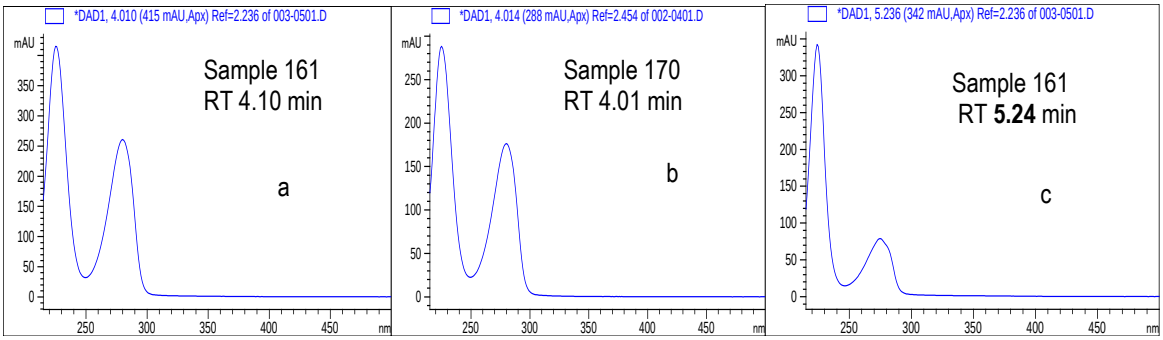



Figure 4.67 UV spectra for peaks detected in *P. robustissimum* Samples 161 and 170 RT 4-6mins

The spectra for peaks RT 4.01 mins were similar for both Samples 161/170 (at 280nm) - Figure 4.67 (a-b) however there was no equivalent well resolved peak in 170 at RT 5.24 mins, Figure 4.67 (c).

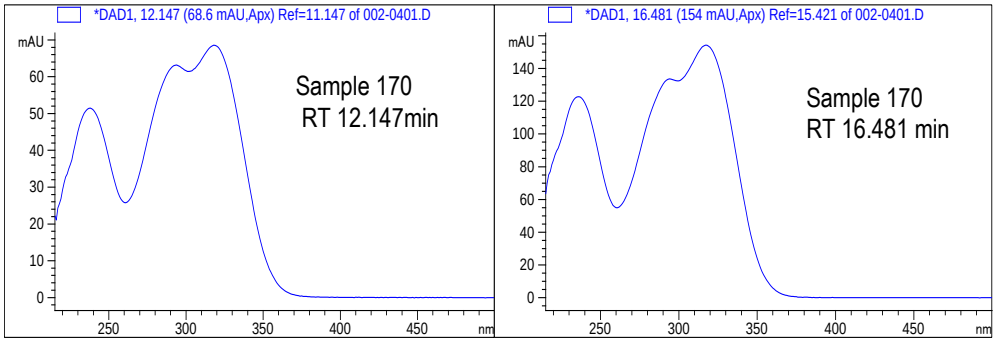



Figure 4.68 UV spectra for peaks detected in *P. robustissimum* Sample 170, RT 12-17mins

There were two peaks with spectra between 12-16 mins in Sample 170 that were not present in Sample 161, Figure 4.68.

## Second methanolic eluent F3

The principal differences were found in the second methanolic fractions (F3), Figure 4.69(a-d). In both cases, these eluents were bright deep yellow coloured solutions. The humped shape of the chromatograms is indicative of many compounds which are not separated and which under these gradient conditions have similar retention times.

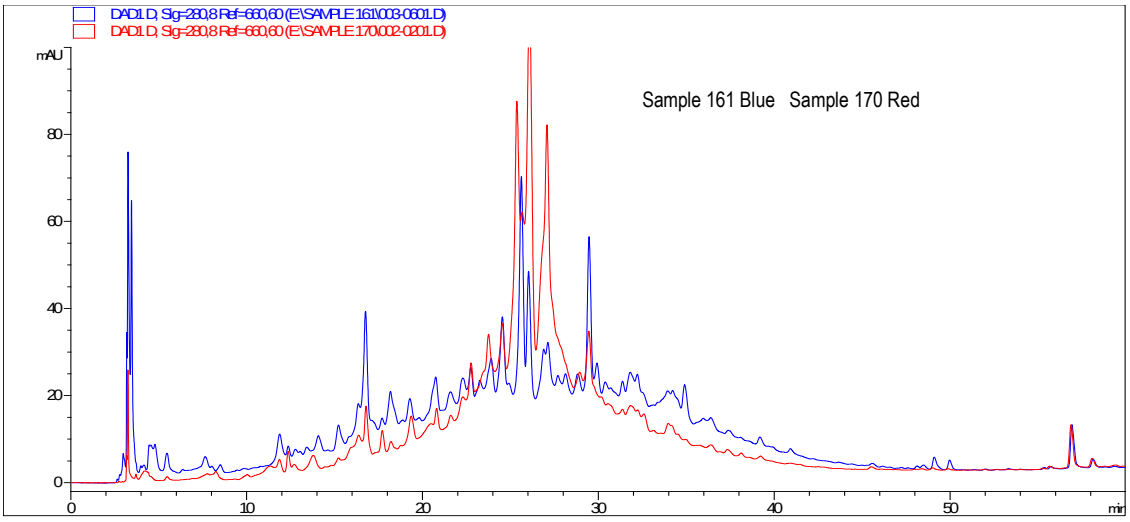



Figure 4.69(a) Chromatogram 280nm detection (F3) *P. robustissimum* Samples 161/170

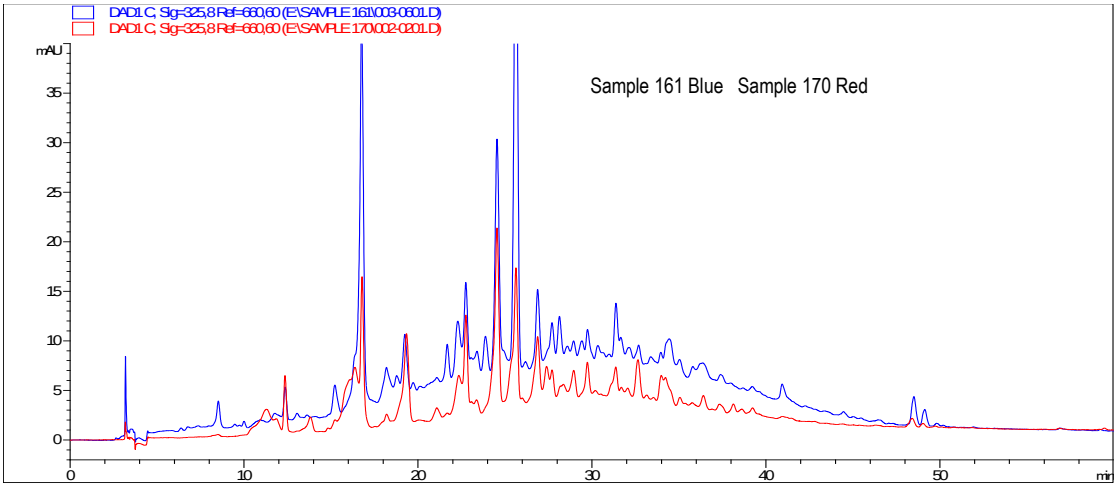



Figure 4.69(b) Chromatogram 325nm detection (F3) *P. robustissimum* Samples 161/170

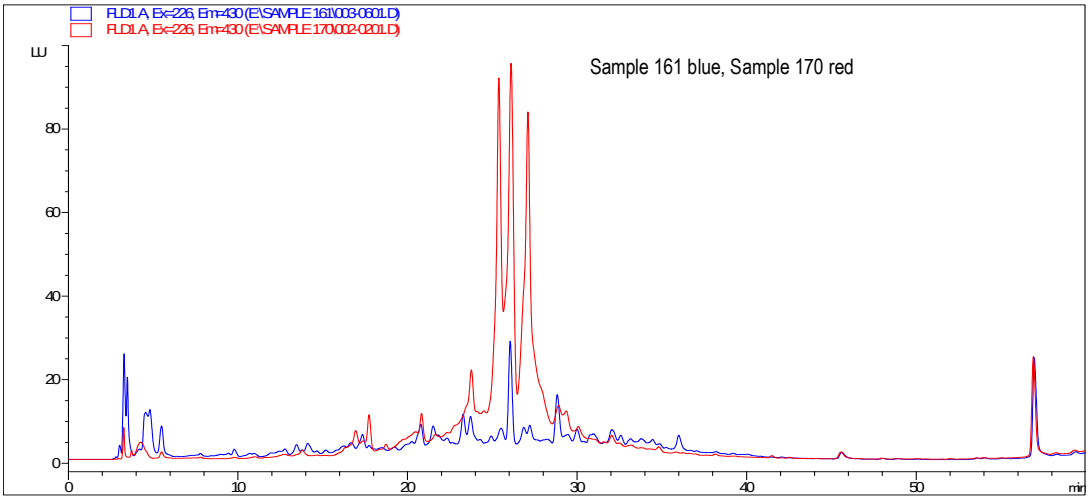



Figure 4.69(c) Chromatogram FL detector Ex 226 Em 430nm (F3) *P. robustissimum* Samples 161/170

The major difference between the samples is best shown by the chromatogram obtained using the fluorescence detector, Figure 4.69(c) and expanded detail Figure 4.69(d).

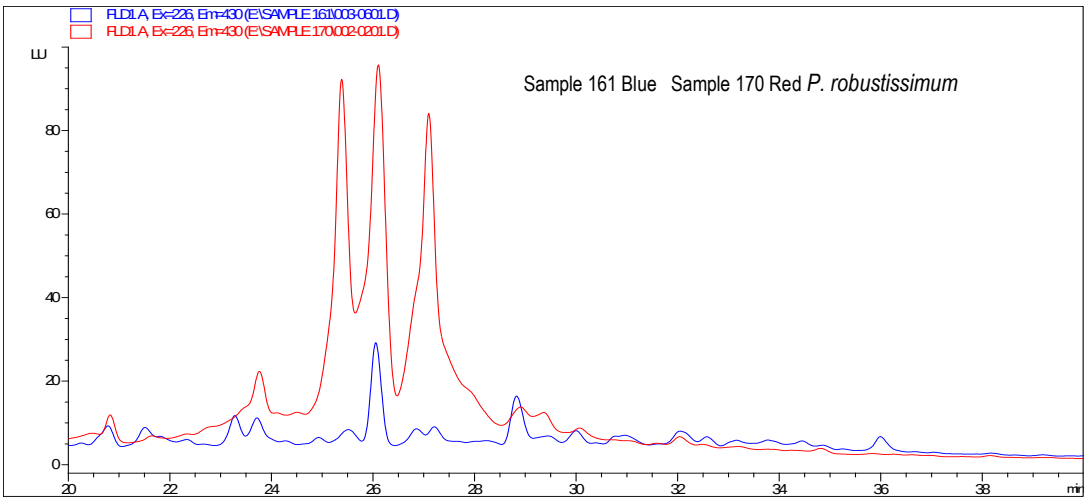



Figure 4.69(d) Expanded detail Chromatogram 4.98c FL detector Ex 226 Em 430nm (F3) Sample 161/170

The difference in constituents Figure 4.69(d) together with the spectral differences Figure 4.67-4.68, indicate that there are differences in the *P. robustissimum* growing on the two host trees. Figures 4.70(a-b) further illustrate the similarities in constituents but variation in concentration of the *P. quadrangulare* eluents.

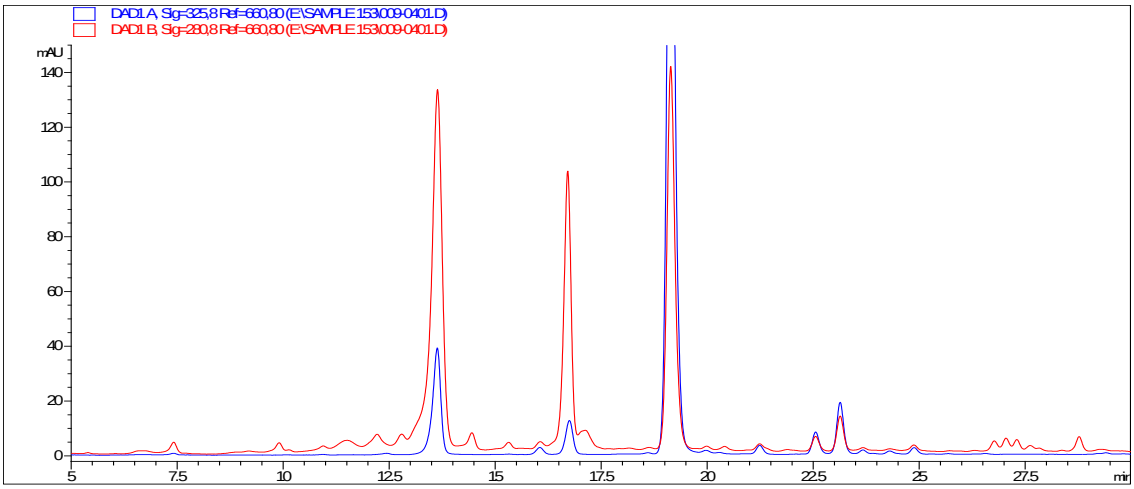



Figure 4. 70(a) Chromatogram (F3) eluent, *P. quadrangulare,* Sample 153 (280 red and 325nm blue).

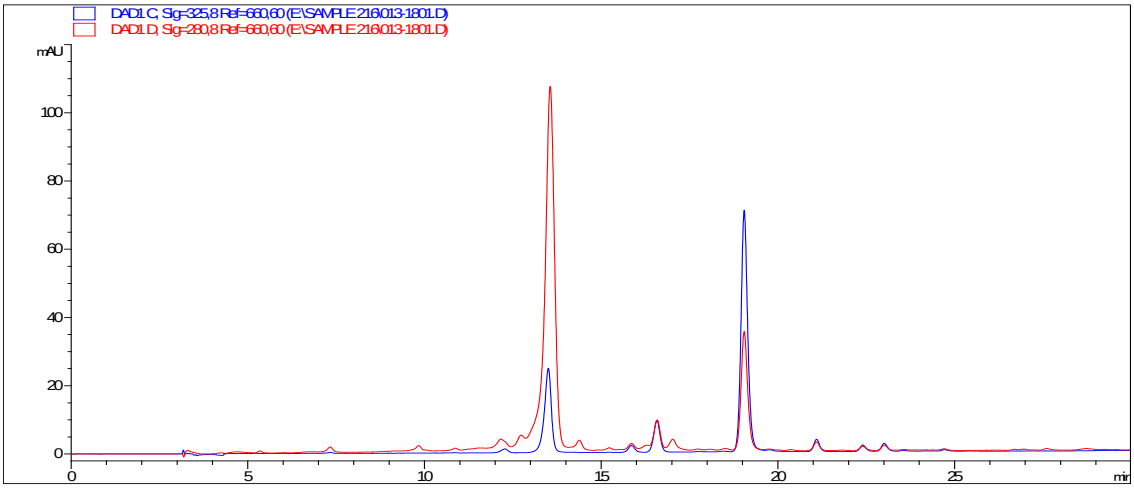



Figure 4.70(b) Chromatogram (F3) eluent, *P. quadrangulare* Sample 216, (280 red and 325nm blue).

A comparison of the first methanolic SPE eluent (F2) of examples of the non-eaten *P. robustissimum* and the eaten *P. quadrangulare* are shown in Figures 4.71(a-d). There are four major peaks in the *P. quadrangulare* samples are not found in the *P. robustissimum* samples.

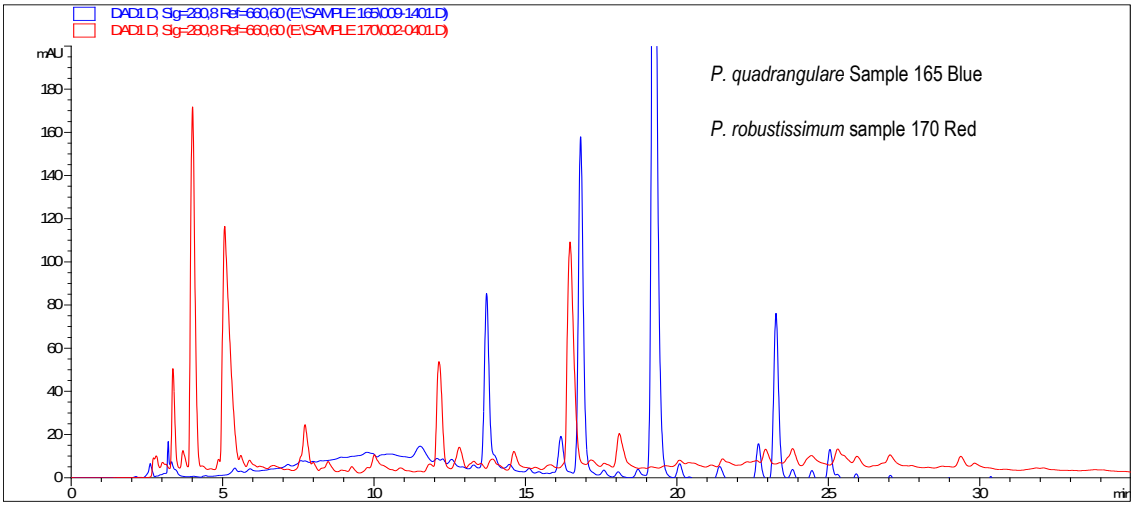



Figure 4.71(a) Chromatogram 280nm (F2).*P. quadrangulare* Sample 165 /*P. robustissimum Sample* 170

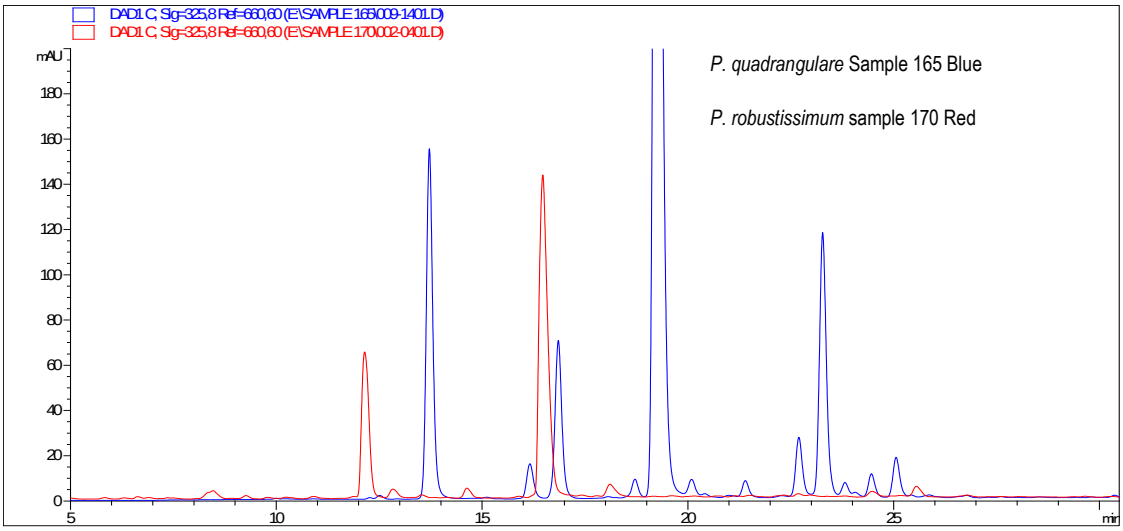



Figure 4.71(b) Chromatogram 325nm (F2).*P. quadrangulare* Sample 165 /*P. robustissimum Sample* 170

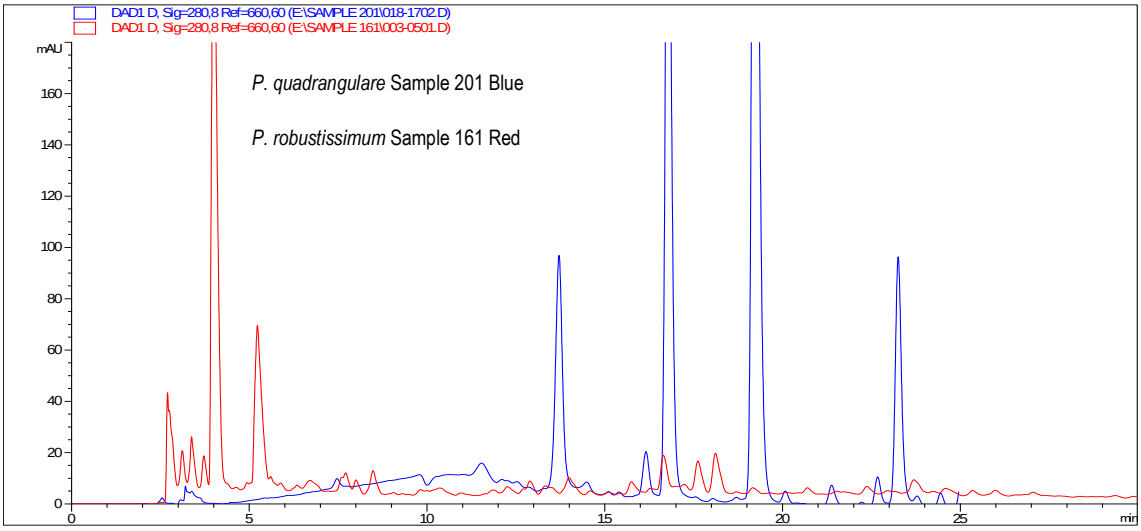



Figure 4.71(c) Chromatogram 280nm (F2).*P. quadrangulare* Sample 201 /*P. robustissimum Sample* 161

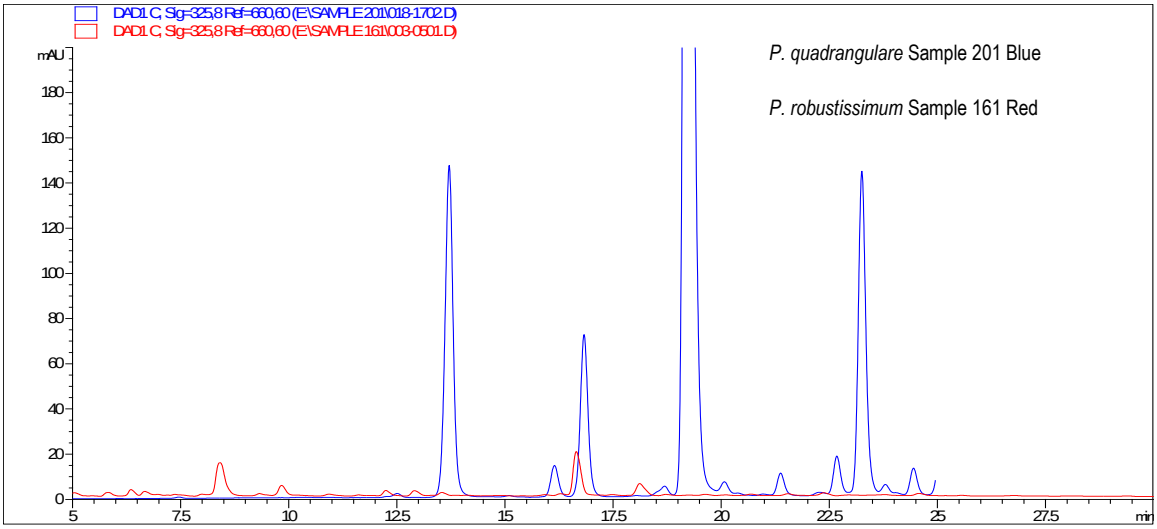



Figure 4.71(d) Chromatogram 325nm (F2).*P. quadrangulare* Sample 201 /*P. robustissimum Sample* 161

*P. robustissimum* chromatograms are different from those of *P. quadrangulare* samples. The humped shape seen in F3 *P. robustissimum* samples, Figures 4.69(a-b), was not seen in the F3 eluents from *P. quadrangulare,* e.g. Figures 4.70(a-b) Samples 153 and 216.

## 4.18.3 HPLC chromatograms *P. quadrangulare*

SPE treatment and subsequent HPLC analyses were conducted on >30 samples. The chosen leaf samples provided sufficient material to permit three replicate digests. The replicates were undertaken to ensure the results were representative and minimise any effect of small sample size. Figure 4.72(a-b) are comparisons chromatograms of the F1, 2 and F3 eluents for Sample 220.

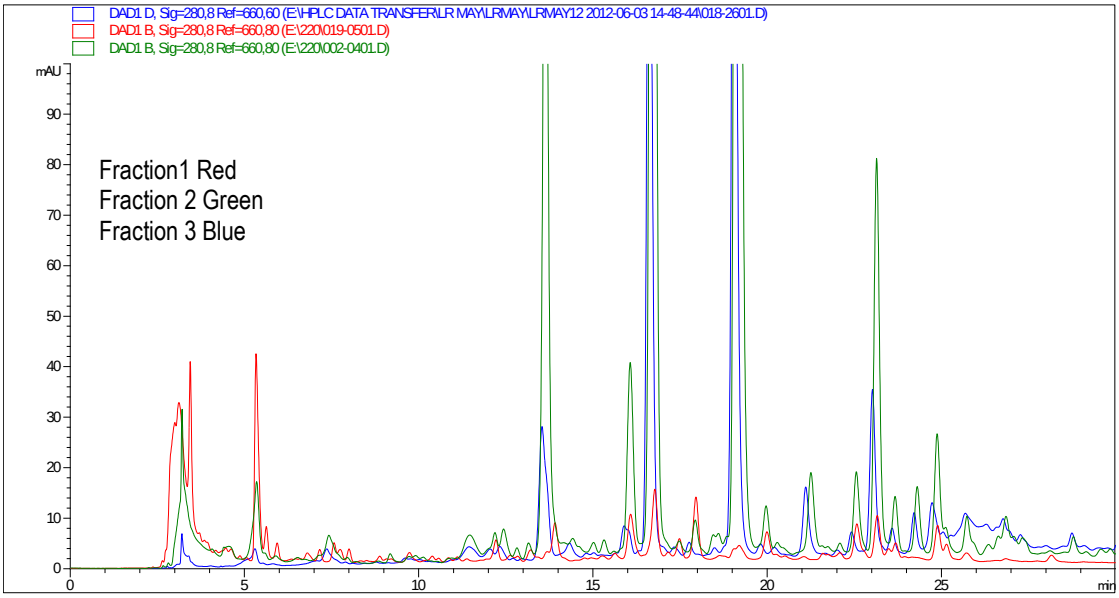



Figure 4.72(a) Chromatograms *P. quadrangulare* Sample 220, F1-F3, 280nm

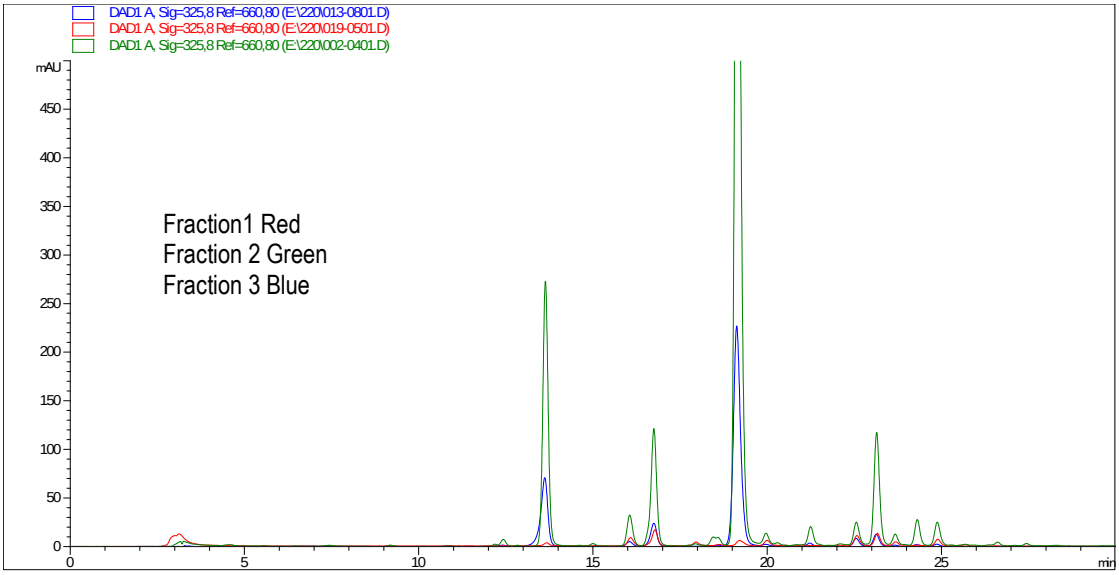



Figure 4.72(b) Figure 4.85a Chromatograms *P. quadrangulare* Sample 220, F1-F3, 325nm

Figures 4.72(a-b) are representative of comparisons for all the *P. quadrangulare*/MC samples analysed. The largest signal/peak area values were obtained from the first methanolic eluent (F2). The remaining comparison chromatograms presented are for this eluate and are **representative** of all the sample results obtained. The chromatograms are for the control tree MC1 Samples 165 (October 2007) and 201 (April 2008) Figures 4.73(a-b). [Norm] axis was used in chromatograms for 4.73a/b. This provides a report in which the peaks are corrected for detector response before calculation of the percentage of each. This enables the alignment of the 3 analysis components to be easily seen, which may not be the case in the chromatograms using mAu axis.

Samples 152, 216, 220 and 226 were *ad hoc* collected samples where >12 individuals had been observed eating on the occasion, Figures 4.74(a-d).

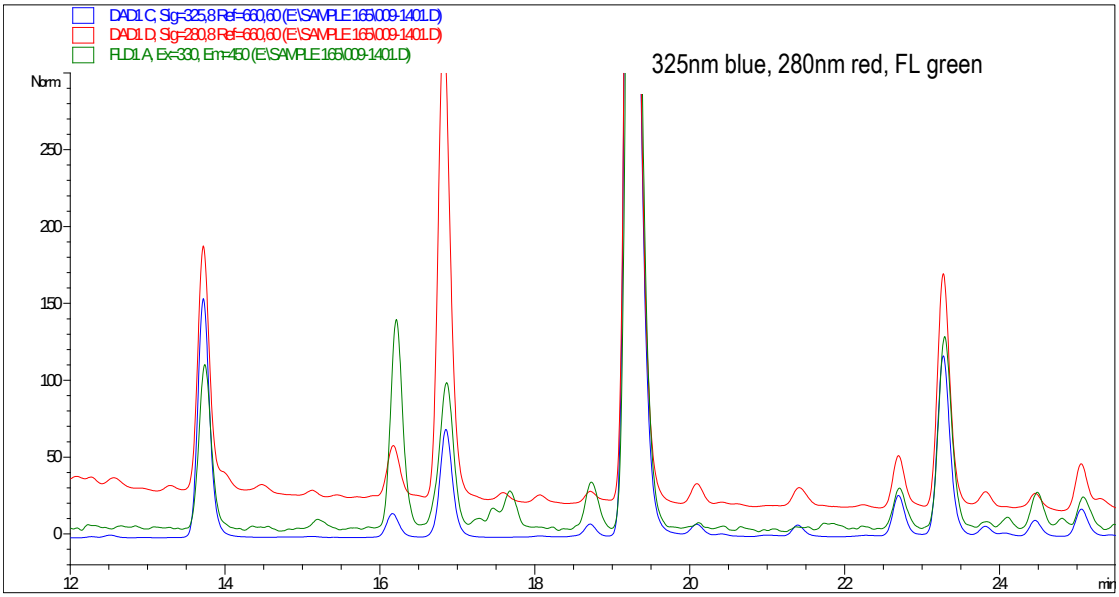



Figure 4.73(a) Chromatogram 280/325nm and FL Ex 226 Em 430nm (F2) *P. quadrangulare* Sample 165

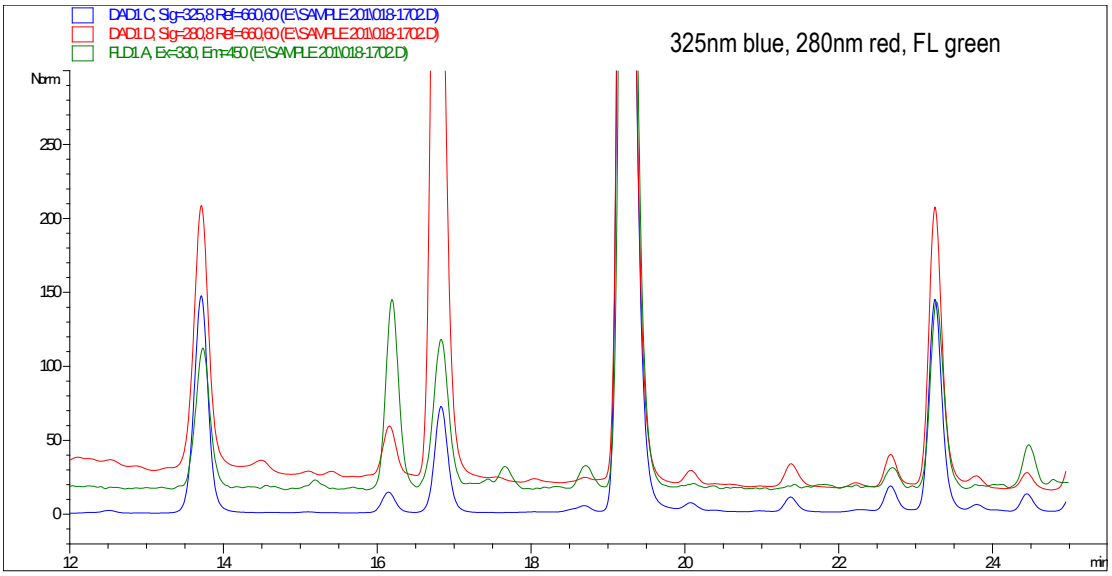



Figure 4.73(b) Chromatogram 280/325nm and FL Ex 226 Em 430nm (Fr2) *P. quadrangulare* Sample 201

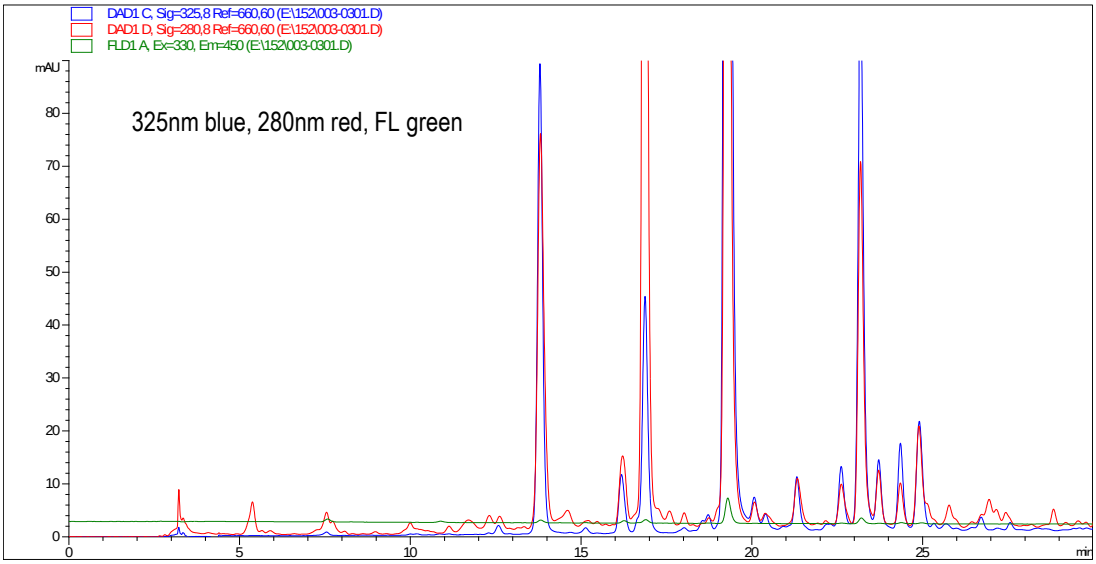



Figure 4.74(a) Chromatogram 280/325nm and FL Ex 226 Em 430nm (Fr2) *P. quadrangulare* Sample 152

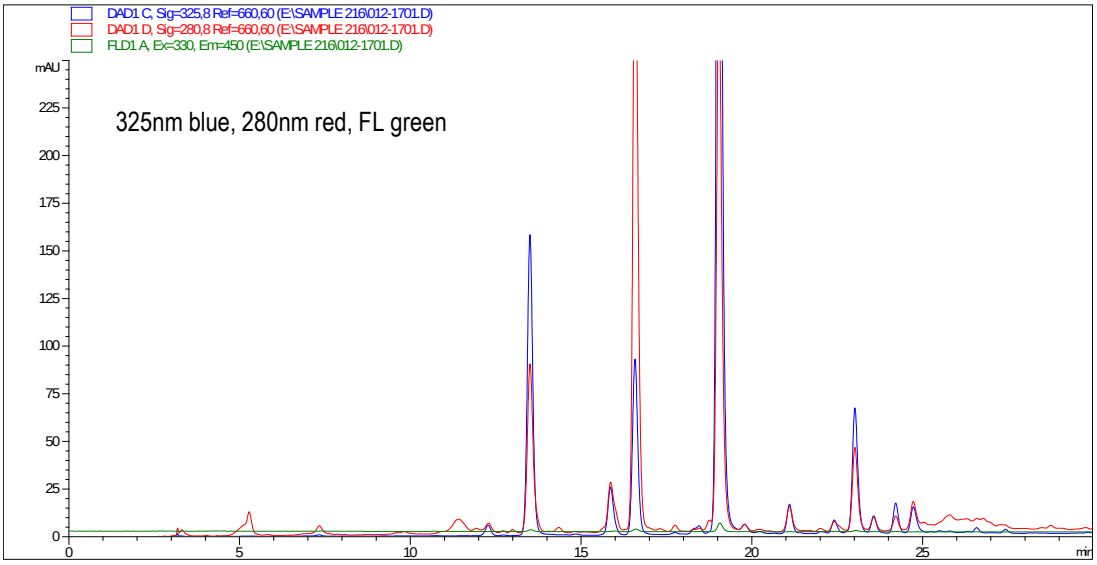



Figure 4.74(b) Chromatogram 280/325nm and FL Ex 226 Em 430nm (Fr2) *P. quadrangulare* Sample 216

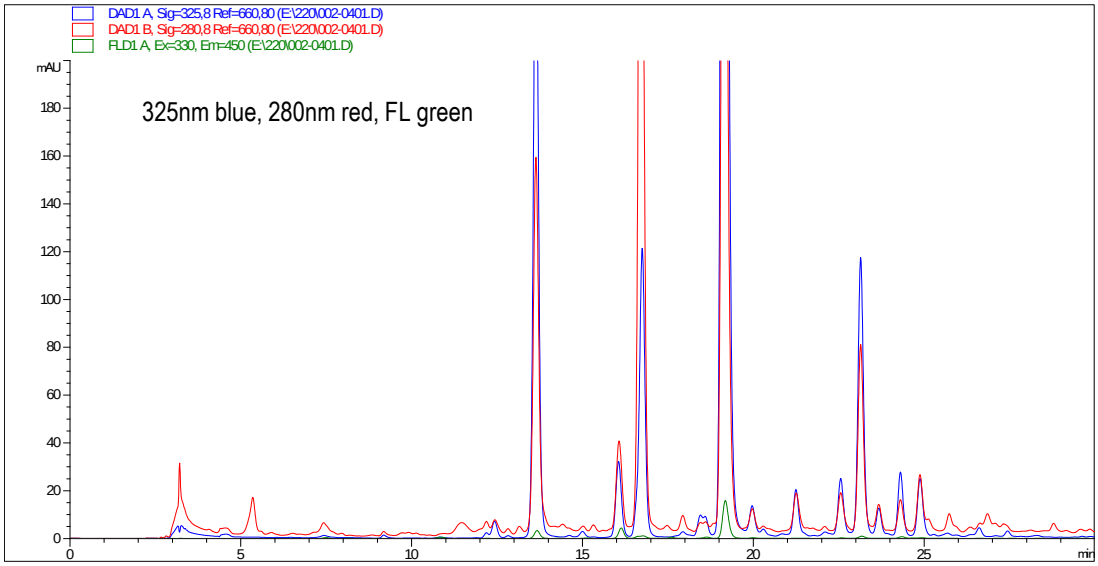



Figure 4.74(c) Chromatogram 280/325nm and FL Ex 226 Em 430nm (F2) *P. quadrangulare* Sample 220

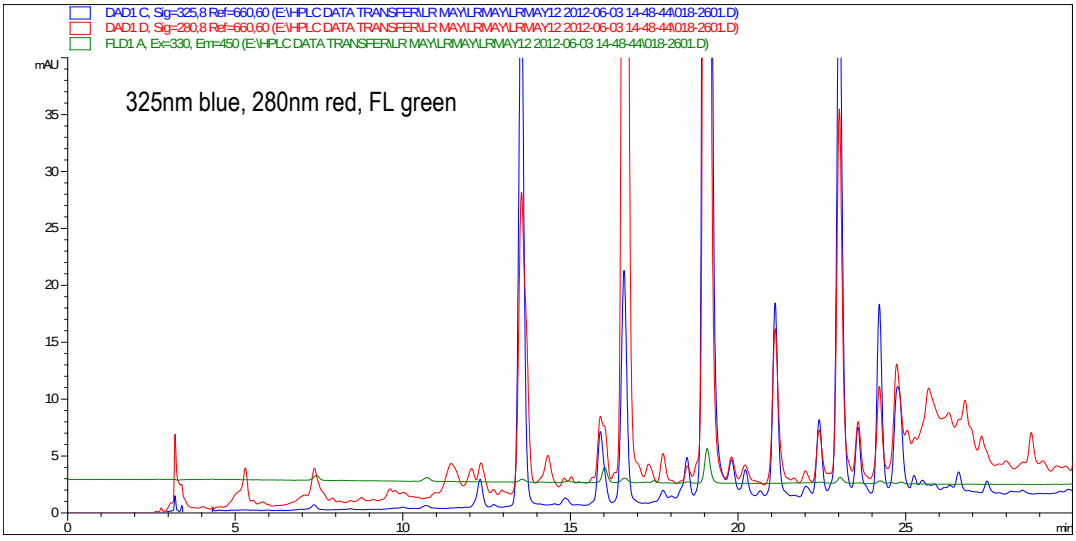



Figure 4.74(d) Chromatogram 280/325nm and FL Ex 226 Em 430nm (F2) *P. quadrangulare* Sample 226

Figures 4.73-4.74 clearly illustrate the consistency of the major constituents based on retention times in the methanolic eluate F2. Similar consistency but lower concentrations were seen in the constituents of the F1

and F3 eluents. The retention times and peak areas for these principal peaks from the 325nm detectors can be found in Table 4.33.

Table 4.33 Peak area mAu *P. quadrangulare* samples, (Fr2) 325nm..

| | mass of sample mg | RT mins | | | |
|---|---|---|---|---|---|
| **Control treesamples** | | **13.617** | **16.713** | **19.152** | **23.151** |
| Peak area Sample 165 | 505.20 | 1691 | 821 | 9785 | 1379 |
| Peak area Sample 201 | 506.17 | 1911 | 899 | 13,313 | 1771 |
| **Eaten Samples** | | | | | |
| Peak area Sample 152 | 502.55 | 951 | 509 | 6843 | 599 |
| Peak area Sample 216 | 504.53 | 1744 | 1011 | 6454 | 750 |
| Peak area Sample 220 | 503.31 | 843 | 466 | 4926 | 642 |
| Peak area Sample 226 | 501.46 | 526 | 227 | 4531 | 567 |

Variation in retention times were all <2% for the samples analysed on a single instrument. Sample 152 was analysed on a second instrument due to maintenance issues, and so there is a greater variation in retention time. (Acceptable variation <2% is standard for this technique, P. Riby LJMU personal communication). The peak area and hence the concentration of the constituents in all the peaks showed variation (Table 4.33).

Comparisons of the UV spectra obtained from the PDA detector can also illustrate variation within the eluents which may indicate variation between the samples.

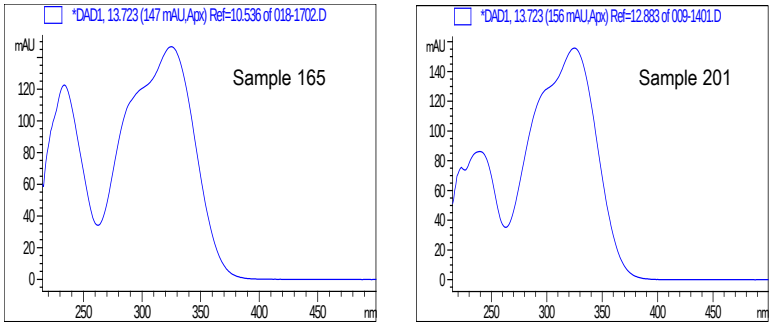



Figure 4.75(a) UV spectra for *P. quadrangulare* Samples 165/201 Host tree MC1, ~13.6 min RT.

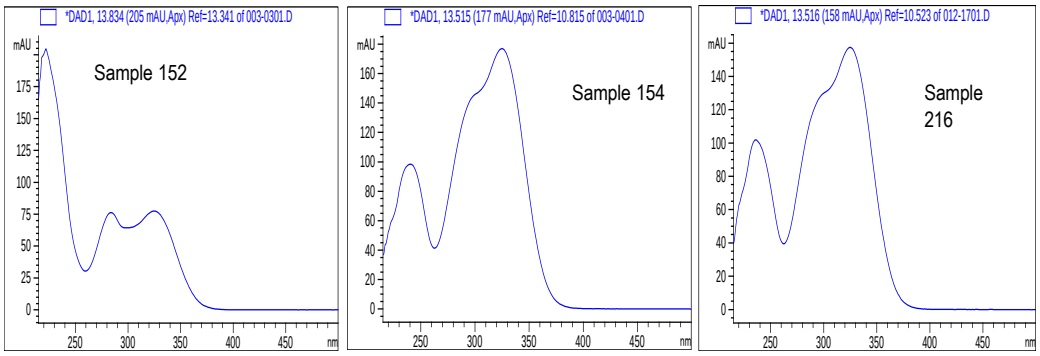



Figure 4.75(b) UV spectra for *ad hoc P. quadrangulare Samples*152/154/216, ~13.6 min RT

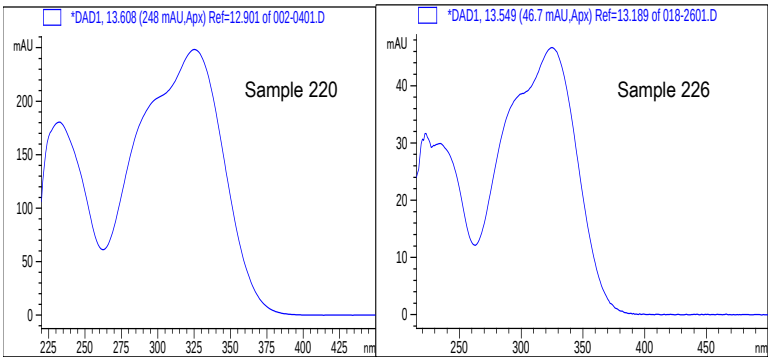



Figure 4.75(c) UV spectra for *ad hoc P. quadrangulare Samples* 220/226, ~13.6 min RT.

There are also some differences within each of these peaks as shown by the UV spectra Figures 4.75(a-c). The shape of spectra seen in Sample 152 was not seen in any of the further samples analysed. The data for the ~13.6min peak for several samples were exported to permit plotting using Excel and comparison of the spectra, Figure 4.76. Sample 152 absorbance was low compared to that of the other samples. It has a different composition from the remaining examples. This is confirmed by the comparison plots Figure 4.76. The max absorbance for all samples except 152 was at 331nm. Hydroxycinnamic acids, flavanones, flavones, flavonols and apigenin 7-glycosides have absorbance at this wavelength.

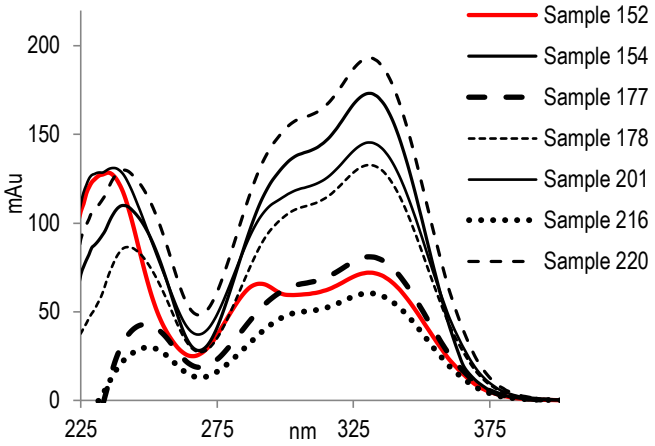



Figure 4.76 Comparison of the spectra for ~13.6min peak, Samples 152,153,154,177, 178,201, 216 and 220.

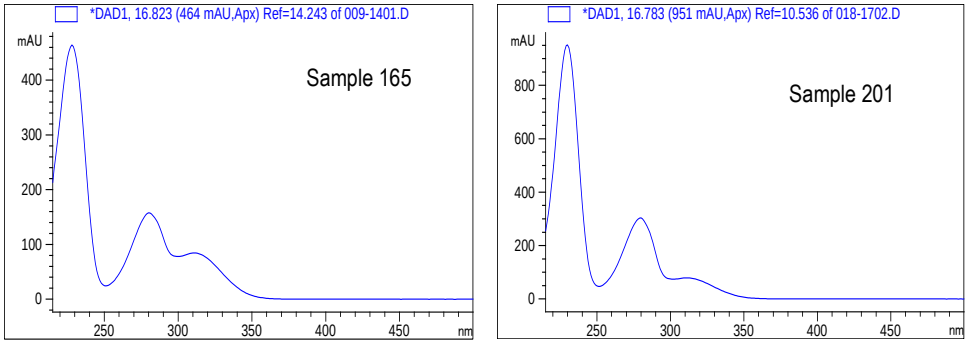



Figure 4.77(a) UV spectra for *P. quadrangulare* 165/201, ~16.6 min RT

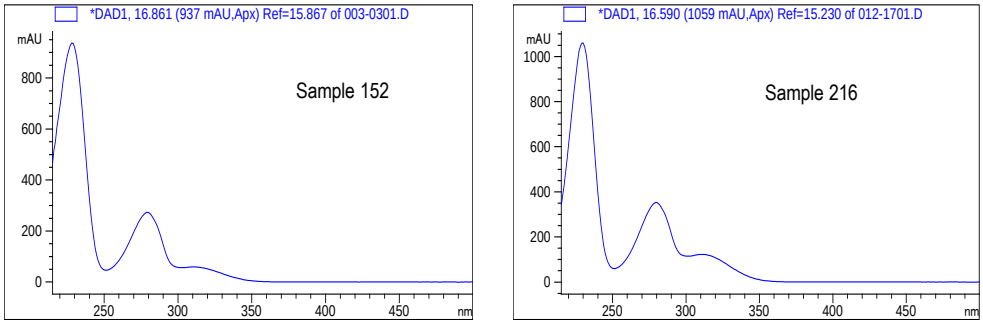



Figure 4.77(b) UV spectra for *ad hoc P. quadrangulare* 152/216, ~16.6 min RT.

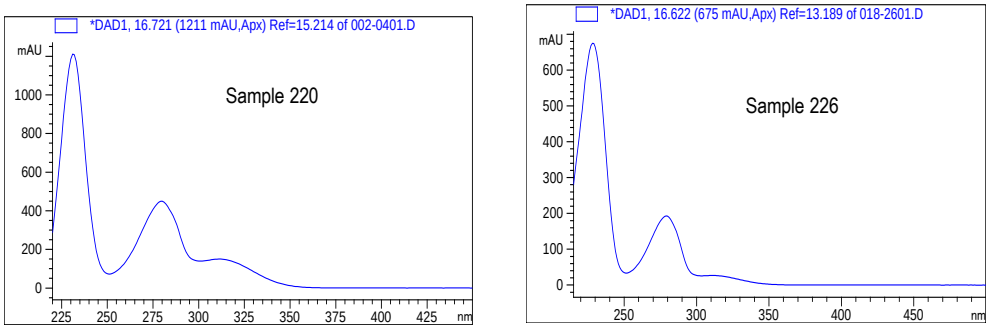



Figure 4.77(c) UV spectra for *ad hoc P. quadrangulare* 220/226, ~16.6 min RT.

The components within the peak ~16.6 mins are broadly similar, with the absorbance at 280nm being higher than that between 300-350nm. There is also a strong absorbance ~ 235nm, Figures 4.77(a-c). The component with the highest concentration in all the *P. quadrangulare* digests had a RT ~19.1 min, Figures 4.78(a-c) and Figure 4.79.

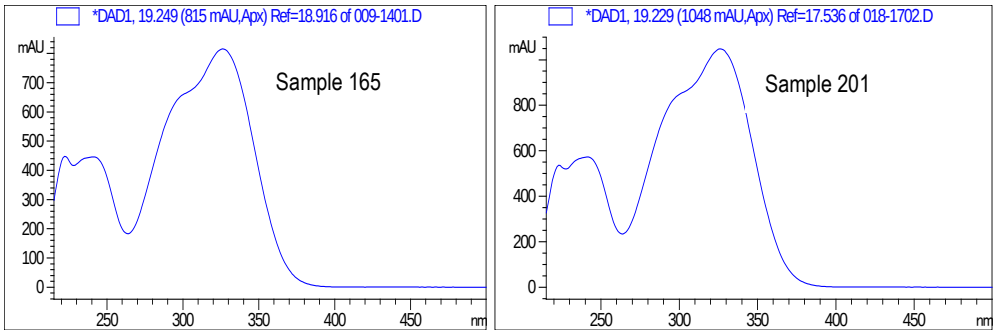



Figure 4.78(a) UV spectra for *P. quadrangulare* 165/201, ~19.1 min RT

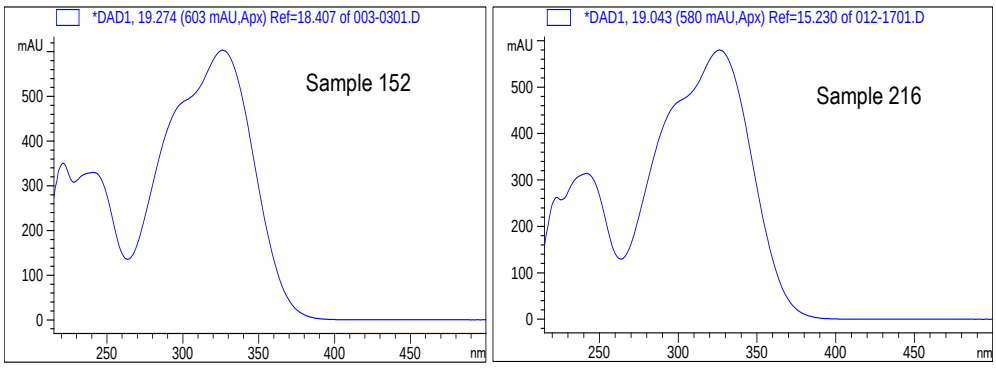



Figure 4.78(b) UV spectra for *ad hoc P. quadrangulare* 152/216, ~19.1 min RT.

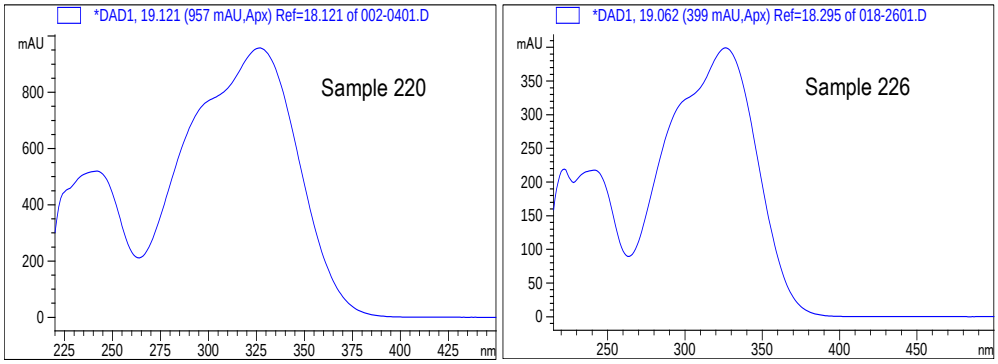



Figure 4.78(c) UV spectra for *ad hoc P. quadrangulare* 220/226, ~19.1 min RT.

The components within the peak ~19.1 mins are very similar in all the samples analysed from any of the host trees or the *ad hoc* examples of *P. quadrangulare.* There was the least variation in spectra of this peak when all samples were compared.

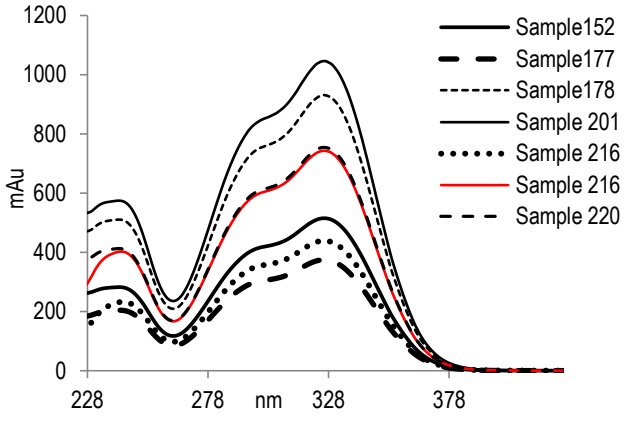



Figure 4.79 Comparison of the spectra for ~19.1min peak, Samples 152,153,177, 178,201,216 and 220.

The data for the ~19.1 peak was exported to permit plotting using Excel and the spectra compared, Figure 4.79, it includes data for further samples. The max absorbance for all samples was at 324-6nm. Thus material eluting in peaks ~ 13 and 19mins whilst having similar shaped UV spectra the peaks contain different components, exhibiting a 5nm difference in the position of the maximum absorbance, $\lambda_{max}$.

The fourth peak in the *P. quadrangulare* chromatograms (RT ~23min) may contain some material similar to that in peaks ~13 and 19 min RT, Figures 4.80(a-c). The $\lambda_{max}$.are at 312nm, 230nm with a shoulder 303nm, Figures 4.80(a-c).

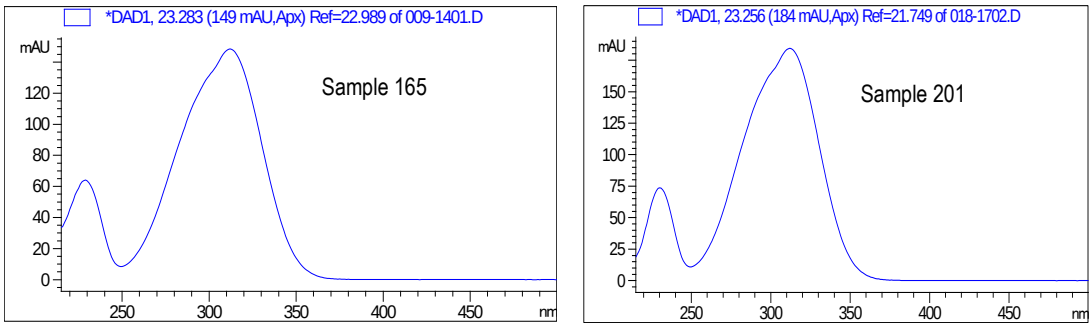



Figure 4.80(a) UV spectra for *P. quadrangulare* 165/201, ~23.1 min RT.

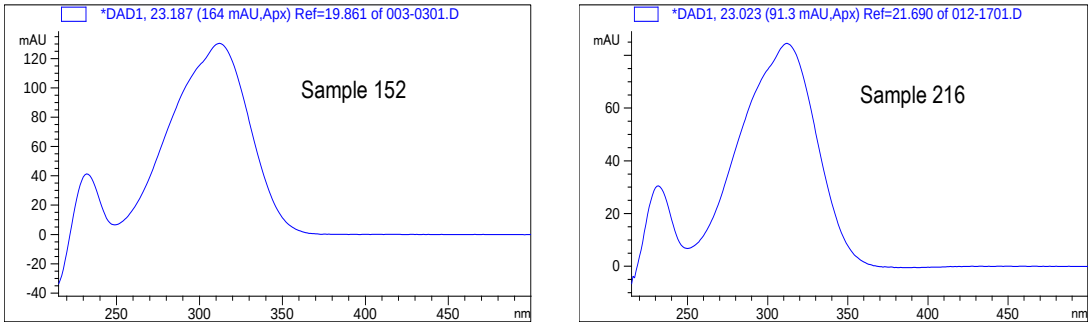



Figure 4.80(b) UV spectra for *ad hoc P. quadrangulare* 152/216, ~23.1 min RT.

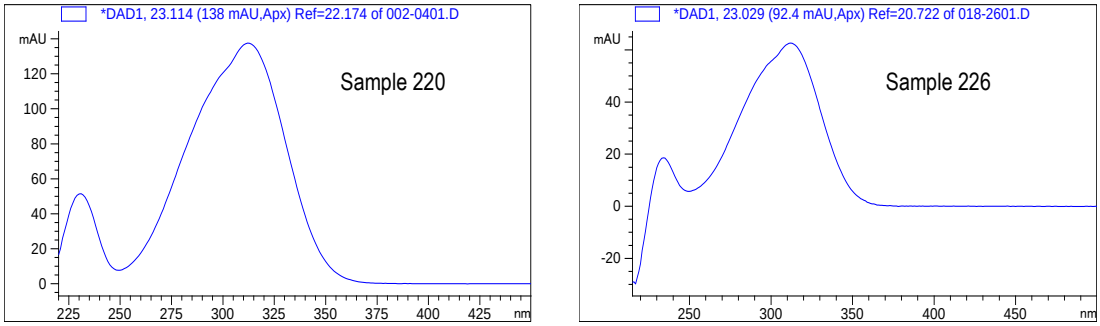



Figure 4.80(c) UV spectra for *ad hoc P. quadrangulare* 220/226, ~23.1 min RT.

## 4.18.4 Digestion of *P. quadrangulare* with geophagy samples

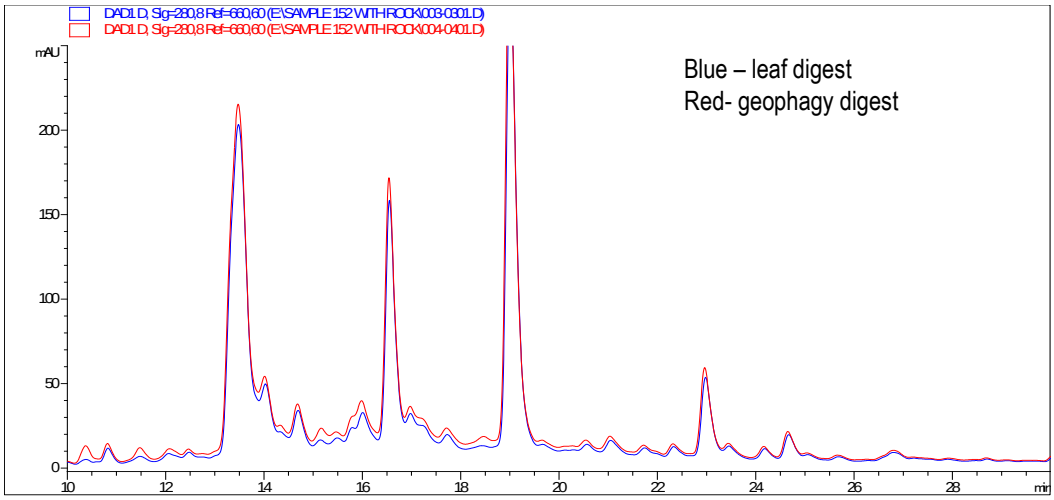



Figure 4.81(a) Sample 152 comparison with geophagy digest 280nm

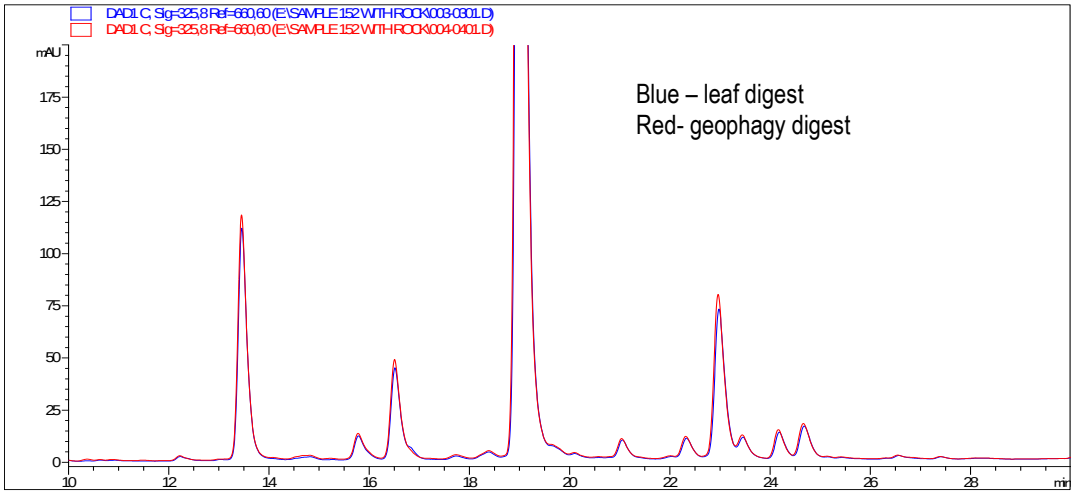



Figure 4.81(b) Sample 152 comparison with geophagy digest 325nm

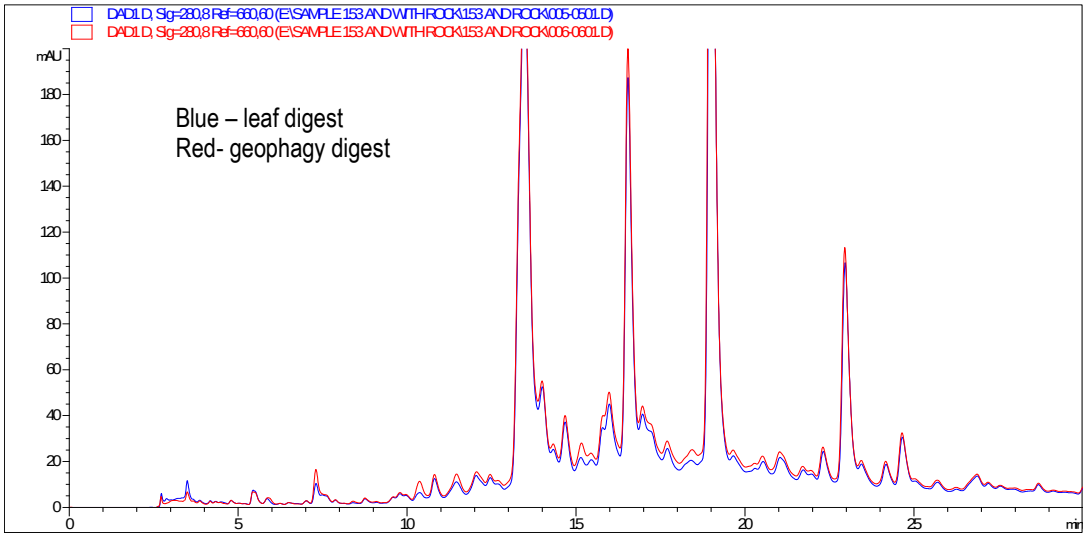



Figure 4.81(c) Sample 153 comparison with geophagy digest 280nm

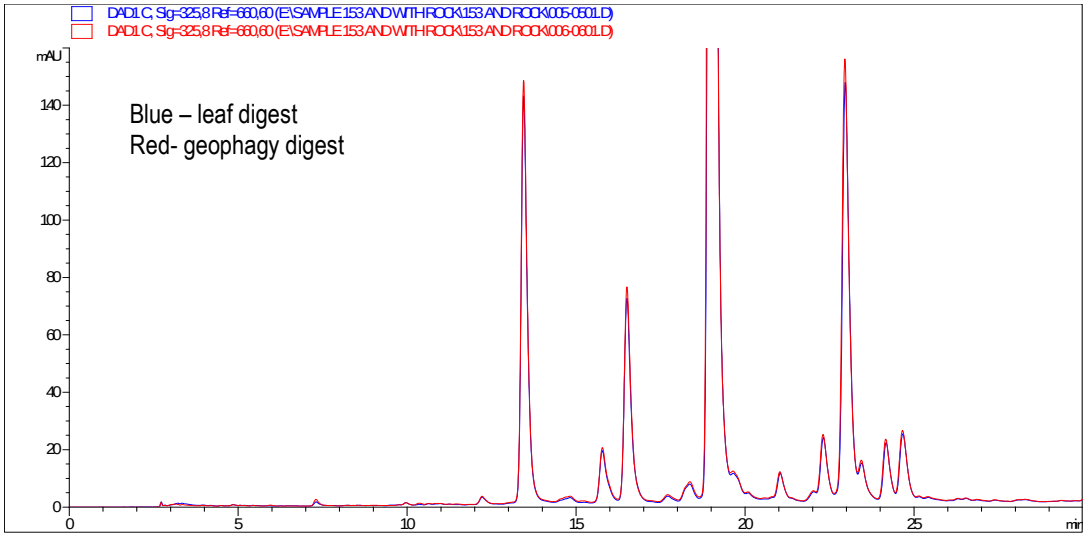



Figure 4.81(d) Sample 153 comparison of digest with geophagy digest 325nm

The effect of digestion upon the UV spectra of both *P. quadrangulare* and *P. robustissimum* has been reported Section 4.15.3. The previously reported UV results had suggested that there was possibly a limited release of more constituents into the digest in the presence of the geophagy sample.

Table 4.34 Comparison of peak areas, Sample 154/153 geophagy digests

| | nm | RT mins | | | |
|---|---|---|---|---|---|
| **ID** | | **13.459** | **16.513** | **19.007** | **23.178** |
| Digest 152 | 280 | 4953 | 2328 | 4670 | 825 |
| digest 152+ rock | 280 | 5326 | 2566 | 5032 | 911 |
| **% change in peak area** | | **7.55** | **10.23** | **7.74** | **10.41** |
| Digest 152 | 325 | 1397 | 644 | 8178 | 1056 |
| digest 152 + rock | 325 | 1475 | 677 | 8662 | 1159 |
| **% change in peak area** | | **5.54** | **5.22** | **5.91** | **9.74** |
| | **nm** | **RT mins** | | | |
| **ID** | | **13.447** | **16.536** | **19.093** | **23.163** |
| Digest 153 | 280 | 5498 | 2830 | 6251 | 1697 |
| digest 153 + rock | 280 | 5728 | 2992 | 6492 | 1716 |
| **% change in peak area** | | **4.19** | **5.72** | **3.85** | **1.12** |
| Digest 153 | 325 | 1805 | 1013 | 10658 | 2164 |
| digest 153+ rock | 325 | 1878 | 1049 | 11111 | 2285 |
| **% change in peak area** | | **4.09** | **3.63** | **4.25** | **5.56** |

These representative results for Samples 152/153 (Figures 4.81(a-d) and Table 4.34) suggested that there potentially was an increased release of more constituents into the digest in the presence of the geophagy sample. Triplicate samples of Sample 178 a, b and c were digested and analysed to assess the reproducibility of the data. However the digests were analysed using an HPLC system without a PDA detector and therefore only data for 325nm was available. Figure 4.82 was representative of the three treatments. There were insufficient results to perform statistical analyses but the increases in peak area Sample 178 (Figure 4.82, Table 4.35) are larger and show more similarity than the results for Samples 152/153 (Table 4.35).

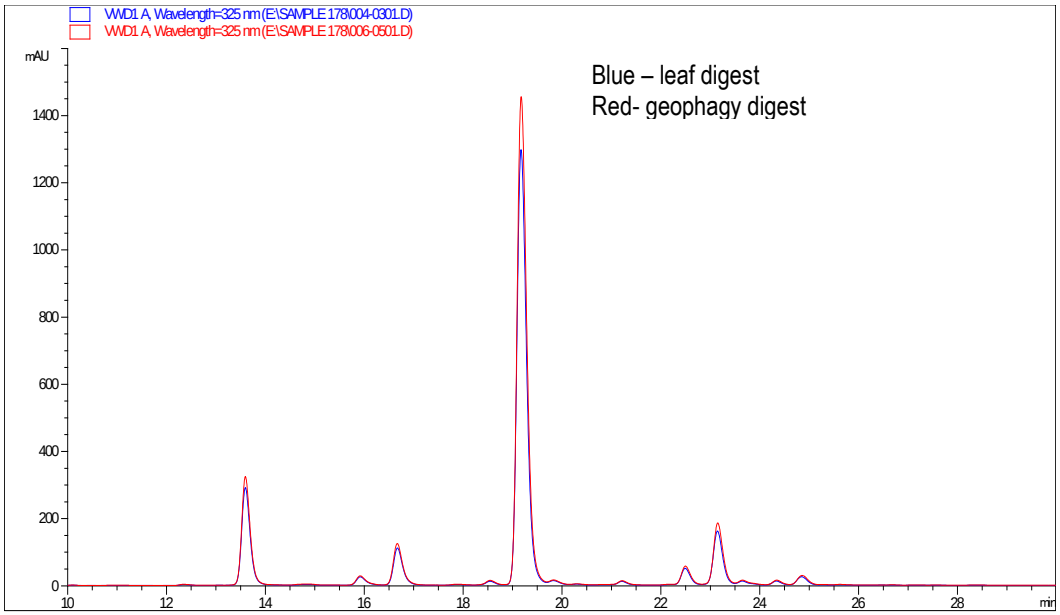



Figure 4.82 Sample 178 (a) comparison with geophagy digest 325nm

Table 4.35 Comparison of peak areas, replicate determinations of Sample 178 geophagy digests

| | nm | RT mins | | | |
|---|---|---|---|---|---|
| | | **13.588** | **16.681** | **19.173** | **23.146** |
| Digest 178a | 325 | 3511.00 | 1462.00 | 17511.00 | 2156.00 |
| digest 178a + rock | 325 | 3911.00 | 1643.00 | 19884.00 | 2479.00 |
| **% change in peak area** | | **11.39** | **12.38** | **13.55** | **14.98** |
| Digest 178b | 325 | 3402.00 | 1482.00 | 177982.00 | 2141.00 |
| digest 178b+ rock | 325 | 3768.00 | 1677.00 | 198657.00 | 2444.00 |
| **% change in peak area** | | **10.76** | **13.16** | **11.62** | **14.15** |
| Digest 178c | 325 | 3328.00 | 1446.00 | 175444.00 | 2179.00 |
| digest 178c + rock | 325 | 3666.00 | 1623.00 | 197322.00 | 2470.00 |
| **% change in peak area** | | **10.16** | **12.24** | **12.47** | **13.35** |

## 4.18.5 Reference standards and Identification of peak ~19 mins

In an attempt to identify the component which eluted at ~ 19 mins in the *P. quadrangulare* samples the UV spectra were determined for the following potential candidate compounds. These included compounds known to be present in the Viscaceae and those reported in *Phoradendron* (Table 4.14, Section 4.9.1).

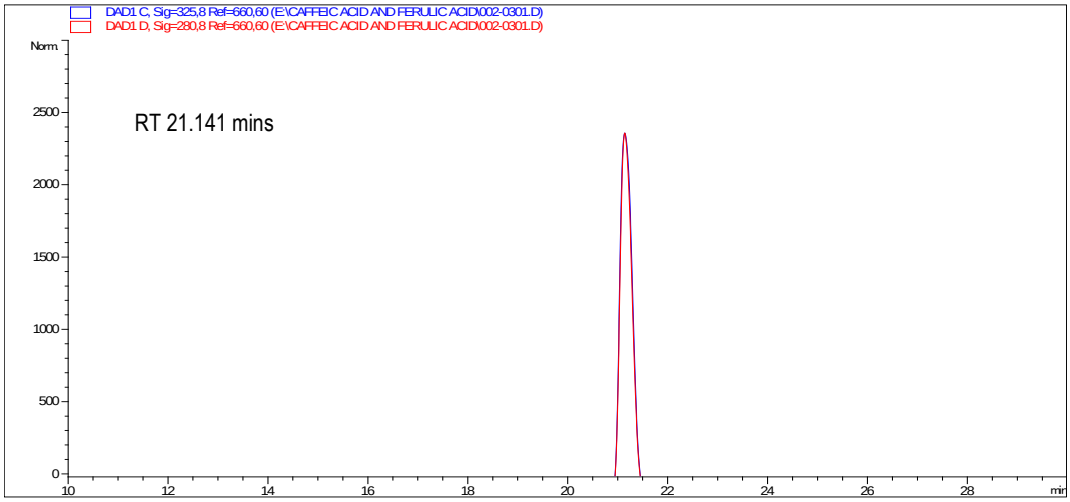



Figure 4.84(a) Chromatogram caffeic acid (3,4-dihydroxycinnamic acid)

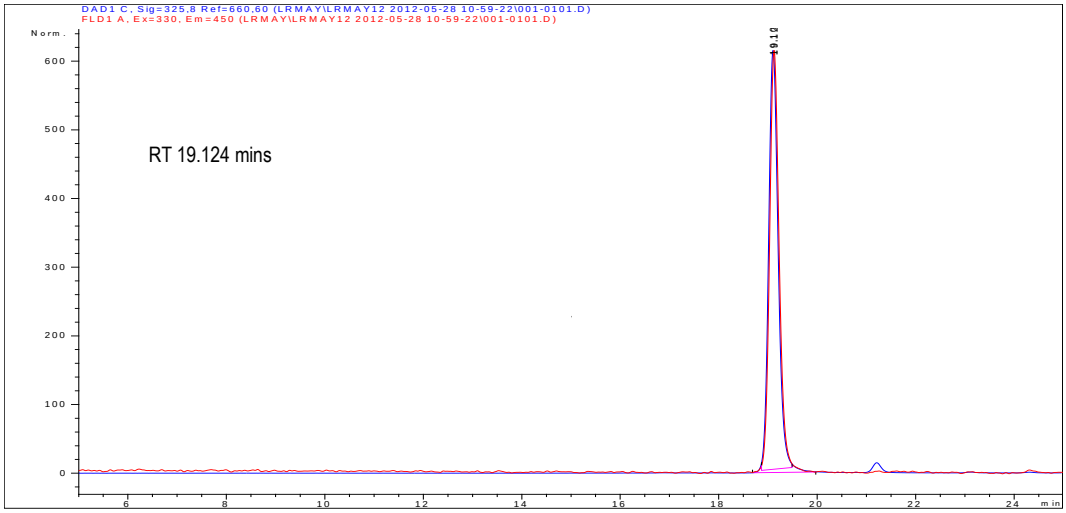



Figure 4.84(b) Chromatogram of chlorogenic acid, (3-(3,4-Dihydroxycinnamoyl)quinic acid)

Only two of the compounds selected for testing from Table 4.14 had RT within the time frame of the analysis. These were caffeic acid (3,4-dihydroxycinnamic acid) and chlorogenic acid 3-(3,4-dihydroxycinnamoyl)quinic acid, Figures 4.84(a-b). Ferulic acid RT was >24 mins.

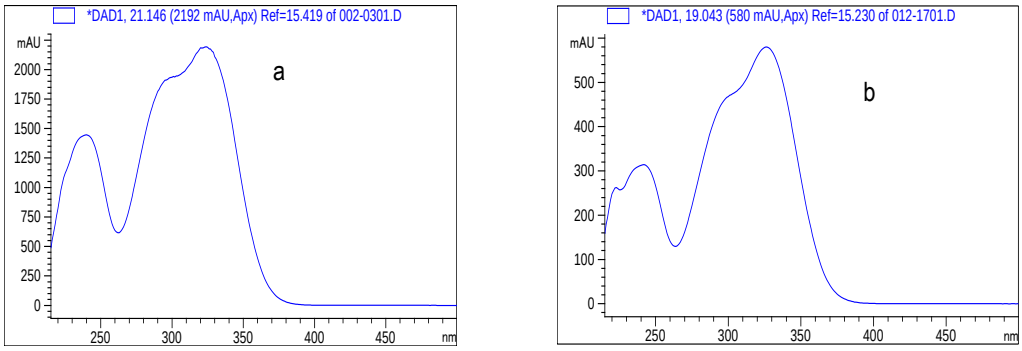



Figure 4.85 HPLC-DAD spectra for caffeic acid (a), chlorogenic acid (b)

Figure 4.85 illustrates the similarity in UV spectra for the two standards. Data for the sample spectra were exported and plotted using Excel to permit comparisons of spectra, Figure 4.86.

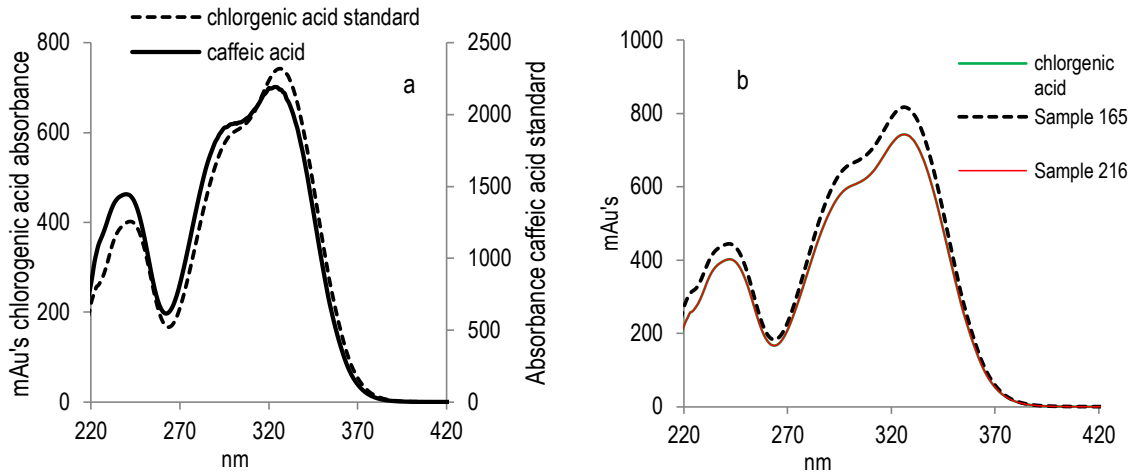



Figure 4.86 Overlay comparisons of spectra chlorogenic acid and caffeic acid standards (a) chlorogenic acid standard and sample peaks eluted RT ~19.1min, Samples 165, 216 (b)

Figure 4.86 (a) illustrates the similarity of the spectra for caffeic and chlorgenic acids obtained from the DAD detector, however these compounds elute at different RT under analysis conditions. Figure 4.86 (b) illustrates the similarities of the spectra for the chlorogenic acid standard spectra with those of two samples with the same RT (spectra for chlorogenic acid and Sample 216 overlay each other).

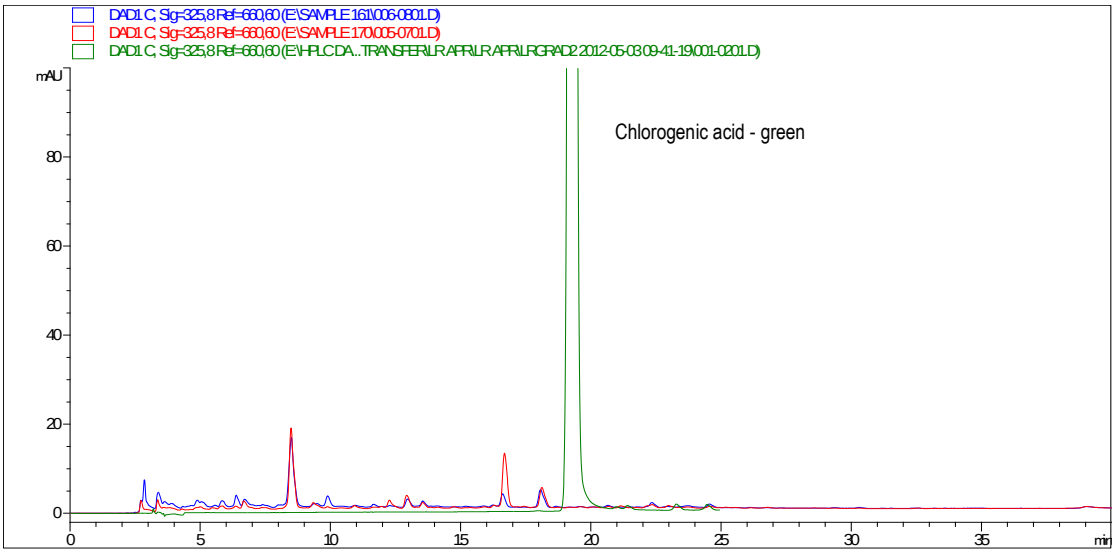



Figure 4.87(a) Chromatogram 325nm detection, *P. robustissimum* Samples 161/170 with chlorogenic acid standard.

Figure 4.87(a) clearly illustrates the lack of detectable levels of a component within the *P. robustissimum* Samples161/170 with components having the same RT as the chlorogenic acid standard. This confirms the major difference between the mistletoe species. Figures 4.87(b-d), show that the retention times of the chlorogenic acid (3-(3,4-dihydroxycinnamoyl)quinic acid) standard and the major component in the representative Samples 153, 165 and 216 are the same.

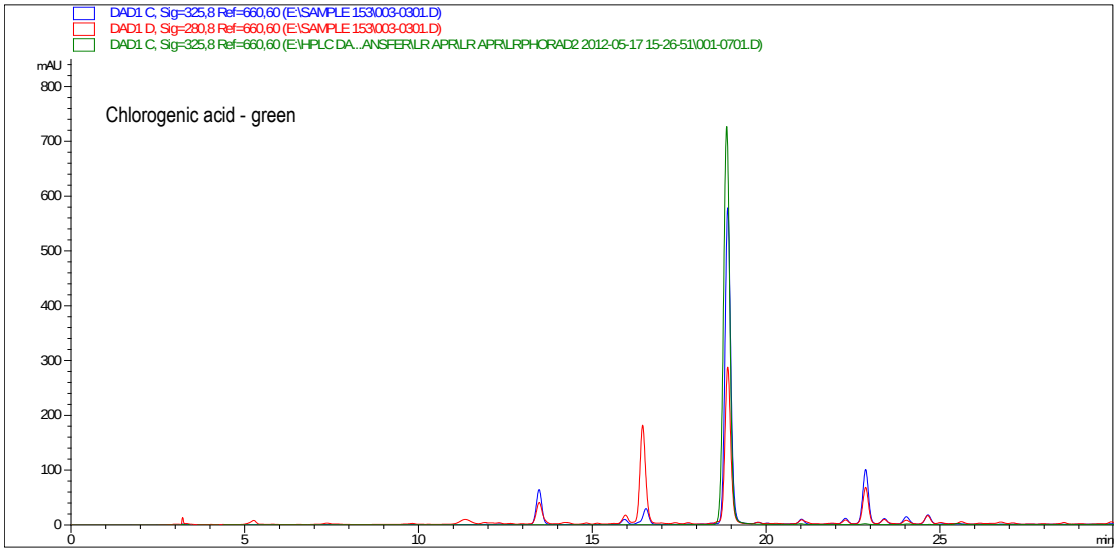



Figure 4.87(b) Chromatogram *P. quadrangulare* Sample 153 with chlorogenic acid standard.

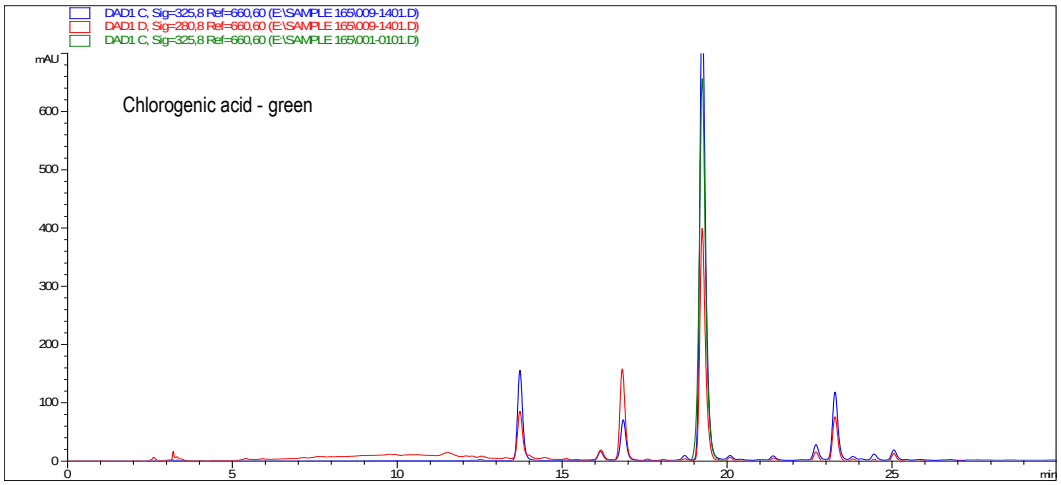



Figure 4.87(c) Chromatogram *P. quadrangulare* Sample 165 with chlorogenic acid standard.

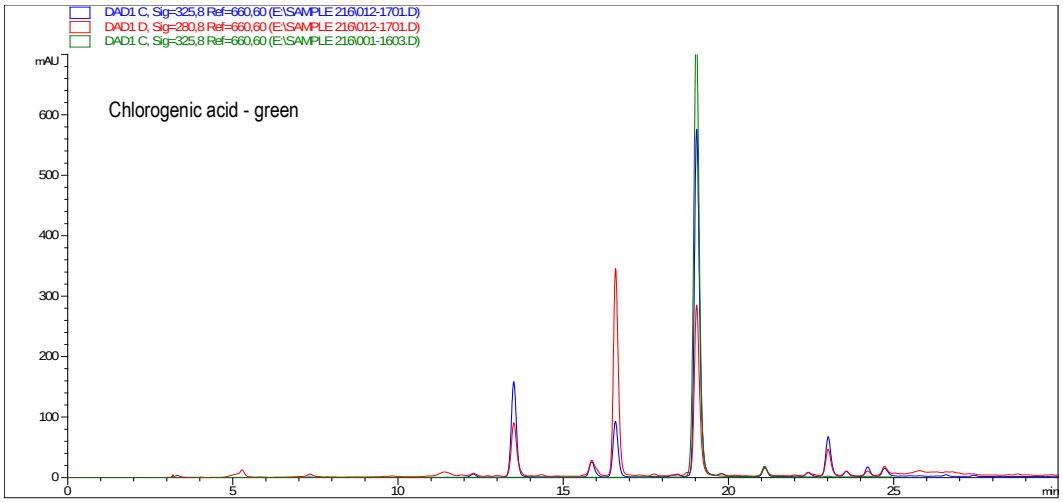



Figure 4.87(d) Chromatogram *P. quadrangulare* Sample 216 with chlorogenic acid standard.

In Summary: HPLC analyses of the aqueous digest, SPE treated eluents showed:

- Differences in the *P. robustissimum* growing on two host trees.
- Differences between *P. quadrangulare* and *P. robustissimum* were shown.
- The major constituent in the 3 SPE eluents (F1, 2 and 3) prepared from *P. quadrangulare* had a RT ~ 19mins.

- Combined digestion of *P. quadrangulare* samples with geophagy material suggested that there was an increased release of the material in the four major HPLC peaks in the presence of the geophagy sample.
- The principal peak and the chlorogenic acid standard have the same are retention time and the DAD spectra of the chlorogenic acid standard show the same features and $\lambda_{max}$ values.

## 4.19 HPLC-MS determination *P. quadrangulare* Sample 216

This technique permits more detailed analysis of the constituents of the major peaks, previously seen eluting from Sample 216. The use of negative ion mode using 60V or lower cone voltage is preferred for some classes of phenolic compounds. It produces a high abundance of the molecular ion $[M-H]^-$ as deprotonation is high. There is little other fragmentation making the interpretation simpler. At higher voltages there is more fragmentation and a base peak due to higher abundance of low molecular mass ion (Pérez-Magariño *et al.* 1999).

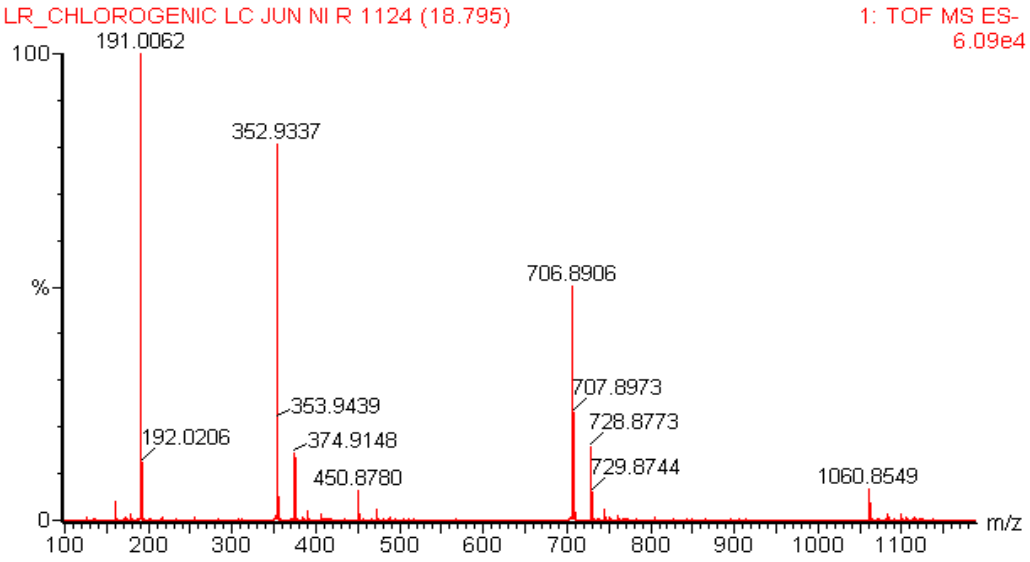



Figure 4.88(a) TOF-MS ES- Chlorogenic acid standard, (3-(3,4-dihydroxycinnamoyl)quinic acid,) RT 18.795 min.

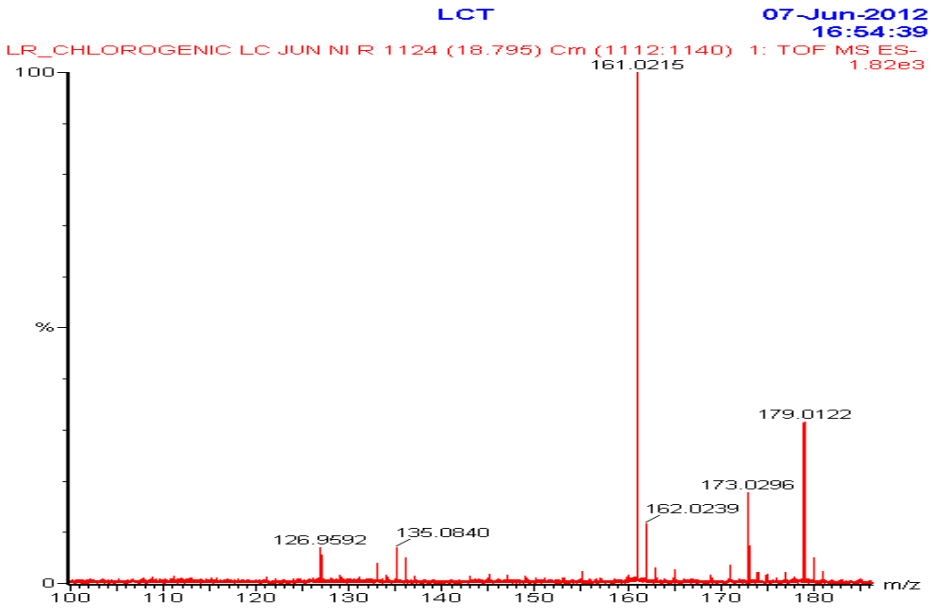



Figure 4.88(b) Expanded section TOF-MS ES- Chlorogenic acid standard, (3-(3,4-dihydroxycinnamoyl)quinic acid,) RT 18.795 min

Figure 4.88(a) is the fragmentation pattern for the chlorogenic acid standard accurate mass determined (ES- mode) as 355.1042 Pubchem Reference database value 354.30872). Figure 4.88(b) is an expanded region of the spectrum for the chlorogenic acid reference standard.

LC-MS instrument output is provided for Sample 216 as representative of the analyses conducted on the samples eaten by the monkeys. Figure 4.89(a) is the mass spectrum for the peak RT 18.864 min provisionally identified as chlorogenic acid from the HPLC results. Figure 4.89(b) is an expanded region of the spectrum for Sample 216.

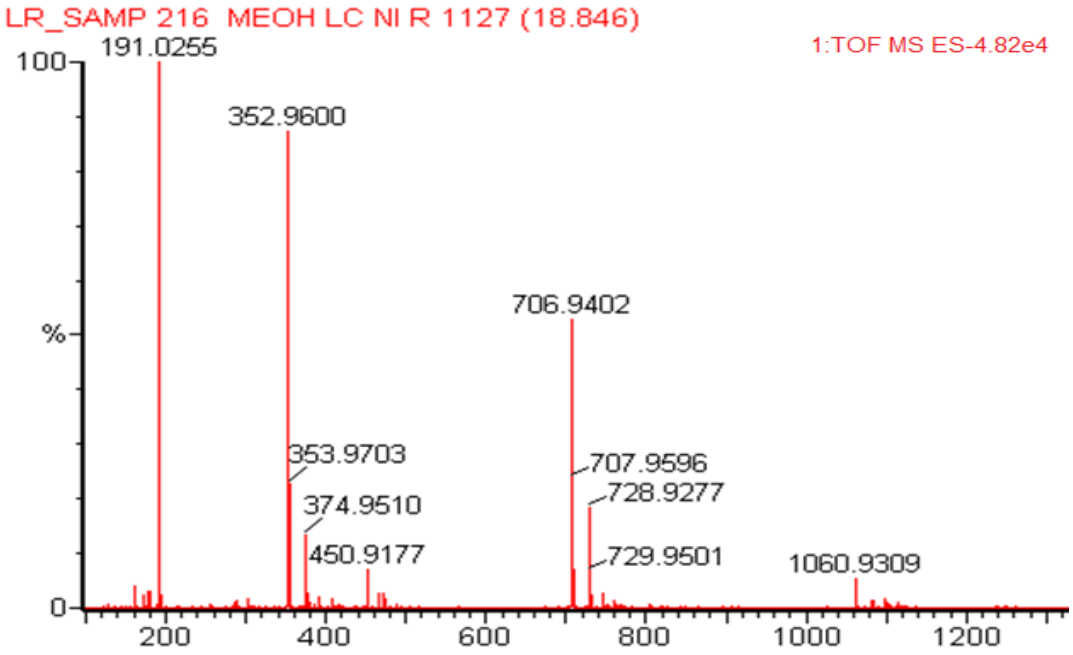



Figure 4.89(a) TOF-MS ES- Sample 216, RT 18.864 mins

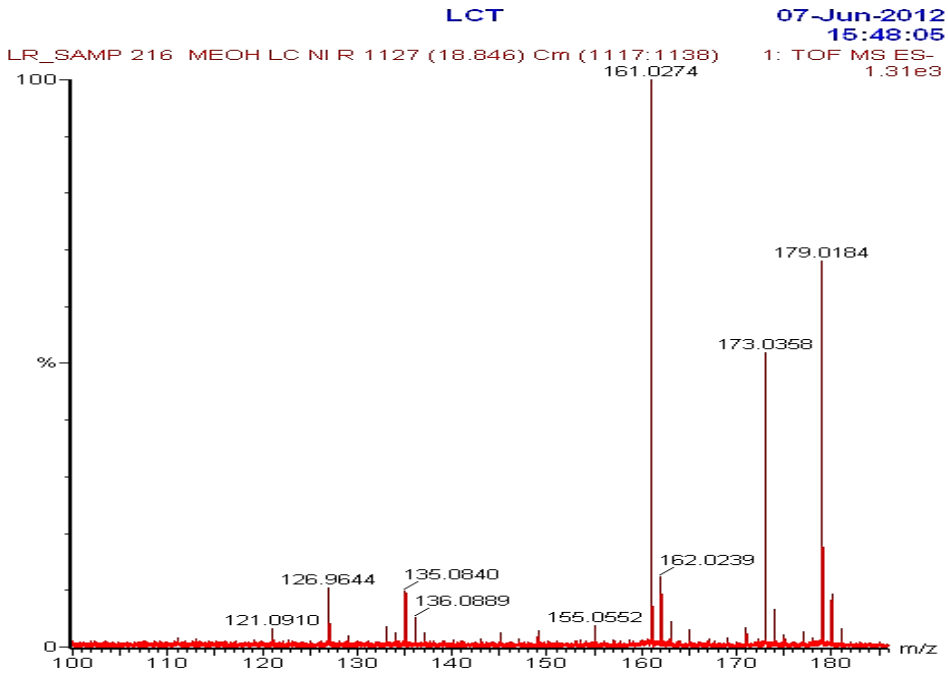



Figure 4.89(b) Expanded area of spectrum 4.89(a) TOF-MS ES- Sample 216, RT 18.864 mins

Comparing the data for the principal *m/z* in Table 4.36 indicates that the constituent of Sample 216, RT 18.846 mins has the same fragmentation pattern as that of the chlorogenic acid standard.

Table 4.36 Comparison of principal mass ions (*m/z*) identified in chlorogenic acid reference standard and Sample 216

| | **chlorogenic acid** | **Sample 216** | |
|---|---|---|---|
| Retention time | **18.795 min** | **18.846 min** | **13.443 minn** |
| *m/z* | 126.9592 | 126.9644 | 126.9485 |
| | 135.0840 | 135.0840 | 135.0840 |
| | 161.0125 | 161.0275 | 161.0274 |
| | 162.0239 | 162.0239 | |
| | 173.0296 | 173.0358 | 173.0296 |
| | 179.0122 | 179.0184 | 179.0184 |
| | 191.0062 | 191.0255 | 191.0255 |
| | 352.9337 | 352.9600 | 352.9439 |
| | 353.9439 | 353.9703 | 353.9688 |

The three peaks from Table 4.36 had similarly shaped DAD spectra from the HPLC analyses (Figures 4.75 and 4.78). Small variations in RT between the HPLC and LC-MS are due to the change of instrument required for these different analyses.

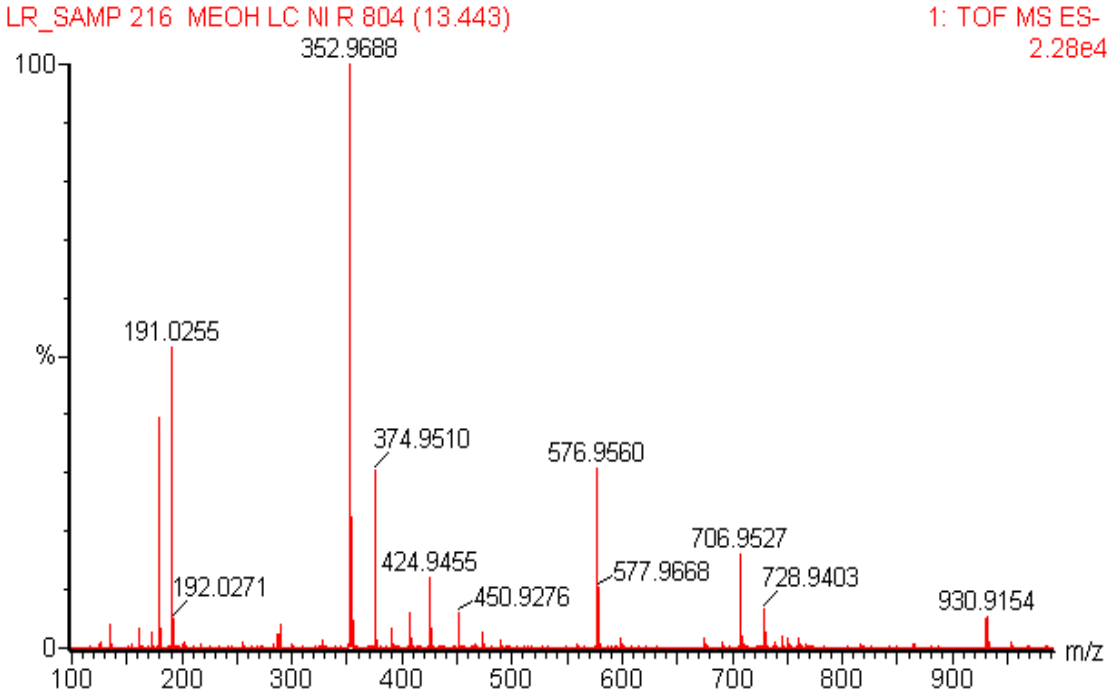



Figure 4.90(a) NI ESI Mass spectrum fragmentation pattern Sample 216, peak RT ~13 mins

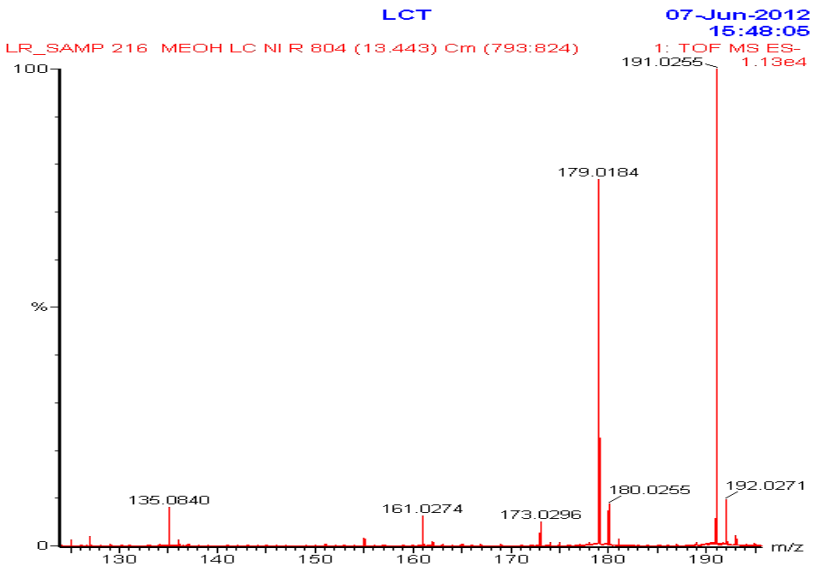



Figure 4.90 (b) NI ESI Mass spectrum fragmentation pattern Sample 216, peak RT ~13 mins

The constituents of the peak RT 13.433 min may be a different isomer of chlorogenic acid. Ibrahim *et al.*

(1989) reported three positional isomers of cinnamoylquinic esters which at pH2 elute with different retention times and that elute in the order 5→4→3. Table 4.37 is a list of the diagnostic mass ions (*m/z*) reported for examples of the isomers of chlorogenic acid (Jaiswal *et al.* 2010, Nollet *et al.* 2012). Whilst the parent ions were not identified for caffeoylshikimic acid and isomers (Table 4.37) several of the *m/z* detected in Figure 4.89(b) and 4.90(b) are formed during mass spectral analyses of caffeoylshikimic acids. The chlorogenic acid certified reference standard used is quoted at ≥95% purity therefore may also contain postional isomers.

Table 4.37 Mass spectra data for caffeoyl quinic acids (CQA) from Jaiswal *et al.* (2010) and ** Nollet *et al.* (2012).

| | **$MS^1$** | **$MS^2$** | | | |
|---|---|---|---|---|---|
| | **parent ion [M-H]** | **base peak** | **secondary peak** | | |
| | ***m/z*** | ***m/z*** | ***m/z*** | ***m/z*** | ***m/z*** |
| **compound** | | | | | |
| 1-caffeoylquinic acid** | 353 | 191 | 179 | 173 | 135 |
| 3-caffeoylquinic acid | 353.1 | 190.9 | 178.5 | | 134.9 |
| 4-caffeoylquinic acid | 353.1 | 172.9 | 178.9 | 190.8 | 135.0 |
| 5-caffeoylquinic acid | 353.2 | 190.0 | 178.5 | | 135.0 |
| *cis*-3-caffeoylquinic acid | 353.1 | 190.9 | 178.5 | | 134.9 |
| *cis*-4-caffeoylquinic acid | 353.1 | 172.9 | 178.9 | 190.8 | 135.0 |
| *cis*-5-caffeoylquinic acid | 353.2 | 190.0 | 178.5 | | 135.0 |
| caffeoyl-*epi*-quinic acid | 352.9 | 190.7 | 178.9 | 172.7 | 135.0 |
| caffeoyl-*epi*-quinic acid[X] | 352.9 | 178.7 | 190.7 | 160.7 | 134.8 |
| | | | | | |
| caffeoylshikimic acid | 335.2 | 160.9 | 178.9 | 134.9 | |
| 4-O-caffeoylshikimic acid | 335.1 | 178.9 | 160.9 | 134.9 | |
| 5-O-caffeoylshikimic acid | 335.1 | 178.9 | 160.9 | 135.0 | |

[X] acylation position uncertain

Chlorogenic acids present in coffee leaves, when exposed to natural UV light, undergo trans-cis isomerisation (Jaiswal *et al.* 2010) therefore it is possible that either form may be the one present in the *Phoradendron.* Figure 4.91 shows the major fragments formed by chlorogenic acids.

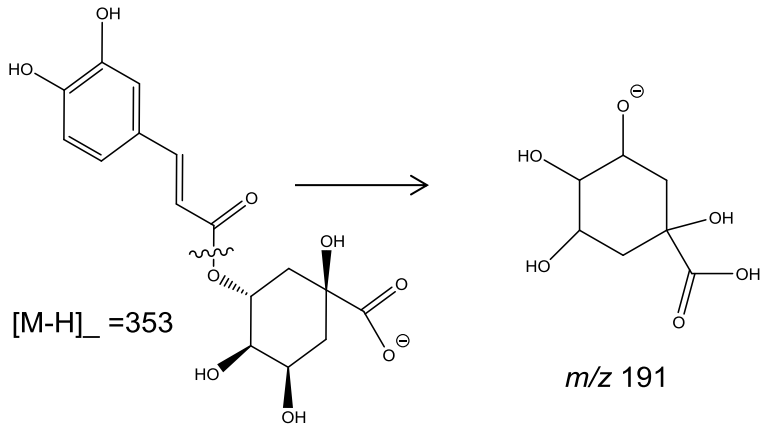



Figure 4.91 Proposed fragmentation pattern for chlorogenic acid showing the formation of the $MS^2$ m/z 191 peak generated from the $MS^1$ parent ion [M-H]− = 353 precursor ion (Callemien *et al.* 2008).

Figure 4.90 also appears to show a fragmentation pattern which may relate to two other potential constituents (a) apigenin-7-O-rutinoside, Figure 4.91, MW = 578 (Figure 4.93) and the $MS^2$ ion m/z 577 *and* (b) proanthocyanidin dimers with MW 578.

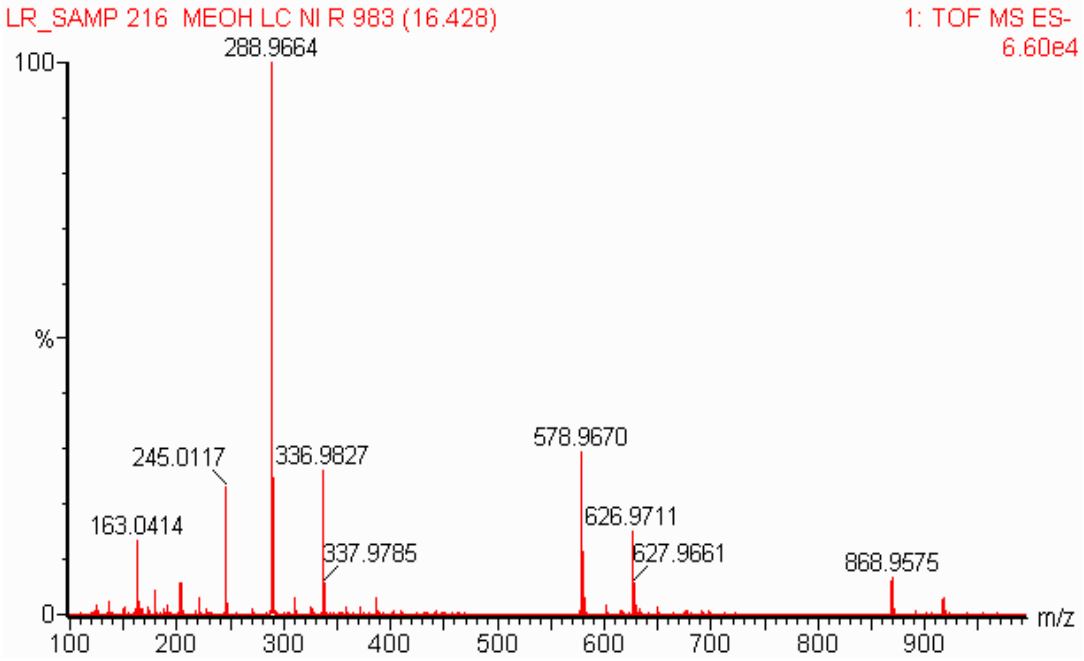



Figure 4.92 NI ESI Mass spectrum fragmentation pattern Sample 216, peak RT ~16 mins

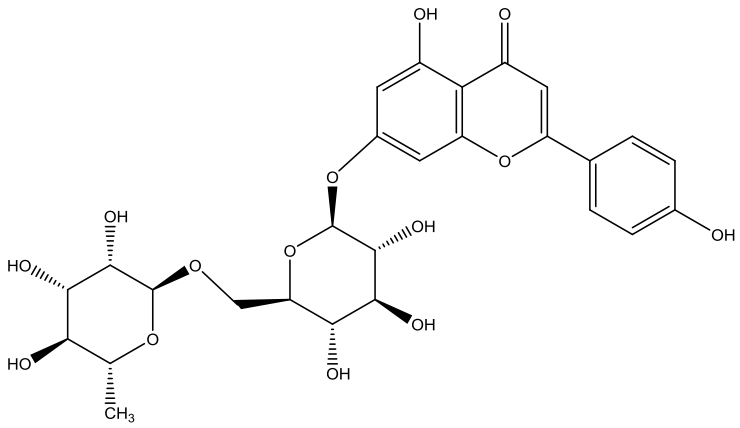
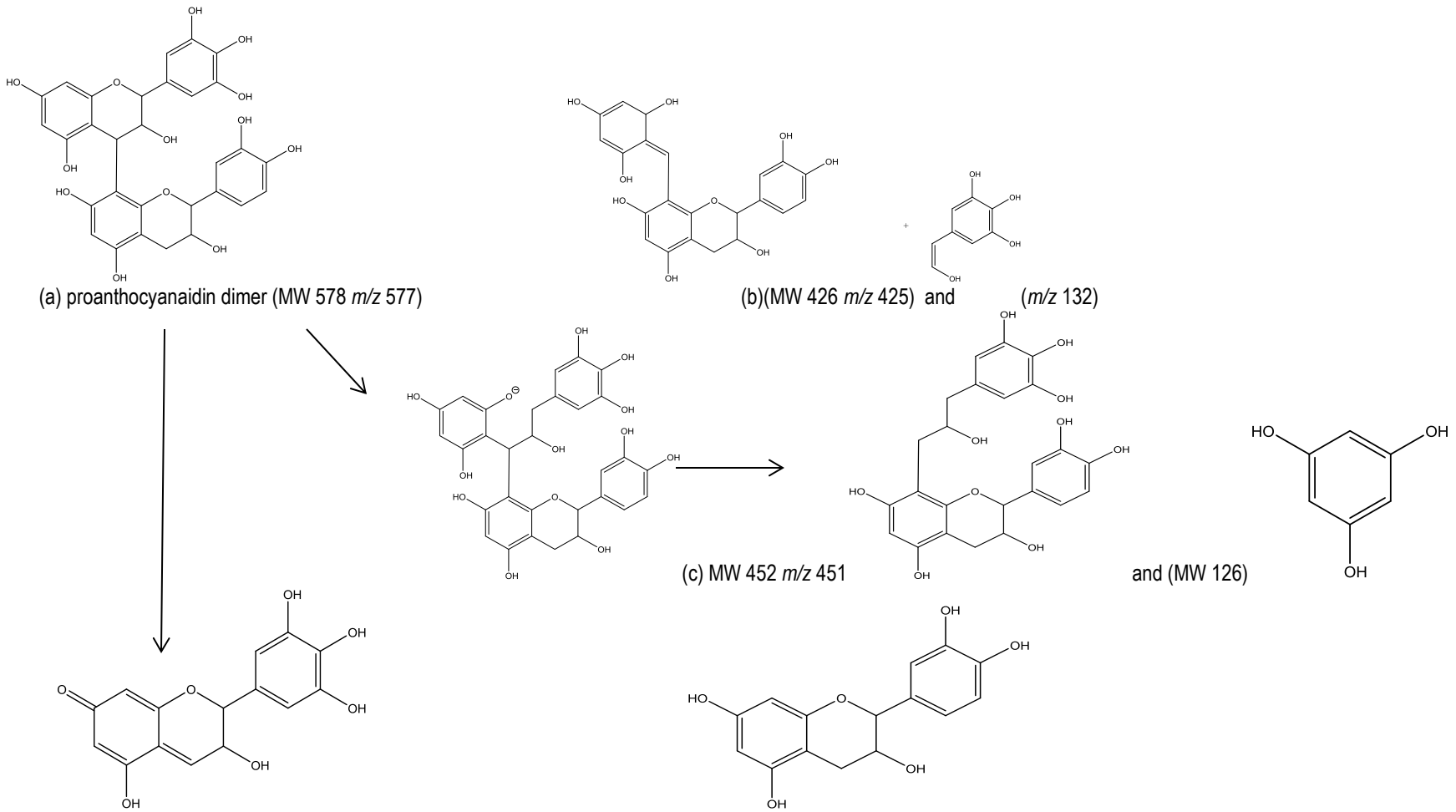



Figure 4.93 apigenin-7-O-rutinoside

The following potential molecules with MW 578 are the procyanidin dimers B1-B8. B1: epicatechin(4β⟶8)catechin, B2: epicatechin(4beta-->8)epicatechin, B3: (+) catechin-(4α-8)-(+)-catechin, B4:(+) catechin-(4α-8)-(+)-epicatechin and B8 - catechin-(4α⟶6)-epicatechin. These procyanidins undergo a variety of fragmentation reactions which generates the following mass ions m/z: 451, 425, 289 and 287 (Callemien *et al.* 2008), possible fragmentation patterns are shown Figure 4.94.

There were no *m/z* characteristic of chlorogenic acid present in the peak with RT 16.42 min (Figure 4.92). As in Figure 4.90(a) a procyanidin B dimer (MW 578, parent ion *m/z* 577) may be present. The *m/z* 868 mass peak is potentially one of the procyanidin C1 or C2 trimers e.g. epicatechin-(4β⟶8)-epicatechin-(4β⟶8)-epicatechin or catechin-(4α⟶8)-catechin-(4α⟶8)-catechin (Callemien *et al.* 2008). The fragment with *m/z* 289 may be the flavan-3-ols catechin or epicatechin.



(a) proanthocyanaidin dimer (MW 578 *m/z* 577)

(b)(MW 426 *m/z* 425) and (*m/z* 132)

(c) MW 452 *m/z* 451

and (MW 126)

(d) 2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-2,3,4,4a-tetrahydro-7H-chromen-7-one (MW 288 *m/z* 287) and 2-(3,4-dihydroxyphenyl)chromane-3,5,7-triol MW 290 *m/z* 289)

Figure 4.94 Possible proanthocyanidin dimer (ESI-MS/MS –ve mode) fragmentation patterns for Figures 4.90(a) and 4.92. From (Callemien *et al.* 2008)



213

Two further compounds which the fragmentation patterns suggest may be present are 3-O-*p*-coumaroylquinic acid (Figure 4.95) and 4-O-*p*-coumaroylquinic acid both have parent ions m/z 337.7 and produce a peak with a *m/z* 163, p-coumaric acid (Clifford *et al.* 2003). Table 4.38 is a summary of potential constituents identified from MS fragments in peak RT 16.428min, *P. quadrangulare* eluents.

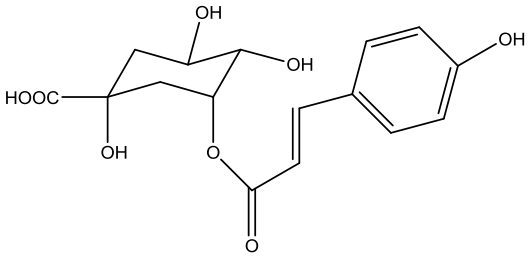



Figure 4.95 3-O-*p*-coumaroylquinic acid

Table 4.38 Compounds potentially identified in peak RT 16.428 min and their diagnostic Ions (*m/z*)

| **compound** | **[M-H]$^-$** | **Diagnostic Ions (*m/z*)** | **Reference** |
|---|---|---|---|
| (+)-catechin | 289 | 246,245, 205, 179 | Plazonic *et al.* (2009). |
| (-)-epicatechin | 289 | 246,245, 205, 179 | |
| (-)-gallocatechin | 305 | 261, 221, 219, 179 | |
| (-)-epigallocatechin | 305 | 261, 221, 219, 179 | |
| gallic acid | 169 | 125 | |
| procyanidin trimers C1, C2 | 577 | 288 | Callemien *et al.* (2008). |
| apigenin 7-rutinoside | 577 | 269 | Cuyckens *et al.* (2001). |
| apigenin | 269 | 151 | |
| *o/p*-coumaric acids | 163 | 119 | Sun *et al.* (2007) |
| 3-*p*-coumaroylquinic acid | 337.1 | 163, 190.0 | Jaiswal *et al.* (2010). |
| 4-*p*-coumaroylquinic acid | 337.1 | 172.7 | |
| 5-*p*-coumaroylquinic acid | 337.2 | 190.9,162.9 | |
| *cis*-3-*p*-coumaroylquinic acid | 337.1 | 162.9, 190.0 | |
| *cis*-5-*p*-coumaroylquinic acid | 337.2 | 190.9, 162.9 | |

In summary**:**

- The RT, relative abundance and accurate mass values for chlorogenic acid standard and peak RT 18.846min in Sample 216 are the same.
- MS fragmentation patterns for peak RT 18.846 min in Sample 216 are consistent with that of chlorogenic acid
- This data confirms the identification of the principal component in the acidic aqueous digests (gastric simulation) RT ~19mins in *P. quadrangulare* samples as chlorogenic acid, 3-(3,4-dihydroxycinnamoyl)quinic acid.
- Other isomers of chlorogenic acid (Table 4.37) may also be present in the digests eluting with different RT.
- MS fragmentation patterns suggest the possible presence of procyanidins and *p*-coumaroylquinic acids, possibly an apigenin-7-O-glycoside and caffeoylshikimic acids.

## 4.20 Properties of chlorogenic acid - 3-(3,4-dihydroxycinnamoyl)quinic acid

The hydroxycinnamic acids, HCA, are a class of phenylpropanoids. Amongst them the most commonly distributed in the higher plants are p-coumaric, caffeic and ferulic acids. The hydroxycinnamic acids, are rarely found in the free form but as conjugates, and occur more commonly as esters than glycosides. Chlorogenic

acid is a conjugate of caffeic and quinic acid (Figure 4.96).

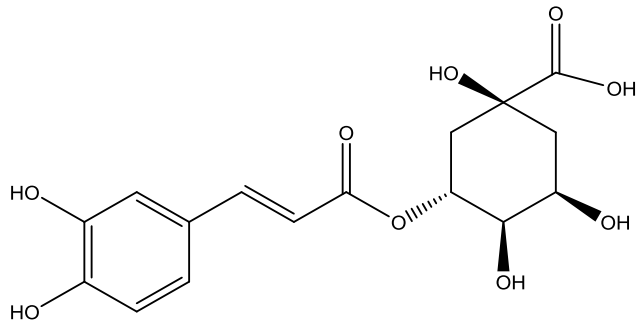



Figure 4.96 chlorogenic acid (3-(3,4-dihydroxycinnamoyl)quinic acid)

## 4.20.1 Response of chlorogenic acid to shift reagents

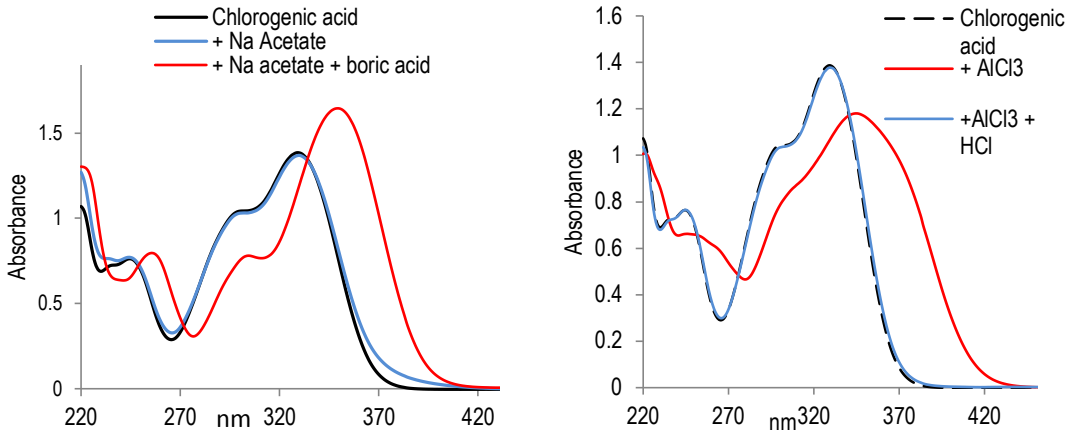



Figure 4.97 UV response of chlorogenic acid to shift reagents

Table 4.39 Shift results for chlorogenic acid standard

| Sample | reagent | Band II λnm | Abs. | Band I λnm | Abs. | shift |
|---|---|---|---|---|---|---|
| Chlorogenic acid | NaAcet | 244 | 0.7621 | 329 | 1.3854 | |
| Chlorogenic acid | NaAcet+$H_3BO_3$ | 244→256 | 0.7960 | 329→349 | 1.6455 | bathochromic + hyperchromic (increased absorbance) |
| | | | | | | |
| Chlorogenic acid | $AlCl_3$ | | | 329→345 | 1.3789 | bathochromic |
| Chlorogenic acid | $AlCl_3$ + HCl | | | 345→329 | 1.1810 | hypsochromic |

The bathochromic shift of chlorogenic acid with $AlCl_3$ is due to interaction with free o-dihydroxyphenyl groups indicative of the presence of a caffeoyl group (Ibrahim *et al.* 1989). Caffeoyl esters differ from the conjugates of coumaric and ferulic acids. Caffeoyl conjugates have a bathochromic shift response to both NaOAc/$H_3BO_3$ and $AlCl_3$, (Figure 4.97 and Table 4.39).

*P. quadrangulare* digests show a clear bathochromic shift with NaOAc/$H_3BO_3$, Figures 4.53-54. There is a suggestion of a shift with $AlCl_3$, Figures 4.58(a-b) but it is less clear and almost absent in some samples Figures 4.58(c-d). The results are consistent with there being more than one class of compound being present in the aqueous digests.

### 4.20.2 Antibacterial testing of chlorogenic acid

Activity was seen against *B. subtilis* and *S. aureus* with the higher concentrations, equivalent to the addition of 37.5µg/ml of chlorogenic acid to the wells however the ZI were smaller than those seen for the *Phoradendron* digests. The ZI were all less than 5mm and it was not possible to distinguish a difference between the sensitivity of the two bacteria. No activity was seen against *E. coli.* This is in accordance with Lou et al (2011) who reported the Minimum Inhibitory Concentration, in a liquid culture, for *E. coli* was 80µg/ml, ~twice that of *B. subtilis* and *S. aureus*. It is therefore possible that insufficient chlorogenic acid was present to inhibit *E. coli* growth using the diffusion test method.

## 4.21 Estimation of chlorogenic acid content of *P. quadrangulare* digests

### 4.21.1. Calibration Plot

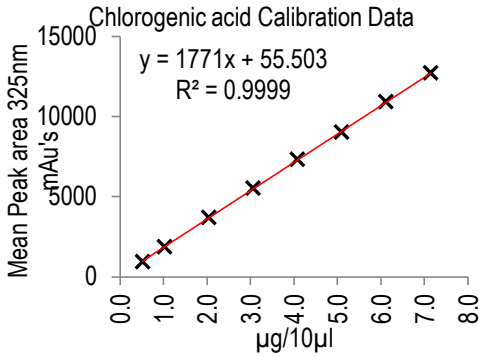



Figure 4.98 Calibration data chlorogenic acid standard solutions

In order to standardise the calculations the peak areas were obtained from the HPLC data exported as an Excel file. The rate of change at the commencement and end of the peak was calculated and a RT time determined for the start and finish points. The sum of the absorbance values within this range was then used as the peak area. The calibration data provided covers the range detected in the majority of the sample eluents.

### 4.21.2 Chlorogenic acid recovery calculation

F1 and F4 SPE fractions had no detectable chlorogenic acid peaks in the recovery work, three replicates of each fraction were analysed.

In order to verify the calculation of the peak area, a series of 10µl injections of aqueous chlorogenic acid solution 7.5µg/10µl were made. The peak areas determined and then the value substituted in the calibration equation (Figure 4.98) and the concentration determined.

Example calculation: Mean peak area 10 injections = 12995.6 Substituting in the calibration equation y = 1771x + 55.503 = 7.31µg/10µl this equates to 97.42% of the expected value. The use of this method of calculating peak area will provide a reasonable estimate of chlorogenic acid content in the digests.

### 4.21.3 Estimation of chlorogenic acid in *P. quadrangulare* samples

The peak areas were calculated as described for the chlorogenic acid and using the calibration equation and a value extracted from the dry leaf mass estimated (Table 4.40).

Table 4.40 Calculated values for chlorogenic acid extracted from *P. quadrangulare* leaf samples

| Host ID | Sample No | Date | chlorogenic acid mg/g dry leaf | No. individuals eating | mean ZI (mm) *B. subtilis* | mean ZI (mm) *S. aureus* |
|---|---|---|---|---|---|---|
| MC1 Control | 147 | Aug-07 | 6.97 | 0 | 8.92 | 15.93 |
| | 155 | Sep-07 | 6.74 | 0 | 9.47 | 16.19 |
| | 165 | Oct-07 | 6.40 | 0 | 9.24 | 16.76 |
| | 178 | Nov-07 | 8.60 | 0 | 9.26 | 16.5 |
| | 195 | Feb-08 | 4.37 | 0 | 8.59 | 15.09 |
| | 201 | Apr-08 | 9.88 | 0 | 9.69 | 17.93 |
| | 221 | Sep-08 | 3.97 | 17 | 8.65 | 14.07 |
| MC76 Control | 180 | Nov-07 | 3.74 | 0 | 8.61 | 15.54 |
| Misc | 153 | Sep-07 | 6.58 | 17 | 9.15 | 16.48 |
| | 154 | Sep-07 | 5.61 | 15 | 9.89 | 16.62 |
| | 179 | Nov-07 | 3.90 | 7 | 8.96 | 15.01 |
| | 220 | Sep-08 | 5.70 | 16 | 9.29 | 13.96 |
| | 216 | May-08 | 5.70 | 13 | 9.63 | 14.42 |
| | 226 | Oct-08 | 3.27 | 21 | 9.48 | 12.47 |
| | 152 | Sep-07 | 5.06 | 17 | 9.09 | 16.66 |
| | 206 | May-08 | 1.91 | 13 | 7.95 | 15.84 |
| | 215 | May-08 | 2.29 | 16 | 9.77 | 16.06 |
| | 177 | Nov-07 | 2.29 | 7 | 8.47 | 15.54 |

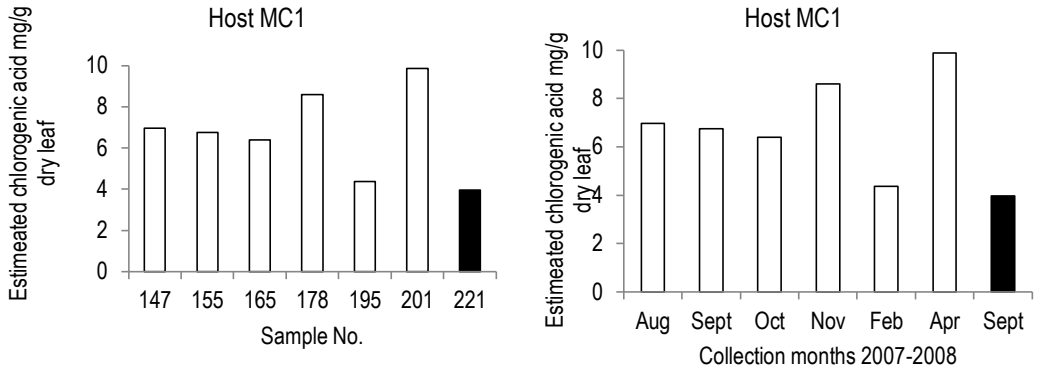



Figure 4.98 chlorogenic acid content *P. quadrangulare* leaf digests from Host MC1.

Host tree MC1 was a control tree where regular collections were attempted (Figure 4.98). The highest chlorogenic acid contents were in samples collected Nov and April, from host Tree MC1.

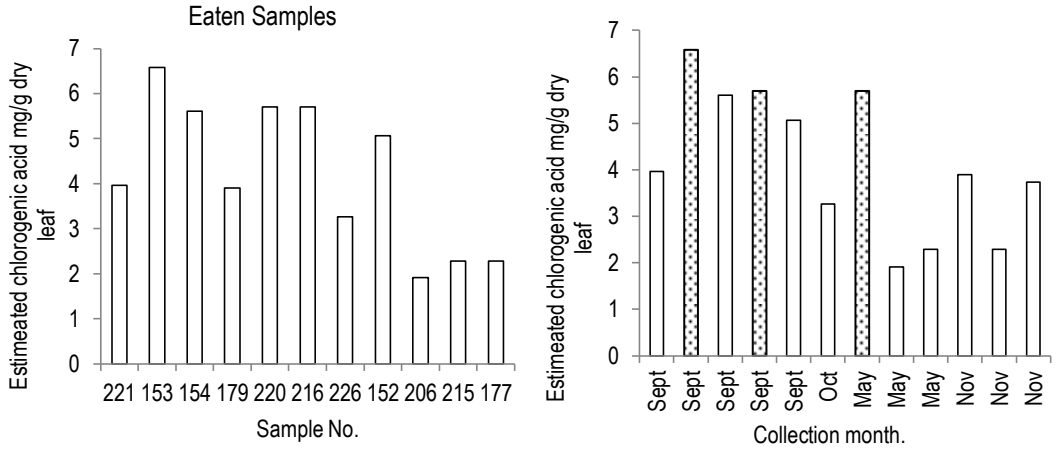



Figure 4.99 chlorogenic acid content eaten samples of *P. quadrangulare* (pattern bars observed as eaten by ≤ 7 individuals, remainder by > 12 individuals)

Sample 221 was collected outside of the regular collection schedule after mistletoe was seen to be eaten. It has the lowest chlorogenic acid content for the samples collected from these samples. The highest chlorogenic acid content in the ad hoc collected samples (Figure 4.99) were in samples collected Sept and May.

Chlorogenic acid determinations for *V. album* growing on a range of host trees have been reported at 0.84-21.74mg% (Luczkiewicz *et al.* 2001). An unspecified chlorogenic acid was also identified in *V. alb*um, levels varying according to host tree and season collected (Vicas *et al.* 2011a,b, Vicas *et al.* 2012). *P. quadrangulare* chlorogenic acid levels appear much higher than those reported for *V. album*. Testing a variety of extraction methods Vicas *et al.* (2011a,b, Vicas *et al.* 2012) found that aqueous extracts with the highest anti-oxidant activity were of *V. album* growing on *Acer campestre* having a TEAC value of 678.72mM equivalent Trolox/g fresh matter.

In Summary:

- There was no discernible pattern to the eating of the *P. quadrangulare* leaf based on the estimated chlorogenic acid
- There was no discernible seasonal pattern to the chlorogenic acid content, however it may relate to mistletoe or host tree phenology
- Only Sample 226 eaten by 21 individuals was rapidly followed by geophagy, this did not contain a high level of chlorogenic acid.

## 4.22 Parasitology Results

The species identified in *Ateles* faecal samples from Santa Rosa included *Strongyloides fuelleborni*-nematode, *Gardia* spp. - flagellated protozoan, *Balantidium coli* - ciliate protozoan, *Entamoeba coli* - non-pathogenic protozoan and a fluke - *Controrchis* spp.

Stuart *et al.*(1990) examined faecal samples from 155 mantled howler monkeys (*Alouatta palliata palliata*) examined at Centro Ecologico La Pacifica, Guanacaste Province, Costa Rica reporting 75 had parasitic infections whereas only 1/9 *Alouatta palliata palliata* from Santa Rosa National Park, were infected. Results for *Ateles geoffroyi* at Santa Rosa indicated that only 3/19 were infected and the organisms identified were *Controrchis biliophilus, Trypanoxyuris minutus,* unidentified strongylid eggs and Isospora sp. Oocysts (Stuart *et al.* 1990).

### 4.22.1 Pathophysiology of identified *Ateles* GI parasitic species - symptoms from Merck Veterinary Manual (Nicholas W. Lerche 2011).

- *Entamoeba histolytica* is the principal pathogenic form in nonhuman primates, rarely been reported as pathogenic in monkeys, mostly in South American spider and woolly monkeys. In a heavy infection, it may cause severe enteritis and diarrhoea.
- *Giardia* inhabit the upper small intestine and may cause watery diarrhoea, dehydration, weight loss and weakness.
- *Cryptosporidium parvum* (related to the Isospora) may also cause diarrhoea in primates, mainly in young animals
- *Strongyloides* and *Trichostrongylus* are invasive—adults may cause enteritis and diarrhoea, larvae may cause pulmonary lesions during migration.

- *Balantidium* infection can cause such symptoms as diarrhoea and abdominal pain.

## 4.23 Discussion and Interpretation of Results

Mistletoe leaves are unlikely to be selected rather than host leaves for possible protein content. Nitrogen levels, (often measured as a proxy for protein content) in mistletoes are consistently reported as being lower than in host leaves and show no enrichment even when host leaf had high a nitrogen content (Ehleringer *et al.* 1986, Bannister *et al.* 2001, March *et al.* 2010). Slow growing/evergreen characteristics are correlated with low foliar nitrogen (Salatino *et al.* 1993) particularly in hemiparasitic plants in mineral poor environments. Total nitrogen determination was not undertaken for these reasons. In addition there have been concerns relating to the use of total nitrogen determination because it assumes that all nitrogen originates from protein. When applied to fruit pulp, this overestimates protein content because pulp typically contains free amino acids and many nitrogenous secondary metabolites (Levey *et al.* 2000).

### 4.23.1 *Phoradendron* leaf Mineral Content

More than 50 minerals are present in mammalian tissues; 16 are classed as essential, 13 important for cell function and many are reported to influence immune responses. Amongst those known to influence immunity are zinc, selenium, iron, copper, magnesium, manganese and sulphur. These functions have been reviewed (McClure 2008). Minerals also play a role in mediating inflammation e.g. Se, Cu, Zn, S and Mn are linked to antioxidant enzymes protecting against oxidative damage to cells and so reducing inflammation. Mg, Zn and S are involved in RNA translation, gene regulation and DNA replication (Keen *et al.* 2004). Decreased Zn levels have been linked to increased rates of infection, due to reduced T cell numbers. Mg deficiency also impacts on T-cell function and increased levels of proinflammatory cytokines. Low Mg levels are also associated with the development of insulin resistance (Rosolova *et al.* 2000). Manganese deficiency has been linked to neonatal mortality, skeletal abnormalities and abnormal carbohydrate, lipid and protein metabolism. It is also thought that minerals may exert synergistic effects (Keen *et al.* 2004).

The significance of minerals in the activity or toxicity of herbal medicines has been investigated by many authors. The use of herbal teas has been proposed as a source of concentrated minerals (Szentmihályi *et al.* 2005). Analysis of herbal teas used to reduce the complications of type-2 diabetes showed variable extraction rates: K (22.5-74.7%), Na (1.9-60.5%), Mg (12.3-52.5%) and Cu (3.7-51.4%) were more readily released but Ca (6.6-28.1%), Mn (6.8-32.3%) and Zn (0-31%) were lower (Szentmihályi *et al.* 2005).

Recently Szentmihályi *et al.* (2013) examined the mineral content of dried leaf of four herbs used in folk medicine for treating inflammation and gastrointestinal conditions. High levels of Ca, K, Mg, P and S and lower levels of Cu, Fe, Mn and Zn were reported. The results of the analysis of the teas produced in the traditional manner (approx. 5g/200ml) were evaluated from nutritional and biomedical points of view suggesting the teas may be a rich source of Ca, Cr, Mn and Mo. Teas also contained variable Fe, Cu, Zn which may have a significant role in the immune defence system.

#### 4.23.1.1 Analytical considerations mineral determination

The instrument available was an Energy-Dispersive-X Ray Fluorescence type. The use of EDX-RF as a non-destructive analytical method for mineral content of plant material was briefly discussed in Section 4.4.1.

One of the accepted limitations is in the differences in the levels elements detectable. The LOD for heavy elements are low ~1µg/g whilst the light elements e.g. Na, Mg and Al are higher at 10-100's µg/g. The limit of detection for the lighter elements is usually mitigated by the high levels of these elements in plant tissue (Marguí *et al.* 2009). The quantification method used by the instrument available was that of Fundamental Parameters (FP). This is a mathematical model used where there is no appropriate standard material available to generate calibration data. It relies upon the spectrometer response to pure elements in predicting the fluorescence intensity for a matrix composition. The precision and accuracy of results from this method are between 5-10%. This level is accepted for biological and environmental studies (Marguí *et al.* 2009).

Guerra *et al.* (2013) compared the EDX-RF results with the results obtained from total plant material digestion followed by ICP-OES (Inductively Coupled Plasma/Optical Emission Spectrometry). The authors reported that EDX-RF produced highly reproducible results and was a useful non-destructive method particularly suitable for dealing with heterogeneity evaluation within samples. Similar correlation of results was found in quantification of Al levels in plants (Campos *et al.* 2014).

In order to place the presented results in context a literature review was undertaken for data relating to the regularly consumed fruit species identified as being eaten by *Ateles* at Santa Rosa (Table 1.1). The data available is presented in Table 4.41. These were detected following digestion of the fruit pulp and analysed by various techniques.

Table 4.41 Mineral content of fruits identified as being regularly eaten by *Ateles* at Santa Rosa (ND = not reported).

| | **determined levels g/kg** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **fruit** | **Na** | **K** | **P** | **Mg** | **Ca** | **Mn** | **Fe** | **Cu** | **Zn** | **Ref** |
| *Spondias mombin* | 0.056 | 2.883 | 0.329 | 0.151 | 0.110 | 0.025 | 0.327 | 0.118 | ND | Tiburski *et al* .(2011) |
| *Manilkara chicle* | 0.12 | 1.93 | 0.12 | 0.012 | 0.210 | ND | 0.008 | ND | 0.001 | USDA (2012) |
| *Cecropia peltata* | 4.60 | 0.17 | 2.400 | 2.200 | 4.60 | ND | 0.098 | 0.014 | 0.0229 | Behie *et al.* (2012) |
| Belize *Ficus* | 0.16-0.4 | ND | 0.05-0.18 | 1.4-4.00 | 7.0-19.0 | 3.23-3.83 | 1.88-6.31- | 0.229-0.879 | 0.43-1.96 | O'Brien *et al.* (1998) |

### 4.23.1.2. *Phoradendron* Mineral results

Table 4.42 Comparison of minerals detected in mistletoes from Santa Rosa and published data *(Ehleringer *et al.* 1985); **(Panvini *et al.* 1993) *** *Ligaria cunifolia*, Argentinian mistletoe (Gimenez *et al.* 2012)

| | ***P. quadrangulare*** | ***P. robustissimum /LS*** | ***P. robustissimum /TO*** | ***P. californicum**** | ***P. leucarpum***** | ***L. cunifolia**** |
|---|---|---|---|---|---|---|
| | **g/kg** | | | | | |
| silicon | 0.74 - 4.95 | 2.3 - 9.4 | 1.91 - 6.23 | 0.189 | ND | ND |
| potassium | 18.66 - 33.93 | 7.7 - 21.9 | 15.84 – 27.34 | 27.6 | 81.53 | 17.7 - 23.1 |
| sodium | 4.01 - 6.31 | 3.1 - 5.6 | 2.26 - 5.31 | 1.15 | 2.06 | 1.83 - 2.14 |
| calcium | 3.75 -9.98 | 2.5 - 6.6 | 3.62 - 7.71 | 15.4 | 17.18 | 9.8 - 12.0 |
| sulphur | 1.53 – 4.51 | 0.46 - 0.72 | 0.361 - 1.09 | ND | ND | ND |
| phosphorus | 0.26 - 0.99 | 0.18 - 0.41 | 0.102 - 0.37 | 2.22 | 3.08 | 0.22 - 2.87 |
| magnesium | 0.20 - 1.22 | 0.42 – 1.17 | 0.31 - 3.74 | 3.01 | 10.40 | 10.1 - 11.9 |
| manganese | ND | 0.30 - 0.58 | 0.098 - 0.26 | 0.013 | 1.27 | 0.14 - 0.19 |
| zinc | 0.004 - 0.01 | ND | 0.005 - 0.013 | 0.025 | 0.06 | 0.17 - 0.24 |
| copper | 0.008 - 0.022 | ND | 0.006 - 0.022 | 0.012 | 0.03 | 0.035 - 0.04 |
| iron | 0.033 - 0.52 | 0.022 -0.05 | 0.024 -0.049 | 0.046 | 0.16 | 1.35 - 2.9 |

The effect of mistletoe ecophysiology and the concentration of elements that this produces were introduced in Section 1.8.7. Table 4.42 provides a summary of the analytical results and a comparison for two further species of *Phoradendron* and the Argentinian mistletoe *Ligaria cunifolia,* widely used as a substitute for *Viscum album* in Argentinian folk medicine (Fernandez *et al.* 1998, Wagner *et al.* 1998, Fernandez *et al.* 2003, Gimenez *et al.* 2012).

### Silicon (Si)

Most plants, particularly dicotyledons, are unable to accumulate high levels of Si in the shoots, levels ranging from 0.1% to 10.0% Si (dry weight). Accumulation of Si is thought to protect plants from multiple abiotic and biotic stresses. Numerous studies have shown that Si is effective in controlling diseases caused by both fungi and bacteria in different plant species and Si also enhances plant resistance to insect pests such as stem borer and other leaf mining insects. This is attributed to Si deposition in the plant tissues providing a mechanical barrier against establishment of bacterial or fungal infections and chewing by insects. Si also alleviates many abiotic stresses including chemical stress (salt, metal toxicity, nutrient imbalance). Increased strength and rigidity of cell walls deceases transpiration from the cuticle and, thus increases the resistance to high temperature, radiation, UV and drought stresses (Ma *et al.* 2006, Cooke *et al.* 2011).

The higher Si levels in *P. robustissimum* would make the leaves less digestible than *P. quadrangulare.* Silicon has been proposed as the primary defence in grasses and is thought to lead to increased abrasiveness of foliage so deterring feeding, as well as reducing foliage digestibility and herbivore performance (Massey *et al.* 2006). Silicon may defend plant material at least in part by reducing mechanical breakdown of the leaf (Hunt *et al.* 2008). Silicon is not considered an essential nutrient for mammals however recently it has been reported that silicon may prevent oral aluminium absorption and retention in mammals, reducing potential aluminium toxicity (Domingo *et al.* 2011).

### Potassium (K)

Potassium levels were high in both *Phoradendron* species. Higher accumulation of K in mistletoe leaf compared to host tissues has been reported in many other studies (Lamont *et al.* 1982, Glatzel 1983, Glatzel *et al.* 2009, March *et al.* 2010, Tennakoon *et al.* 2011, Tennakoon *et al.* 2014). There have been several hypotheses for this accumulation. There may be active uptake of this element essential for osmoregulation and control of stomal opening (Lamont *et al.* 1982, Lamont 1983, Tennakoon *et al.* 2011). Potassium enrichment observed may be associated with uptake of host photosynthesis products and consequently K is accumulated and cannot be exported due to lack of phloem connection (Glatzel 1983, Glatzel *et al.* 2009). It is also possible that the low levels reported for host leaf is due to translocation of K from the host-leaf tissues via the phloem to other host tissues such as the roots, flowers, and fruits.

Potassium homeostasis in mammals is tightly controlled and is governed by the daily consumption of potassium and the renal excretion mechanisms. High potassium levels are associated with acute cardiovascular changes with ECG abnormalities, muscular weakness, and nausea and vomiting. Ingestion of high potassium is more significant where there is impaired renal function (Saxena 1989). The potassium may potentially have detrimental effects depending upon the amount of leaf consumed.

### Sodium (Na)

There is some indication that Na may be accumulated in the *Phoradendron* leaves. Sodium levels are generally considered to be low in fruits.

### Calcium (Ca)

The levels detected in the Santa Rosa *Phoradendron* species were much lower than the other reported levels (Table 4.42). However calcium levels in both host and mistletoe will be determined by the available calcium in the soil. Calcium is not recycled via the phloem in the host. Results in Section 3.5.10.1 only detected calcium at µg/g levels in the geophagy samples, suggesting that there may be very low levels of available calcium in the local soils. Applying a 25% recovery value 2g of *P. quadrangulare* would potentially provide 5mg calcium.

### Sulphur (S)

Sulphur levels were much higher in *P. quadrangulare* at g/100g dry material compared to *P. robustissimum at* mg/100g.

Mistletoe thionins are peptides with 46 amino acid residues. Phoratoxins (thionins) are known to contain six cysteine residues (Samuelsson *et al.* 1970a, Li *et al.* 2002, Johansson *et al.* 2003), and are stored in vesicles in the leaves. There are seasonal variations in the levels and this is considered a mechanism for conservation of limited resources e.g. S, P (Urech *et al.* 2011). Thionins are also considered part of the plant defence system against plant pathogens, including bacteria and fungi, by working directly at the cell membrane. The high levels of S may therefore be due to the thionin stored content. A further contribution to the sulphur levels may be mistletoe lectins. ML3 consists of 262 amino acid residues including ten cysteine residues (Wacker *et al.* 2005). Mistletoe lectin ML1, B chain has 264 amino acid residues with seven cysteine residues (Soler *et al.* 1998).

Izhaki (1998) reported that in frugivorous birds much fruit pulp consumed was deficient in the sulphur-containing amino acids methionine and cysteine (41–61% below requirement). *Ficus obtusifolia* one of nine *Ficus* species eaten at Santa Rosa, contained low levels of almost all amino acids with the sulphur-containing methionine and cysteine the lowest (Wendeln *et al.* 2000). *Spondias mombin,* another commonly consumed fruit at Santa Rosa is reported as having measured total protein of 1.06g/100g edible portion (Tiburski *et al.* 2011) lower than that of the figs *(*Wendelen *at al.* 2000 and *Cecropia* (Herrera 1981). Analysis of the fruit pulp of a further regularly consumed fruit *Brosimum alicastrum* yields 84% water content, 2.5% total protein, with high levels of lysine 2.34-4.0% and tryptophan1.2-2.3% but again low cysteine (Ortiz *et al.* 1995). As stated previously there are issues with the use of total protein. Analysis of *Manilkara chicle* reported amino acid content at 0.371g/100g fruit pulp, with cysteine levels of 1mg/100g (Hall *et al.* 1980).

If the higher sulphur levels are entirely as a result of thionin storage or mistletoe lectin contents the *P. quadrangulare* leaves are not potentially a source of these important amino acids. Both peptides are acid and proteases resistant (Van Damme *et al.* 1998, García-Olmedo 1999, Stec 2006, Sharon 2008).

*Ateles* are unlikely to be exposed to thionins and lectins in diet fruits but would be exposed to both from the mistletoe as both are extracted in aqueous acidic conditions.

### Phosphorus (P)

Phosphorus is one of the minerals commonly seen at higher levels in mistletoe leaf than in host leaf in parallel with potassium, (Lamont *et al.* 1982, Glatzel 1983, Glatzel *et al.* 2009). As with potassium, phosphorus is readily translocated from host leaf to root tissues, thus lowering measured values in host leaf (Ehleringer *et al.* 1985). Host leaf levels of phosphorus are at the highest in rapidly growing leaf and lowest just before leaf senescence when there is an up-regulation of acid-phosphatase genes associated with salvaging phosphorus (Buchanan-Wollaston 1997).

*P. quadrangulare* and *P. robustissimum*/LS indicates some phosphorus enrichment but *P. robustissimum*/TO does not. This variation may be related to host/mistletoe leaf ages. Both Santa Rosa species have much lower levels than *P. californicum* and *P. leucarpum.* All three samples levels are only in the µg/g range and how much of this would become available is uncertain.

### Magnesium (Mg), copper (Cu), zinc (Zn) iron (Fe) and manganese (Mn).

*P. quadrangulare* and *P. robustissimum* had lower levels of magnesium, zinc, copper than the reported values for *P. californicum*, *P. leucarpum* and *L. cunifolia* and iron levels lower th*an P. leucarpum* and *L. cunifolia.* Manganese levels in *P. quadrangulare* did not reach LOD value. Where detected manganese, copper, zinc and iron levels were all at low mcg/g dry leaf.

The Argentinian mistletoe *L. cunifolia* growing on host tree *Ulmus pumila*, had the highest Fe, Cu and Zn levels but Na levels were lower than *Phoradendron* at Santa Rosa, (Table 4.42). Sodium, K and P are present in the *P. quadrangulare* samples at higher levels/100g than in the fruit pulp (Table 4.41) for which data is available. This would be significant if similar amounts of *Phoradendron* and fruit were eaten, but the consumption of 1-10 leaves only amounts to approx. 3.2g fresh leaf and 16g fresh leaf dried to 5.12g, leaf drying results Section 4.13.2 leaf drying results. (3.2g fresh leaf = 1.024g dried leaf).

Applying a 50% extraction efficiency, 1g dried *P. quadrangulare* (highest value*)* would provide 15-18mg of potassium and would potentially provide 3mg sodium. Using 25% extraction efficiency, 1g *P. quadrangulare* would potentially provide 2-3mg calcium. *P. quadrangulare* would therefore provide > K and Na than all the fruits in Table 4.41 and < calcium than *Ficus* but > than the three remaining fruits of *S. mombin*, *M. chicle* and *C. pallata* for which data is available.

## 4.23.2 Phoratoxin and mistletoe lectin

### 4.23.2.1 Thionins

An introduction to phoratoxin, Type III thionins (cationic peptides of 45-46 amino acids) occurrence in mistletoes and biological activities is provided in Section 1.8.2 Tables 1.9-1.10. Phoratoxin had previously been identified in *P. quadrangulare,* (Larsson 2007) and Santa Rosa samples analysed at RBG Kew (Table 4.7). Thionins are classified as pathogenesis-related proteins due to their toxicity against phytopathogens and also the ability to accumulate in plants and to contribute to plant innate immunity. Thionin toxicity is mediated by binding electrostatically and hydrophobically to patches of negatively charged phospholipids phosphatidic acid (PA) or phosphatidyl serine (PS). The formation of this peptide-lipid complex causes solubilisation of the membrane and its lysis (Markman *et al.* 1993, Stec 2006).

As already stated, the author was not able to compare HPLC data of Santa Rosa samples with that determined by RBG Kew, Section 4.5.1.1. HPLC detection and resolution may have been possible with a larger sample size but there were no certified standards available to aid with identification. Nor would there be sufficient material for identification of individual thionins present using fractionation and subsequent Edman degradation, trypsin enzymatic digestion and EI tandem mass spectrometry sequencing (S. Larsson personal communication). The HPLC peak area/mass ion data provided from the analysis undertaken at RBG, Kew (Appendix 1.7, Table 2.1) may be an indication of different phoratoxin profile and/or concentrations between the two species. The phoratoxin profiles of the two *Phoradendron* species and host related influences would potentially be an area of future work.

The main feature of thionins is a toxic effect on biological systems. Thionins have activity against Gram -ve and Gram +ve bacteria, fungi, parasites and viruses that have envelope membranes, such as the human immunodeficiency virus and *Herpes simplex* (Taveira *et al.* 2014). Type III thionins are not widely distributed, only being identified in two families of the Lillipsida i.e. the Poaceae and Liliales and in three families within the Magnoliopsidaie i.e. the Brassicaceae, Ranunculaceae and Santalaceae (Stec 2006). *Ateles* exposure to these compounds is therefore limited to the mistletoes (Appendix 1.1 Table 2.1).

#### 4.23.2.2 Mistletoe lectins

An introduction to mistletoe lectins, mechanism of activity and known biological activities is introduced in Section 1.8.3 and Table 1.11.

Ribosome-inactivating proteins are widely present in the plant kingdom, with Type 1-RIPs found more frequently. Mistletoe lectins belong to Type 2-RIPs along with ricin. Most RIPs were isolated from plants belonging to the Angiosperma, preferentially distributed among plants belonging to Caryophyllaceae, Cucurbitaceae, Euphorbiaceae (Stirpe 2005). Lectins are commonly found in storage tissues such as tubers, corms, bulbs, root stocks, seeds and bark. In a few cases lectins are found in stems and leaves e.g. mistletoes where the function may be as a storage protein. Lectins however are more commonly regarded as plant defence compounds (Pusztai *et al.* 2005).

Mistletoe lectins have well defined agglutination characteristics. It was possible to infer lectins as present in the *Phoradendron* digests from the inhibition of agglutination results. Lectins are divided into groups according to cytotoxicity. Lectins also vary in selectivity. Type 2- RIPs are highly but non selectively toxic (Pusztai *et al.* 2005). MLI is toxic at ng/ml levels to mouse derived brain cells (Eifler *et al.* 1993, Stirpe 2005) therefore the presence of lectins in the aqueous digests may potentially be significant to the *Ateles* .

#### 4.23.2.3 Analytical considerations lectin agglutination

*In vitro* Erythrocyte Aggregation is a standard method for preliminary investigation of pharmacological activity (Vogel 2006) and is also used diagnostically for quantification for viruses or bacteria and anti-body titres. It is widely used as it is rapid, relatively easy to perform and very sensitive.

The detection of lectins in crop plants regularly used agglutination of erythrocytes (D'Mello *et al.* 1991) and an early use of this property was in the typing of human erythrocytes (Judd 1980, Sharon *et al.* 2004). Rat erythrocytes share agglutination types in common with human anti-A and anti-B serotypes, i.e. have α-N-acetyl-D-galactosamine (GalNAc) and d-galactose (d-Gal)/D-galactosamine specificities. The alternative available

erythrocytes from the rabbit have d-mannose specificity therefore were unsuitable. MLI, the most abundant of the three mistletoe lectins, is more than 100 times more specific for Gal than GalNAc, MLIII shows more affinity for GalNAc, and MLII has intermediate specificity (Pevzner *et al.* 2004).

Luther (1980) reported agglutination of erythrocytes with mistletoe lectin concentration of <4µg/ml and Franz *et al.* (1981) inhibited erythrocyte protein synthesis with ML1 at 2.6µg/ml. ML1 is toxic to K562 cells (human erythroleukemia) cells or EL-4 (mouse thymoma) cells at picogram range (Urech *et al.* 1995).

Identification of agglutination type is dependent upon being able to inhibit agglutination with a free sugar. The assay results are not quantitative and because there is a degree of subjectivity there may be variation in interpretation of marginal results. This is a potential problem where there is incomplete agglutination. A sugar-polymer based enzyme-linked adsorbent assay with improved sensitivity has been reported by (Wang *et al.* 2009), however the strong colour of the digests would have masked the yellow indicator.

The agglutination reaction was very obvious in the *P. quadrangulare* digests within 30 minutes; agglutination was seen to a lesser degree within 60 minutes with the *P. robustissimum* digests. This variation may be due to species difference or due to the phenology of the mistletoe. ML levels vary seasonally (Urech *et al.* 2006, Urech *et al.* 2009). There was no agglutination for the host tree digests. The inhibition assay is specific for agglutination due to the presence of lectins. The results suggest the presence of lectins ML1 and either ML2/ML3, in *Phoradendron* but not the host tree leaves

Type 2-RIPs are rarely found in fruits (Stirpe 2005) and so mistletoe toxin type molecules are a class of compound to which *Ateles* would rarely/unlikely be exposed.

## 4.23.3 Initial Phytochemical Results

The principal aim of this section of work was to see if there was any detectable phytochemical difference between the two *Phoradendron* species available to the *Ateles* . During the initial work to develop a HPLC analysis method the chromatograms showed that there were distinct differences between *Viscum album/Phoradendron* and between the two *Phoradendron* species. As a result of initial work it was decided that sample clean up would be appropriate. A standardised method for the use of Polyamide SPE Cartridges was developed. The results from the preliminary HPLC and UV analyses showed that there were differences in the UV signature of the two *Phoradendron* species. UV analysis of the SPE fractions indicated several types of compounds present in *P. quadrangulare* samples with strong UV absorbance ~280, 320 and shoulder around 325nm with absorbance tailing off 360nm. The *P. robustissimum* samples had strong absorbance at 280nm but then slowly declining absorbance between 300-430nm. It did not show the features present in *P. quadrangulare* Figures 4.19-4.21. Examination of the fractions under 366nm UV light and the results of ferric chloride and gelatine tests confirmed that there were differences between the phytochemistry of the species, Tables 4.17-4.18.

These preliminary results indicated there were different suites of compound in the digests obtained from the two *Phoradendron* species. There were strong indications that plant peptides and phenolics were potential important contributors to the mistletoe phytochemistry. Section 1.8.4, Tables 1.12-1.13 provide a brief introduction of plant phenolics, reported biological functions and known occurrence in mistletoes.

## 4.23.4 Extended Phytochemical Screening

### 4.23.4.1 Analytical Considerations phytochemical screening

The range of tests performed were those widely used in preliminary phytochemical screening and many have a long history of use. This permitted a wide range of results to be obtained on a very small amount of aqueous digest. The same batch of digest was then analysed by UV and used for microbiology testing and HPLC. This provided a coherent set of results for each sample. The tests were performed on the aqueous acidic digests in all cases as the aim was to determine the nature of the phytochemicals likely to be present in the *Ateles* digestive tract.

UV spectroscopy is usually applied to purified isolated individual compounds. The complexity of the digests and sample size made traditional extraction/isolation inappropriate. The use of UV absorbance profile together with shift reagent responses was found to provide a reliable reproducible stable trait for individual species enabling it to be used for comparisons (Martin 1970, Tosserams *et al.* 1997, Hespanhol *et al.* 2014). Accepting the limitations created by UV analysis of complex digests it was possible to distinguish between the two *Phoradendron* species. The use of the shift reagents and the fluorescence behaviour again suggested a significant presence of plant polyphenols and these were different in the two species. There were also detectable host related differences in condensed (CT) and hydrolysable tannins (HT). The common tests specific for CT and HT are not suitable for complex mixtures containing other phenolic compounds (Porter 1989b, Schofield *et al.* 2001, Dai *et al.* 2010).

The use of HPLC analysis of whole plant digests without any attempt at isolation of individual constituents is now widely accepted. Chromatographic fingerprint techniques are used to characterise marker compounds and unknown compounds in such complex systems. This can be combined with chemometric analysis (Gan *et al.* 2006). Chromatographic finger prints are used as a standard method in quality and consistency of botanical products by US Food and Drug Administration (US Food and Drug Administration 2004), European Medicines Evaluation Agency (EMEA) and the State Food and Drug Administration of China (Zhao *et al.* 2011).

HPLC-UV fingerprinting and subsequent chemometric statistical analyses were successfully applied to *Viscum coloratum,* resulting in identification and *t*he use of 18 common peaks in quality control of 37 samples. The use of this approach for comparing herbal extracts has been extensively reviewed (Tistaert *et al.* 2011, Gad *et al.* 2013, Goodarzi *et al.* 2013). The author was unable to undertake the chemometric statistical analysis; however there were obvious visual similarities and/or differences between the chromatograms of the two *Phoradendron* species.

The use of the UV-DAD detector for the HPLC analysis on the fractions obtained from the SPE sample clean up procedure enabled the author to generate UV spectra for individual peaks. This data again suggested that classes of plant phenolics were the major constituents and differences between aqueous digests of the two *Phoradendron* species. This is consistent with Pietrzak *et al.* (2013) who recently reported extracts prepared with water-polar solvent mixtures displayed the highest polyphenols (TPC) and flavonoids (TFC) and antioxidant activity, while organic polar solvents were the least efficient extractants.

A literature review provided a list of potential plant phenolic candidate compounds which had been detected in mistletoes and which had properties consistent with the phytochemical screening and HPLC

analyses (Table 4.14). Subsequent HPLC analysis was able to identify the principal difference between the *Phoradendron* species as chlorogenic acid. Chlorogenic acid was confirmed by accurate mass determination by LC-TOF-MS.

Examination of the mass spectral fragmentation patterns for two further peaks, only identified in *P. quadrangulare,* suggested the potential presence of chlorogenic acid isomers, catechins and gallocatechins, procyanidin dimers and trimers, 3- or 4-O-p-coumarylquinic acids and an apigenin-7 glycoside (Tables 4.37 and 4.38).

#### 4.23.4.2 Dietary considerations polyphenols

There is a large body of literature on the health benefits of dietary polyphenols e.g. antiproliferative and anticancer (Xiao *et al.* 2011), as antioxidants (Morel *et al.* 1994, van Acker *et al.* 1998, Pietta 2000, Heim *et al.* 2002), anti inflammatory (Korkina *et al.* 2011) and cardio protective functions (Wen-Feng *et al.* 2006, Testai *et al.* 2013). In order to place these results in context the literature was reviewed for such compounds and activities in the fruits or closely related species consumed by *Ateles*. The pulp of *Maclura tinctoria* has been analysed and was found to contain antioxidant chalcone glycosides and flavanones (Cioffi *et al.* 2003) and 4 flavonoids, one prenylated flavonoid and four prenylated isoflavones (de Oliveira Oyama *et al.* 2013). Identification of specific compounds was limited; most data only quantified antioxidant activity (Table 4.43).

The TEAC (Trolox equivalent antioxidant capacity) assay is a spectrophotometric technique based on scavenging of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical anions (ABTS−). It is however susceptible to solvent effects and so direct comparisons are only possible when standard conditions have been used (van den Berg *et al.* 1999). The treatment of the source and juice extraction also affect constituents and assay results (Fischer *et al.* 2013). The blue/green ABTS+ chromophore radical is produced through the reaction between ABTS and potassium persulfate. In the presence of an antioxidant the ABTS+ radical changes from blue/green to colourless depending on antioxidant capacity of components. The dark colour of the *Phoradendron robustissimum* digests in particular did not permit observation of the colour changes to enable the author to measure antioxidant activity using this technique.

Table 4.43 Measured Trolox Equivalent Antioxidant capacity for fruit pulp

| **fruit pulp** | **TEAC/100g** | **reference** |
|---|---|---|
| *Spondias mombin* | 175 | Tiburski *et al.* (2011) |
| *Spondia purpurea* | 368 | Murillo *et al.* (2012) |
| *Annona muricata* | 975 | |
| *Eugenia uniflora* | 275 | |
| *Genipta americana* | 78 | |
| *Manilkara zapota* | 87 | |

Flavonoid anti-oxidant activity is thought to be related to metal ion chelation and free radical scavenging abilities, with greatest activity seen in the flavonols. From a structural point of view the presence of a 3'OH or adjacent 3',4' OH and 2-3 double bond in the flavonoid molecule gave the highest activity (Hendrich 2006).

Flavonoid anti-oxidant activity is increased in the presence of carotenoids and ascorbic acid (Hendrich 2006). *Spondias mombin* contains β, α and γ-carotene which exert pro-vitamin A activity. 100g portion of the fruit provides 37% RDI for human adult. The presence of carotenoids and anthocyanidins antioxidants in *Ateles*

diet is inferred by the colours of the fruits at time of consumption. Hiramatsu *et al.* (2008) reported the colours of 33 species of immature fruit consumed by *Ateles* at Santa Rosa. At time of consumption 20/33 examples were no longer green, the colours ranging from white→yellow→orange→red→purple. The colour charges associated with ripening are due to a reduction in chlorophyll and an increase in carotenoids and anthocyanidins. There are both qualitative and quantitative changes in antioxidant capacity associated with ripening and colour change (Gould *et al.* 2008). Carotenoids are responsible for the red, orange, and yellow colours of fruits, vegetables, fungi, flowers, plumage and fish. *In vivo* and *in vitro* studies have shown that carotenoid photo protective role is related to its antioxidant activity or with modulation of other cellular antioxidants (Delgado-Vargas *et al.* 2000). Anthocyanins are responsible for many of the attractive colours, from scarlet to blue, of flowers, fruits, leaves, and storage organs e.g. purple carrot. Apigeninidin is a widely distributed example which has a yellow colour. Anthocyanins have similar function to many of the other flavonoids i.e. antioxidant, photo protection.

This data from a limited number of diet species suggests that the *Ateles* diet is not deficient in antioxidant capacity when there are a suitable variety of fruits available.

#### 4.23.4.3 Dietary considerations chlorogenic acid 3-(3,4-dihydroxycinnamoyl)quinic acid

Chlorogenic acid (3-(3,4-dihydroxycinnamoyl)quinic acid) was identified as the principal difference between the species. Examination of 22 tropical fruits consumed in Brazil found that 5-caffeoylquinic acid was the major chlorogenic acid present in most of the tropical fruits studied and was generally accompanied by small amounts of 4-caffeoylquinic acid and 3,4-dicaffeoylquinic acid and 3,5-dicaffeoylquinic acid (Pontes *et al.* 2002). An unspecified chlorogenic acid was identified in *Manilkara chicle* fruits (Mathew *et al.* 1969).

As with the flavonoids the *Ateles* would not appear to have a requirement for chlorogenic acid as an additional anti-oxidant.

### 4.23.5 Biological activity Testing

#### 4.23.5.1 Analytical considerations microbiological testing

The difficulties encountered with selecting a suitable method have been reported briefly in Section 4.7.2. There are other problems associated with the use of dilution and microdilution microbiological methods in extract testing. In the microdilution method the pH of the medium may be changed by the addition of the acidic extract, thus having an impact on bacterial growth giving a false indication of activity. The inoculum level needs to be sufficient that changes in turbidity can be measured, growth below $10^5$ CFU will not register (Valgas *et al.* 2007, Reller *et al.* 2009). The accuracy of spectrophotometric readings may be hampered by (i) additives or antibacterial compounds that affect the spectral characteristics of growth media, (ii) the aggregation of bacteria, or (iii) bacterial pigments (Eloff 1998). Organic solvent extracts may precipitate when diluted in aqueous agar solutions and the pH of such solutions may induce changes in the structure of flavonoids e.g. galangin at alkaline pH (Cushnie *et al.* 2005). Klancnik *et al.* (2010) suggested that microdilution is appropriate for fast screening but that it should be accompanied by serial dilution and plating (at set time points) to provide accurate numbers of CFU.

Testing of flavonoid and flavan-3-ol compounds has further limitations. These compounds cause whole bacterial cells to aggregate leading to an artificially low estimation of viable CFU. The reduced surface area and

oxygen consumption would also give a false result with reagents such as tetrazolium salts used as colorimetric indicators of viability of aerobic bacteria (Cushnie *et al.* 2011).

Agar diffusion methods require standardised inoculum levels to enable comparisons to be made. The ZI is proportional to the growth rate of the organism and the differential diffusion rates of active compounds in the extract. Difficulties may occur when comparing a range of compounds that have large differences in molecular size/shape which will determine the rate of diffusion so affecting the size of the ZI achieved in the set time. Valgas *et al.* (2007) compared activity of natural products from various sources using four different techniques. The well diffusion method was more sensitive than the disc method and concluded this was because the method provided more suitable conditions for the bacterial growth.

The generation of fractions from digests and the subsequent testing may give false negatives where the activity of the whole digest is due to synergistic effect of components which may act in different ways.

#### 4.23.5.2 Antibacterial properties of polyphenolic compounds

Table 4.44 highlights the anti-infective properties of flavonoid compounds on wide range potential infective organisms.

Table 4.44 Examples of Anti infective properties of flavonoid compounds

| **polyphenolic** | **organisms** | **Reference** |
|---|---|---|
| flavonoids | animal viruses, *S. aureus, E. coli, Bacillus* spp. cariogenic *Streptococci* and oral bacteria | Bohm (1999) |
| | *E. coli, P. aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter baumannii, S. aureus, B. subtilis, Enterococcus faecalis, Candida albicans* | Ozçelik *et al.* (2006) |
| | *E. coli, K. pneumoniae, Salmonella typhi, Enterococcus faecalis, P. aeruginosa , Shigella flexinerrii, S. aureus , Shigella sonneii* | Gautam *et al.* (2012) |
| | anti fungal *Trichophyton mentagrophytes, Cryptococcus neoformans* | Sathiamoorthya *et al.* (2007) |
| | anthelmintic | da Silva *et al.*(2008) |
| | *Strongyloides stercoralis* | El-Sherbini *et al.* (2013) |
| | *Trypanosoma cruzi* | Marin *et al.* (Marin *et al.* 2011) |
| | antitrypanosomal and antileishmanial | Tasdemir *et al.* (2006a) |
| chalcones | anti cariogenic *Streptococci* and oral bacteria | Sato *et al.* (1997) |
| | anti fungal | El Sohly *et al.* (2001) |
| prenylated flavonoids | antiplasmodial, antitrypanosomal | Bourjot *et al.* (2010) |
| flavan-3-ols | anti microbial and anti viral | Aron *et al.* (2008) |
| | *Trichostrongylus colubriformis* | Kerboeuf *et al.* (2008) |
| flavanone | Methicillin-Resistant *Staphylococcus aureus.* (MRSA) and vancomycin resistant *Enterococcus faecium* | Cushnie *et al.* (2011) |
| (-)-epigallocatechin | *Proteus vulgaris, S. aureus* | Shinozuka *et al.* (1988) |
| | methicillin-resistant *Staphylococcus aureus. (MRSA)* | Hatano *et al.* (2005) |
| chlorogenic acid | *Stenotrophomonas maltophilia* | Karunanidhi *et al.*(2013) |
| | *S. aureus, Streptococcus pneumoniae, B. subtilis, E. coli, Shigella dysenteriae,Salmonella typhimurium* | Lou *et al.* (2011) Li *et al. (2014b)* |
| | *E. coli* | Kabir *et al.* (2014) |
| phenylpropanoid glycosides | respiratory syncytial virus. | Kernan *et al.*(1998) |

There has been considerable research into the mechanisms responsible for the antibacterial activities of flavonoids. These include (Hendrich 2006, Cushnie *et al.* 2011):

- cytoplasmic membrane damage due to perforation or changes in membrane fluidity (flavonol,

flavan-3-ol). Membrane property changes also affect ion channels and this may be related to changes in efflux of $K^+$ and $Ca^{2+}$ induced by some flavonoids.

- disruption of the cell membrane will affect transport of solutes into the cell causing various consequences e.g. synthesis of DNA, peptidoglycan. Inhibition of ATP-driven membrane transport proteins such as ABCB1 which is responsible for elimination of potentially toxic molecules from the cell would allow toxic agents to accumulate in the cell. This mechanism is thought to be responsible for increased sensitivity to antibiotics and chemotherapeutic agents.
- inhibition of cell wall synthesis and cell membrane synthesis (inhibition of various enzyme systems)
- inhibition of DNA synthesis due to topoisomerase inhibition/dihyrofolate reductase (flavan-3-ol, isoflavones) e.g. apigenin in *E. coli* (Collin *et al.* 2011)
- Inhibition of enzyme DNA gyrase will trigger apoptosis and lysis
- inhibition of energy metabolism by inhibition of NADH-cytochrome c reductase (flavone, flavon-3-ol, flavone)

Other flavonoid/phenylpropanoid effects limit the establishment of infections. Flavonoids including procyanidins inhibit the action of Gram –ve *P. aeruginosa* released signal molecules. Signal molecules initiate the formation of biofilms (Vandeputte *et al.* 2010, Trentin *et al.* 2013) and (−)-epicatechin gallate disrupts biofilm formation in *S. aureus* (Shah *et al.* 2008). Chlorogenic acid reduced biofilm formation and acid production by bacterial species responsible for dental caries *Streptococcus mutans* and *S. sanguinis* (Ferrazzano *et al.* 2009). *In vivo* anticaries activity has been shown by cranberry procyanidins, cocoa extracts procyanidins, and propolis apigenin (a flavone) (Gazzani *et al.* 2012). The prevention of bacteria adhesion to epithelial surfaces and hard surfaces e.g. catheters as a potential mechanism of infection control is an expanding area of research (Bavington *et al.* 2005).

Toxins play an important role in bacterial pathogenesis, an often fatal disease long after the bacteria themselves have been killed. Choi *et al.* (2007) demonstrated that polymerised catechin flavonoids neutralises the effect of *S. aureus* α-toxin both *in vitro* and *in vivo.* Delehanty *et al.* (2007) have shown that polymers of catechin and epicatechin neutralise endotoxin lipopolysaccharide (LPS) from multiple species. Endotoxin lipopolysaccharide (LPS) is an integral structural component of the outer membrane of Gram-ve bacteria. LPS is released from the bacteria during cell division and cell death. Flavonoids blocked the interaction between LPS and its receptors and the development of septic shock. (−)-Epicatechin gallate prevents the secretion of α toxin by *S. aureus (*Shah *et al.* 2008*)* as does chlorogenic acid (Li *et al.* 2014b).

Chlorogenic acid has a high level of activity against two important pathogens Gram +ve *Streptococcus pneumoniae* and Gram –ve *Shigella dysenteriae.* The outer membrane of Gram –ve organisms is a mixture of lipopolysaccharides and proteins stabilised by the presence of divalent cations. Chlorogenic acid is thought to bind to the outer membrane and chelate $Mg^{2+}$ ions depolarising the membrane, disrupting barrier function and increasing permeability (Lou *et al.* 2011). Large effluxes of $K^+$ ions and nucleotide leakage were observed with both bacteria. This increased permeability would be potentially synergistic with other antimicrobial compounds. *In vivo* antibacterial activity has recently been reported (Yusuf *et al.* 2013).

The majority of polyphenols appear to be released from plant material in the stomach e.g. 65% of total

apple phenolics and flavonoids were released in the stomach, and 10% further in the small intestine. Absorption of orally administered chlorogenic acid has been measured as 33-50% (Olthof *et al.* 2001).

Enzymatic activity in the intestine generates a range of phenolic acids depending upon the original molecules of which about 75% are available for absorption. Further microbiological fermentation decreases the bioavailable fraction of the original polyphenols. This may produce more bioactive metabolites e.g. those with catechol structures (Pietta 2000, Bohn 2014). In humans the metabolism of procyanidins to phenolic acids by colonic microflora in anoxic conditions takes approx. 48 hours (Déprez *et al.* 2000) Therefore due to the rapid transit time in *Ateles* procyanidin metabolism is likely to be less extensive due to the much reduced residence time in the colon.

Procyanidin dimers B1 (epicatechin-(4β⟶8)-catechin) (Sano *et al.* 2003) and B2 (epicatechin-(4β-8)-epicatechin) have been shown to be absorbed in humans (Holt *et al.* 2002). Recent studies suggest, however, that only low-molecular-weight oligomers with degree of polymerisation <3 are absorbed intact in the gastrointestinal tract. Investigation of the stability of procyanidins found that procyanidins are not degraded under the acidic conditions of the stomach *in vivo* and procyanidins reach the small intestine intact and would be available for local activity or absorption (Rios *et al.* 2002). Procyanidin dimers and trimers have potentially been identified by the author in the mass spectral analysis of *P. quadrangulare* samples and also in *P. liga (Varela et al. 2004)* and an Argentinian mistletoe *Ligaria cuneifolia* (Wagner *et al.* 1998). Procyanidins are reported to improve endothelial function, reduce platelet aggregation, prevent atherosclerosis and have cardio protective properties (Rasmussen *et al.* 2005).

There is also the possibility of beneficial local activity. Pathogenic bacteria cultured with the tea phenolics, epicatechin, catechin, showed reduced growth of bacteria such as *Clostridium perfringens, Clostridium difficile* and *Bacteroides* spp. with little effect on commensal anaerobes like *Bifidobacterium* and *Lactobacillus* (Lee *et al.* 2006).

## 4.23.6 Potential pathogens of *Ateles*

Habitat fragmentation caused by human intervention may create the circumstances for higher risks of parasite infections (Chapman *et al.* 2005, Gillespie *et al.* 2006) due to reduced ability to exploit variation in routes chosen and concentration at reduced resource. Reduced habitat increases population density and greater contact with conspecifics and potential for increased exposure to parasites. Changes in host social interactions, such as aggregation at fruiting trees, will increase the potential for both intra and interspecies contact, which may influence infection rates. In Santa Rosa the wet season has the highest abundance of fruit availability (Asensio *et al.* 2009) with both *Ateles geoffroyi* and capuchin *(Cebus capucinus)* using the same fruit trees such as F*icus* spp. (Di Fiore *et al.* 2008, Parr *et al.* 2011). Parasite transmission from fruit eating bats to primates including humans has been reported in Africa (Leroy *et al.* 2004, Leroy *et al.* 2009). *Carollia perspicillata,* a species of fruit eating bats found at Santa Rosa, is known to eat *Ficus* (Charles-Dominique 1991*)* one of the species of fruits regularly consumed by *A. geoffroyi. Carollia perspicillata i*s reported as a potential reservoir of *Leishmania chagasi* (Lima *et al.* 2008).

Primates have been considered significant as a source of zoonotic pathogens (Gillespie *et al.* 2006, Davies *et al.* 2008). Recently there have been confirmed reports of transmission of potential pathogens from humans and domestic animals to primates (Graczyk *et al.* 2002, Gillespie *et al.* 2008, Pedersen *et al.* 2009,

Tegner 2013) with reports of transmission causing the decline of endangered species (Nizeyi *et al.* 2002, Kaur *et al.* 2008, Köndgen *et al.* 2008).

Host physiology may also play a significant role in susceptibility. In males elevated testosterone levels and increased immunosuppressive hormones have been found to be associated with breeding (Hosseini *et al.* 2004). Male infection loads are often reported as higher than females (Zuk *et al.* 1996, MacIntosh *et al.* 2010, Guerra-Silveira *et al.* 2013). Placental mammal females lower their immune responses during pregnancy to protect the foetus (Robinson *et al.* 2012). The severity of diseases caused by inflammatory responses e.g. autoimmune disease is reduced and the severity of diseases that are mitigated by inflammatory responses e.g., infection is increased during pregnancy (Robinson *et al.* 2012). Births and juveniles also represent a potential source of new uninfected individuals for parasite colonisation.

The incidence of parasite infections and infectious diseases is highly seasonal (Nelson *et al.* 1996, Cattadori *et al.* 2005, Altizer *et al.* 2006). Parasite prevalence is dependent upon several different mechanisms related to parasite life history e.g. type and life history of vectors. Arthropod vectors are sensitive to temperature and humidity and parasite susceptibility to desiccation may reduce incidences in dry season. Trejo-Macais *et al.* (2012) reported higher prevalence of wet seasonality in infection with *Entamoeba* sp., *Nematoda* sp., *T. minutus* and detection of Ascaridae eggs but *Trichuris trichiura* and *Strongyloides fuelleborni* showed no seasonality.

## 4.23.7 Anti infective activity of identified constituent classes

### 4.23.7.1 Anti-infective properties of lectins

Lectins consist of two non-identical peptide chains (A and B) joined by a disulphide bond, with a net positive charge. The B-chain binds to a sugar/the receptor on the cell surface then crosses the cell wall by endocytosis to enable the A-chain to reach the cytosol. Once in the cytoplasm the A-chain binds to a nucleotide of the 28S rRNA of the ribosomes causing inactivation of the ribosomes. ML display individual differences in biological activity. ML3 is the most active followed by ML2 and ML1 in the induction of apoptotic cell death of cultured human lymphocytes (Pevzner *et al.* 2004). Hajto *et al.* (1989) reported that the carbohydrate-binding B-chain of MLI is responsible for the stimulation of natural killer (NK)-cell activity *in vivo* while the A-chain appeared to be completely inactive at the same concentration since it has no capacity of selectively binding to cellular receptor glycoproteins. The proportion of ML present will therefore have an impact on the antibacterial activity according to the type of bacterial specificity.

Biological activity of mistletoe lectins was introduced Section 1.8.2, Table 1.10. Lectins have direct antibacterial activity, (Gaidamashvili *et al.* 2002, Oliveira *et al.* 2008) due to disruption of the bacterial cell wall. The higher activity of lectins for Gram +ve organisms is thought to be due to the multiple layers of peptidoglycan in Gram +ve wall providing more binding opportunities. Gram-ve bacteria have an outer lipopolysaccharide membrane outside of the peptidoglycan layer. This forms a barrier to binding. This may be part of the mechanism seen in the microbiology results of the tested digests where greater activity was seen against Gram +ve organisms (Section 4.17.2 Tables 4.30(a-c)).

The reported anti-viral activity is through inhibition of protein synthesis in the infected cell, due to ribosome inactivation in the infected cell, denaturing viral RNA (Stirpe 2005, Puri *et al.* 2012). Damage to rRNA causes an effect called ribotoxic stress which results in the activation of cell signalling involved in further

antiviral responses (Laskin *et al.* 2002). Ribotoxic stress has been also been linked to the toxicity of ricin (Suntres *et al.* 2005).

There are examples in the literature of lectin activity against other important types of organisms. The trophozoites stage of *E. histolytica* and *E. invadens* encyst before exiting the body and this involves binding to mucins or terminal galactose on a glycoprotein, (Eichinger 2001, Boettner *et al.* 2002). The embryonic development stage of the parasitic worm *Onchocerca volvulus* binds strongly to N-acetyl-D-galactosamine specific lectins. Mistletoe lectins could potentially compete with this binding process, so reducing the numbers of *Entamoeba* spp. shedding cysts and also preventing the development of the adult form of *Onchocerca volvulus.*

#### 4.23.7.2 Anti-infective properties of phoratoxin and AMPs

Biological activity of mistletoe cationic peptides (thionins) was introduced in Table 1.11. The presence of phoratoxin was inferred from data supplied to the author as previously stated. To illustrate the potential difficulties imposed by sample size Schaller *et al.* (1996) extracted viscotoxins from 12kg/ dried leaf of *Viscum album* grown and achieved yields of total viscotoxin 0.016-0.23mg/g, with qualitative difference in the concentrations of the VA1, VA2 and VA3 from each host tree. Thionins were the first antimicrobial peptides to be isolated from plants. The thionins belong to a much larger important group the Antimicrobial Peptides, AMPs.

All plant AMPs isolated so far contain 4, 6, or 8 cysteines which are all connected by disulfide bridges, thus providing high stability to the peptides. Based on homologies at the primary structure level, plant antimicrobial peptides can be classified into distinct families including thionins, plant defensins, lipid transfer proteins, and hevein- and knottin-type antimicrobial peptides (Egorov *et al.* 2012). Induction of genes responsible for the production of mistletoe and other thionins has been reported as a response to infection of the leaves by microbial pathogens (Broekaert *et al.* 1997). AMPs are toxic towards both Gram+ve and Gram-ve bacteria, fungi, yeast, and various mammalian cell types. Antimicrobial peptides kill susceptible bacteria *in vitro* at concentrations ranging from 0.25 to 4 µg/ml (Hancock *et al.* 1998).

"*Antimicrobial peptides (AMPs) are evolutionarily conserved molecules involved in the defence mechanisms of a wide range of organisms. Produced in bacteria, insects, plants and vertebrates, AMPs protect against a broad array of infectious agents. In mammals these peptides protect against bacteria, viruses, fungi, and certain parasites. Recently, novel biologic effects of AMPs have been documented such as endotoxin neutralization, chemotactic and immunomodulating activities, induction of angiogenesis and wound repair. Thus these ancestral molecules are crucial components of the innate immune system and attractive candidates for novel therapeutic approaches*" (Guaní-Guerra *et al.* 2010).

AMPs, including thionins, exhibit differential binding properties and hence toxicity to bacteria and multicellular animals. This is due to the nature of the bacterial outer layer of the bi-layer. This consists of large amount of lipids with negatively charged head groups of phospholipids and lipopolysaccharides (LPS) which bind electrostatically and hydrophobically to the thionin. In multicellular animals the outer layer has no net charge, the charged head groups are concentrated on the inner surface of the bi-layer facing into the cytoplasm (Zasloff 2002). Resistance to AMPs in general is seen in species such as *Serratia* which have a very low density of negatively charged binding sites in the outer layer (Zasloff 2002).

The high net positive charge of thionins makes them very soluble in water >300mg/ml (Stec *et al.* 2004). Mistletoe thionins bind to phospholipids and lipopolysaccharides (LPS) in outer membrane of Gram-ve and teichoic acid in the peptidoglycan wall of Gram+ bacteria. Thionins also interact with plasma membrane lipids through specific receptors at the surface of the cell (Stec *et al.* 2004). Consequently, peptide binding to the membrane can activate several pathways that will cause cell death, by the formation of membrane pores, resulting in leakage of ions and metabolites, depolarisation and interruption of the respiration process, biopolymer synthesis and cell death (Barbosa Pelegrini *et al.* 2011). Plant thionin compounds have inhibitory activity against bacteria and leishmanicidal activity (Castro *et al.* 2005, Berrocal-Lobo *et al.* 2009) and against the following human pathogenic fungi *Saccharomyces cerevisiae, C. albicans,* and *C. tropicalis* and reduce the growth rate in *E. coli* and *P. aeruginosa* (Taveira *et al.* 2014). Aqueous extracts of viscotoxin have shown activity against human parainfluenza virus type 2 (Karagoz *et al.* 2003).

#### 4.23.7.3 Anti infective properties of phenolics

Flavonoid activity against protozoan parasites has been widely investigated and flavonoid activity is thought be due to multiple mechanisms (Tasdemir *et al.* 2006a, Tasdemir *et al.* 2006b, Kerboeuf *et al.* 2008). The mechanisms thought to be significant include host-parasite interactions due to inhibition of attachment, and disruption of parasite metabolism (Tasdemir *et al.* 2006b) or maturation (Min *et al.* 2003, Iqbal *et al.* 2007). Flavonoid and condensed-tannin containing plant materials have been used traditionally as vermifuges against cestodes and trematodes (Athanasiadou *et al.* 2001, Kerboeuf *et al.* 2008).

The most commonly documented antiparasitic effect of CT (proanthocyanidins) is a reduction in faecal egg count and female nematode fecundity (Brunet *et al.* 2006, Novobilsky *et al.* 2011). Low levels of CT (< 6% dry matter) has had positive effects associated with an increased proportion of dietary protein reaching the intestine (Molan *et al.* 2000, Molan *et al.* 2002, Hoste *et al.* 2006, Waghorn 2008). High protein intake has been associated with increased immunocompetence, weight gain and milk production (Hoste *et al.* 2006). CTs have been shown to have a direct effect on nematode parasites (Athanasiadou *et al.* 2001). Signalling from the Decay-Accelerating Factor - DAF-2/insulin receptor to the DAF-16/FOXO transcription factor controls longevity, metabolism, and development (Lee *et al.* 2003, Tullet *et al.* 2014). Apigenin inhibits larval growth of *Caenorhabditis elegans* by activating DAF-16 which in turn inhibits larval growth (Kawasaki *et al.* 2010). Herbivore selection of varied dietary items would be less difficult than for a frugivore with a reliance on a more highly seasonal availability of fruits. Tropical fruit trees may exhibit mast fruiting years; this is the synchronous intermittent production of large seed crops in perennial plants (Janzen 1967), leading to a degree of uncertainty in available resources.

The multiple classes of compounds likely to be present in the *Phoradendron* digests have activities in many areas and together there is the potential for synergistic activities. It is not necessary for there to be an overwhelming action from one compound; it is more likely that activity would arise from attack against *Ateles* pathogens at many different functional levels.

## 4.23.8 Lectins and mistletoe thionins as immune stimulants

Hajto *et al.* (1989) extracted the β-galactoside-specific mistletoe lectin (ML 1) from a proprietary extract of mistletoe (Iscador®) that has US federal approval for clinical application. The lectin (ML 1) was administered

to immunological compromised rabbits and was monitored *in vivo*. Injections of nontoxic doses of the purified lectin (0.25–1.0 ng/kg) into rabbits yielded significant increases in natural killer (NK) cell cytotoxicity, numbers of large granular lymphocytes, and increased phagocytic activity of granulocytes. The changes in these parameters were also determined in cancer patients after extract (Iscador®) injection both s.c. and i.v. emphasizing the potential relevance of the lectin (Hajto *et al.* 1989). Iscador® and a second product Lektinol® (standardized for mistletoe lectin-1) has been found to increase release of tumour necrosis factor (TNF)-α (Hajto *et al.* 1990, Boneberg *et al.* 2001). TNF-α is involved in many situations: antitumour activity, immune modulation, inflammation, septic shock, viral replication and haematopoiesis. TNF-α is mainly produced by macrophages in response to immunological challenges such as bacteria (lipopolysaccharides), viruses, parasites, mitogens and other cytokines.

*In vitro* tests using murine thymocytes (the precursors of T-cells) at low doses equivalent to 0.8ng/kg body weight purified ML1 stimulated T cell differentiation and maturation but at higher levels equivalent to 100ng/kg body weight the effect caused was cytostatic/apoptosis. ML1 prepared from aqueous mistletoe extracts was shown to have identical effect *in vivo* tests (Hajto *et al.* 2003). These effects occurred at levels well below the ML1, $LD_{50}$ of 5µg/kg by injection (Pusztai *et al.* 1998). The dose of ML1 required to stimulate the immune response is 1ng /ml and is lower than the dose required to exert a cytotoxic effect (Nehmann *et al.* 2009). There have been several reports recently on pre-clinical trials or clinical trial reports of the use of plant lectins including those from several mistletoes (Liu *et al.* 2010, Bar-Sela *et al.* 2013, Longhi *et al.* 2014, Weissenstein *et al.* 2014).

Reports examining the outcome of a total of more than 2000 incidences of accidental intake of mistletoe have shown that there is no medical evidence suggestive of toxicity in humans (Hall *et al.* 1986, Spiller *et al.* 1996, Krenzelok *et al.* 1997). Many lectins resist digestion, survive gut passage, and bind to gastrointestinal cells and/or enter the circulation intact, maintaining full biological activity (de Mejia *et al.* 2005). Pryme *et al.* (2007) has reviewed the effects on the immune system and the successful use of ML1 in an oral form as an immunomodulatory stimulant for treating cancers.

Intestinal epithelial cells (IECs) respond to GI parasites by physical and biochemical responses; these include adaptations to maintain barrier function, secretion of mucin and polysaccharide-rich glycocalyx. IECs express several types of antimicrobial peptides including defensins. ICEs also influence adaptive and innate immune cell function, including tumour-necrosis factor (TNF) and T cell α-chemoattractant (Artis *et al.* 2008). This type of activation in response to bacteria and viruses has been reviewed by (Abreu *et al.* 2005, Kelly *et al.* 2005, Takeuchi *et al.* 2006). TNF stimulation is a property of mistletoe lectins (Pusztai A. *et al.* 1995, Pusztai *et al.* 1998, Boneberg *et al.* 2001, Fernandez *et al.* 2003, Huber *et al.* 2006).

Defensins are small, cationic peptides that are composed of three structural subclasses, α-, β-, and θ-defensins, differentiated by the spacing and pairing of the six disulfide-bonded cysteines, θ-defensins are the only known cyclic protein motif expressed in animals. Mammalian defensins exhibit strong viral-neutralizing activities by directly interacting with viral envelope proteins (Hazrati *et al.* 2006, Doss *et al.* 2009). θ-Defensins were first identified in neutrophils and monocytes of the *Rhesus* monkey. There are three Rhesus θ-defensins, RDT1-3 which show activities against *E. coli, S. aureus,* and *C. albicans* (Tran *et al.* 2008), fungi, Human Immunodeficiency Virus Type 1 and herpes simplex virus (Garcia *et al.* 2008). θ-defensins directly interact with specific viral receptors on the host cell, antagonizing viral attachment, entry, or intracellular shuttling (Cole *et al.*

2002, Yasin *et al.* 2004, Gallo *et al.* 2006). A phylogenetic survey revealed the existence of intact θ-defensin genes in Old World monkeys and two apes the siamang and orangutan, but humans, chimpanzees and gorillas do not produce θ-defensin peptides nor do New World monkeys or prosimians (Nguyen *et al.* 2003, Li *et al.* 2014a).

The respiratory epithelium is the largest surface of the human body in contact with the external medium, exposed to a large number and type of pathogens. An increased susceptibility to respiratory infections due to a dysfunction of the epithelial barrier and/or low levels of AMPs has been hypothesised (Guaní-Guerra *et al.* 2010). Arnett *et al.* (2011) showed θ-defensin and other AMPs interact with macrophages to control intracellular proliferation of infecting bacteria and hypothesised that

"*at the site of infection defensins and possibly other AMPs and macrophages play an important role in the innate immune defences*".

A similar activity of θ-defensin was reported for macrophages (Welkos *et al.* 2011) and neutrophils (Tongaonkar *et al.* 2011). Natural killer (NK) cells are traditionally considered part of tumour surveillance and viral defence, but recently a role in bacterial infections, particularly those caused by enteric pathogens, has been reported. Infection with an enteric pathogen showed that NK cells were recruited to the GI mucosa and secreted a wide range of immune-modulatory factors and reduced bacterial loads (Hall *et al.* 2013).

Mistletoe thionins also have immunomodulatory activity (Elluru *et al.* 2006, de Souza Cândido *et al.* 2014). Viscotoxins exert a strong immunomodulatory effects on human granulocytes (Stein *et al.* 1999) and activate NK cells (Tabiasco *et al.* 2002). Mistletoe thionins are active at levels of 1-100 ng/ml, induce apoptosis of activated neutrophils (Lavastre *et al.* 2004) and human lymphocytes (Bussing *et al.* 1999) stimulate the production of IgG antibodies (Klein *et al.* 2002) and have activity against solid tumours (Johansson *et al.* 2003, Stan *et al.* 2013).

The θ-defensin deficiency may impact upon the innate abilities of deficient New World primates including *Ateles geoffroyi* to respond to viral and bacterial infections leaving them with an increased dependence upon stimulation of immune response by flavonoids and lectins and the activity of plant AMPs such as mistletoe thionins.

### 4.23.9 Further potential physiological effects of lectins and chlorogenic acid

Administration of a low oral dose of mistletoe lectin to young rats caused hypertrophy of the pancreas, lungs and small intestine, increased circulation of TNFα and reduced body fat (Pusztai *et al.* 1998). Local effects included increased turnover and loss of gut epithelial cells, damage to the luminal membranes of the epithelium, interference with nutrient digestion and absorption, stimulated shifts in the bacterial flora and modulated the immune state of the digestive tract (Vasconcelos *et al.* 2004).

Chlorogenic acid has been linked to many different potential health benefits. e.g. anti-oxidant (Kono *et al.* 1998) anti-hypertensive (Watanabe *et al.* 2006, Mubarak *et al.* 2012), anti fungal (Sung *et al.* 2010), anti bacterial and anti viral (Hemaiswarya *et al.* 2011, Lou *et al.* 2011, Karunanidhi *et al.* 2013), immune system stimulant (Graus *et al.* 2003) and cardio vascular protection (Bonita *et al.* 2007).

Chlorogenic acid has been shown to facilitate maintenance of the intestinal barrier (Ruan *et al.* 2014). An intact intestinal mucosal barrier is essential for preventing both gut-related diseases and ensuring adequate provision of dietary nutrients. Chlorogenic acid has been reported to induce human lymphocytes and human

peripheral blood leukocytes to produce IFN-c and IFN-a and enhanced levels of immunoglobulins IgE, IgG, and IL-4 *in vivo* (Ruan *et al.* 2014). Chlorogenic acid has been shown to regulate the intestinal mucosal immune function, intestinal flora and exert antioxidant activities against ischemia and reperfusion injury in the rat small intestine (Sato *et al.* 2011). Recently Ruan *et al.* (2014) showed that dietary supplementation with chlorogenic acid improves intestinal structure and metabolic function, decreases intestinal mucosal damage and enhanced intestinal mucosal integrity.

Chlorogenic acid has been undergoing clinical trials for the treatment of diabetes, (U.S. National Institutes of Health, Identifier: NCT01523028 (Jan 2012). Chlorogenic acid prevents glucose release from the liver by inhibiting glucose-6-phosphatase (Hemmerle *et al.* 1997). This enzyme is involved in glycogenolysis (the breakdown of glycogen polymers into glucose) and gluconeogenesis (the synthesis of glucose in the liver from non-carbohydrate substrates). The effects of inhibiting this enzyme by lowering release from the liver are reduced blood glucose levels and this potentially has an important function in preventing overproduction of glucose in the liver. The effect on the enzyme is dose dependent with 1mM (354µg/ml) chlorogenic acid causing a 40% reduction in activity. This is considered important for treating Type 2 diabetes (Bassoli *et al.* 2008). A similar concentration has been shown to inhibit glucose uptake in the intestine, preventing rapid rises in blood sugar reducing glucose transport by 80% (Bassoli *et al.* 2008). Administration of a commercial product containing ≥45% chlorogenic acid to insulin resistant fat-fed, diabetes induced mice had no effect on body mass, but improved glucose clearance during glucose tolerance tests. The same product was seen to reverse the depressed brain mitochondrial respiration rates caused by the high fat diet (Ho *et al.* 2012) by induction of genes involved in mitochondrial respiration. Chlorogenic acid also increases levels of glucagon-like-peptide-1 (McCarty 2005). The antioxidant activity reduces oxidative stress and reduces production of N-nitroso compounds in the GI tract, a known factor in development of Type-2 diabetes, whilst the metal chelation properties reduce Mg levels in the liver which improves glucose tolerance (Van Dam *et al.* 2002, Tunnicliffe *et al.* 2008).

Other reports of chlorogenic acid potential antidiabetic effects include (Johnston *et al.* 2003, Thom 2007, Bassoli *et al.* 2008, Ho *et al.* 2012, Ong *et al.* 2013). Chlorogenic acid is present in the leaves of *Cecropia obtusifolia* and *Cecropia peltata. A* cold aqueous infusion of the leaves when consumed over the course of a day improved glycaemic control. This was achieved by blocking the hepatic glucose output (Nicasio *et al.* 2005, Andrade-Cetto *et al.* 2010). Chlorogenic acid also rapidly lowers blood pressure (Mubarak *et al.* 2012) and influences lipid levels (Onunogbo *et al.* 2012).

There are specific instances in the literature of folk medicine of the use of *V. album* extracts in diabetes and reports of its effect in diabetic models (Eno *et al.* 2008, Adaramoye *et al.* 2012, Onunogbo *et al.* 2012). Variations in glycaemic effects due to the effect of seasonality and host tree have also been demonstrated (Osadebe *et al.* 2004, Orhan *et al.* 2005, Osadebe *et al.* 2010). Anti-diabetic effects have also been shown for *P. tomentosum* in STZ-diabetic rats (Calzado-Flores *et al.* 2002, Careaga-Olivares *et al.* 2006). Several *Phoradendron* species are used as anti-diabetic treatments in herbal repertoires *P. longifolium, P. bolleanum* and *P. serotinum* in Mexico, Andrade-Cetto *et al.* 2005) and *P. piperoides* in Venezuela (Rodríguez *et al.*, 2008).

Such widespread reports of activity to regulate blood sugar levels may be an important property of *Phoradendron* spp. for a frugivore such as *Ateles.*

### 4.23.10 Summary of the findings and Conclusions

- At the levels detected in combination with the amounts of *P. quadrangulare* consumed there is no evidence for use as a mineral/micro-mineral supplement.
- The preliminary screening indicated a lack of detectable alkaloids and the presence of mistletoe lectins, saponins and polyphenolic compounds
- There were detectable phytochemical differences between the eaten and non-eaten *Phoradendron* species particularly in the HT and CT signatures
- There were also host tree related differences in UV signatures
- There was limited adsorption of *P. robustissimum* constituents by geophagy material
- There was no detectable adsorption of P. quadrangulare constituents by geophagy material and there was a potential increase in constituents released from the mistletoe leaf. This was confirmed by an increase in peak area of the principal peaks in the HPLC analysis of the combined digests
- There were differences in the antibacterial activity with *P. quadrangulare* having higher activity against *B. subtilis* and *P. robustissimum* no measurable activity against *S. aureus*
- HPLC analyses showed host tree related difference in both species and between *P. quadrangulare and P. robustissimum.* The major difference was a compound eluting RT ~19min, this was later shown to have the same retention time and UV-DAD spectra as a chlorogenic acid standard
- LCMS analyses identified the principal component RT ~19 mins as having the same fragmentation pattern as the chlorogenic acid standard, 3-(3,4-dihydroxycinnamoyl)quinic acid.
- MS fragmentation patterns also suggested the presence of chlorgenic acid isomers, procyanidins and p-coumarylquinic acids, possibly an apigenin-7-O-glycoside and caffeoylshikimic acids.
- Antibacterial activity was not related to the estimated levels of chlorogenic acid
- There was no discernible pattern to the eating of P. quadrangulare based on the estimated chlorogenic acid content. Consumption of Sample 226 with a relatively low chlorogenic acid content (eaten by 21 individuals) was rapidly followed by geophagy.

## In conclusion:

There were detectable differences in the phytochemical signatures of the two *Phoradendron* spp. available for consumption. The classes of compounds identified suggest the digests may have multiple potential anti-oxidant, antimicrobial, antiparasitic and immunomodulatory activity. There is the potential for both localised GI and systemic activity. It is probable that consumption is linked to the multiple potential synergistic activists related to changes in anti-oxidant demand or changes required in immune responses. These would be individual responses to both internal and external factors.

A potential hypothesis for the infrequency of observed geophagy may be suggested by the proximity of geophagy following consumption of Sample 226. Consumption may be linked to the taste modifying properties of chlorogenic acid. There are several patents registered for the use of chlorogenic acid to modify astringent or off tastes e.g. *(2012) US Patent Application No: 2012/0189, 750 -Taste Modifiers Comprising a Chlorogenic Acid*. The low level of chlorogenic acid in Sample 226 may not mask bitter or astringent tastes of the other leaf constituents, making leaves less palatable and hence the geophagy response.

# Chapter 5 Conclusions and Future work

## 5.1 Introduction

The three specific aims for this project were:

**Specific Aim 1** To determine a possible function for the consumption of the geophageous material

**Specific Aim 2** To determine whether differences in phytochemistry influence the selection of the *Phoradendron* species consumed and if such a selection suggests a possible function for *Phoradendron* consumption.

**Specific Aim 3** To determine whether there is any interaction when geophagy follows *Phoradendron* consumption.

The results of the individual objectives are presented and how they relate to the stated aims.

**Specific Aim 1** To determine a possible function for the consumption of the geophageous material

**Objective 1** To identify any clay or other minerals present in the samples using Munsell© colour characteristics, determination of Water content, Loss on Ignition (LOI), X-ray Diffraction spectroscopy (XRD), X-Ray Fluorescence (XRF), Infra Red spectroscopy (IR), and the pH of sample material.

### Mineral characterisation

The results of Colour characterisation, Water content and Loss on Ignition are presented in Sections 3.5.1 -3.5.3. The colour characterisation Section 3.5.1 was consistent with the findings of (Alvarado *et al.* 2004). The tuffs and ignimbrites adjacent to Rincón de la Vieja are described (Alvarado *et al.* 2004) as having angular-sub angular grainy material of variable composition derived from the underlying rocks. The reported material contained red-grey pumice fragments and lithic fragments in a fine-coarse sand matrix. Ash material was dark to light grey containing plagioclase fragments up to mm size. The material sampled by the *Ateles* was from the uppermost layer of the 'mesa'. Zamora *et al*. (2004) describe the upper 1.5m layer of the ignimbrite plateau as pumice 12%, plagioclase 5%, quartz 5% including magnetite crystals < 3mm in size. Magnetite was indicated by the XRD results. The results indicated that colours of Santa Rosa geophagy samples were markedly different from those in the bulk of the published material and that they were unlikely to contain iron. The low water content indicated a lack of hydrated minerals. The Santa Rosa Samples LOI results did not suggest that there was any organic carbon present such as plant root or fungal hyphae as has been seen in other literature reports. The LOI also indicated that there was unlikely to be significant amounts of inorganic carbonates or sulphates present.

XRD, IR and XRF techniques were used to determine the mineral characteristics of the geophagy samples. The results are presented in Sections 3.5.4-3.6.6. The results of the XRD analyses suggested a high degree of homogeneity across the sites sampled (Table 3.21) and this was confirmed by statistical tests. The XRF Total alkali/silica (TAS) classification showed the material to be an ignimbrite, acidic in nature and derived from rocks with andesite-dacite composition. This is consistent with the findings of (Chiesa *et al.* 1987, Chiesa

1993, Hannah *et al.* 2002, Vogel *et al.* 2004). The results indicated that the minerals identified from the multiple peak matches are consistent with material from volcanic origin or weathering of volcanic material (Wada *et al.* 1985, Wada 1987).

The XRD and IR results suggest that, if present, kaolinite mineral is only at a low percentage and that there was unlikely to be any montmorillonite present. The absence of montmorillonite may be attributed to the particular physical and environmental conditions at the sites. Smectites/montmorillonites commonly result from the weathering of basic rocks. The formation of montmorillonites are favoured by level or gently sloping terrains which are poorly drained (Deer 2001). Poor drainage is necessary to prevent the leaching of cations during the formation process. The terrain of the sample sites is not gently sloping or poorly drained. This combination of climate and condition do not favour the formation of smecties/montmorillonite. Island Arc activity and hydrothermal conditions at Santa Rosa (Chiesa 1993) are the type of conditions under which kaolinites are usually formed.

### pH determination

The acidic nature of the material in suspension was confirmed by the pH determination, Results Section 3.5.7. The majority of the Santa Rosa samples had a pH <5.2. This would provide conditions in which there would be exchangeable $Al^{3+}$ ions and, if present, possibly high levels of soluble manganese both of which are potentially toxic. The literature values for pH of geophagy material Section 3.6.4, Table 3.37 showed considerable variation from acidic pH 5.01 to alkaline pH10.1, there were however more sites with pH in the acidic range.

**Objective 2.** To develop a suitable *Ateles* gastric model in order to investigate the behaviour of geophagy samples in physiological conditions.

When compared to the human the transit time of *Ateles* was a major factor in choosing to develop a new model system. The use of such a model would provide information about the physical behaviour of geophagy material in *Ateles* GI conditions; in particular pH may influence the behaviour of colloidal and clay materials. The results indicated that the geophagy samples did not behave in a similar manner to either of the reference clays in simulated GI conditions. This observed behaviour (Section 3.5.8.1) impacts on the use of geophagy material for several of the functional hypotheses e.g. the ability to adsorb water/toxins in mitigation of symptoms of diarrhoea.

**Objective 3.** To identify the physico-chemical characteristics of the geophagy material using Laser Diffraction Particle size analysis, Ultra Violet Spectrophotometry (UV) and Inductively Coupled Plasma - Mass Spectrometry (ICP-MS).

The results for the particle size analysis are presented in Section3.4.8. The 17- 26% content of particles >500mm in the *Ateles* eaten samples differed from the other sites analysed and from much of the published work which had high level of clay sized particles, Section 3.63 Table 3.34. The behaviour of the samples in gastric media did not resemble that of either of the kaolin or montmorillonite reference clays. This is further evidence of the lack of both kaolinite and montmorillonite in any appreciable amounts.

The results of the UV analysis of the adsorption of test PSM compounds in simulated gastric media are presented in Section 3.4.10. The site samples consistently adsorbed less mg/g than the montmorillonite CRM. The kaolinite CRM adsorbed more ephedrine than the geophagy samples but the results were variable for the other test compounds. There were also differences between the sites (Table 3.30). The acid pH and the relatively low values for particles <31µm in the *Ateles* chosen sites are both important factors in the ability to absorb PSM.

The ICP-MS results are presented in Section 3.5.10. Only seven of the detected elements met LOQ values determined; these were potassium, magnesium and calcium which varied significantly between sites but aluminium, barium, manganese and zinc did not vary significantly between sites.

**Objective 4**. To test the gastric digests for antibacterial activity against a range of bacterial species.

The results are reported in Section 3.5.11. No antibacterial activity was observed with either direct contact with geophagy material or aqueous leachates.

5.1.1 **Conclusions relating to Specific Aim 1** - To determine a possible function for the consumption of the geophageous material

### Detoxification of PSM

The conclusion reached was that the results suggest that the Santa Rosa geophagy samples would be of potential use in reducing exposure to PSM to varying degrees according to class of PSM and constituents in the samples. The amounts of the test compounds adsorbed by as little as 25mg of the montmorillonite CRM suggests that any geophagy sites with relatively small amounts of a montmorillonite would be very efficient in adsorption of potential toxic PSM but potentially beneficial molecules would also be affected. The highest adsorption was seen with gallic acid, tannic acid and naringin and binding may be related to the number of free hydroxyl groups present in these molecules. The classes of PSM detected in the *Phoradendron* digests had poor adsorption to the geophagy samples and it is therefore not possible to support this hypothesis.

### Mineral supplementation

The conclusion reached was that the quantities released from Santa Rosa samples digested in acidic aqueous conditions do not support the nutrient supplementation hypothesis.

### Anti-infective and Antiparasitic activity

Direct antiparasitic activity was not undertaken as this would have required the use of live animals. No bacterial or fungal organisms were cultured from the geophagy samples. They would not therefore be a source of antimicrobial compounds as has been found for samples from termite mounds (Ketch *et al.* 2001). The large sized particles may physically irritate the mucosal lining resulting in increased gastric secretion disrupting biofilm formation and also impair parasite attachment so reducing infection load and its health impacts. It was not possible to reject this hypothesis.

The limitations imposed by the relatively small amounts of kaolinite minerals together with the lack of any montmorillonite minerals are discussed Section 3.6.7. The samples present little opportunity for the adsorption of water and so reducing watery diarrhoea.

The samples may however reduce the impact of bacterial toxins. Whilst not undertaken, there is much literature relating to the mitigation of other classes of toxins e.g. bacterial endotoxins (Brouillard *et al.* 1989), the mycotoxins aflatoxin (Phillips 1999) and zearalenone toxin (Sprynskyy *et al.* 2012) and the toxic lectin ricin (Jaynes *et al.* 2005). Ricin and mistletoe lectins have similar structures, belonging to the same class of compounds. This may be an important property. Gastro-intestinal symptoms may also be related to dietary changes. The possibility of this was noted in the discussion as clay minerals and clay sized particles can adsorb free fatty acids (Theng 2012).

The conclusion reached was that the samples do not have swelling clay properties and due to the limited amount of kaolinite minerals present they are unlikely to have a direct effect on osmotic diarrhoea. They may through interaction with organic acids and potential toxins/irritants have an indirect effect. The presence of Al, Ba, Ca and Mg in the samples may, despite the acidic nature of the extracts, have limited antacid properties.

Non-adaptive hypotheses - Only the use of geophagy as a famine food could be addressed by this work. This hypothesis cannot be supported. This is not as a result of any determination but as a result of the recorded observations occurring at times of high fruit availability.

In conclusion it was not possible to identify with any degree of certainty a possible function for the geophagy in *Ateles* at Santa Rosa. Geophagy is not related to food scarcity and it occurs in a frugivorous primate at a time of abundant fruit. The apparent seasonality and its relatively rare occurrence suggest it may be a response to an uncommon set of circumstances and have varying functions e.g. an abrasive effect due to the proportion of coarse particles present, limiting iron availability to gut parasites or reducing the effects of fructose-induced gastro-intestinal disturbances. There was no consistent pattern relating to mineral supplementation seen in the primate literature (Section 3.6.8) suggesting that mineral requirement, as a driver of geophagy, may be facultative and site or diet specific. Data for frugivorous bats reported that they visited geophagy sites more than insect eating species (Voigt *et al.* 2007). Bravo *et al*. (2012) related geophagy to diet (in frugivorous bats) and the low sodium content of *Ficus* species. Geophagy was linked to higher site use by pregnant females of material with a high sodium content with omnivorous *Chacma* baboons (Pebsworth *et al.* 2012(b)). There are also reports of higher numbers of pregnant or lactating frugivorous bats visiting geophagy sites with high calcium content (Bravo *et al.* 2008, Voigt *et al.* 2008, Ghanem *et al.* 2013).

The results highlight how problematic it is to deconvolute geophagy to a single function; however it is unlikely that mineral supplementation or detoxification of PSM is the drivers at Santa Rosa. The limited observations of an apparently deliberate behavioural change still suggest that there is a possible function.

**Specific Aim 2** To determine whether differences in their phytochemistry influence the selection of the *Phoradendron* species consumed and if such a selection suggests a possible function for *Phoradendron* consumption.

**Objective 5.** To determine the mineral content of the *Phoradendron* leaf using XRF. To identify the

phytochemical characteristics of digests of the eaten and non-eaten *Phoradendron* species produced from the gastric model. This will be achieved using Ultra Violet Spectrophotometry (UV) and standard phytochemical screening of the gastric digests and Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC). Microbiological testing is used as a proxy for biological activity. The gastric digests are to be tested against a variety of bacterial organisms.

### Mineral content

The results of the XRF analysis of minerals detected in dried *Phoradendron* leaf are presented in Section 4.14. Higher silicon was observed in the thicker and physically more robust *P. robustissimum* samples. This contrast can be seen in the images in Appendix Table 1.2(b). K, Na, Ca and S were higher in the *P. quadrangulare* samples at mg/g levels. P, Mg, Mn, Zn, Cu and Fe, when detected in either *Phoradendron*, were at mcg/g dried leaf.

### Analysis of gastric digests

During the period of method development several issues arose relating to the analysis of the simulated gastric extracts. The limiting factors which restricted the use of the simulated gastric fluid have been reported in the relevant methods/results sections. These limitations relating to analytical methods were the interference with UV determination due to the biological constituents i.e. mucin and enzymes. The viscosity of the digests and salting out of electrolytes created problems in RP-HPLC, LC-MS and ICP-MS equipment. Additionally the biological constituents present acted as an additional source of nutrition to the bacteria. Literature reviews had been conducted indicating that the mistletoe lectins and thionins were successfully extracted in acidic aqueous media, with a similar pH to that of the simulated gastric media. The decision was taken to use a simplified pH2 adjusted, deionised water as the digestion media.

### Determination of lectin content

Results are presented in Sections 4.15.2-4.15.3. Inhibition of agglutination indicated the presence of more than one ML with different affinities in *P. quadrangulare* samples. The response of the *P. robustissimum* samples suggested ML presence but possibly at much lower levels. There are documented variations in ML content dependent upon species, host tree and age of the leaf. These factors need to be taken into account in any future comparisons.

### Preliminary Phytochemical screening of *Phoradendron* digests

The results of the preliminary screening of acidic aqueous digests are presented in Sections 4.13.3 and 4.15.1-4.15.4. The screening tests indicated the presence of phenolic compounds, saponins and did not detect alkaloids. Digests when observed under UV light indicated the presence of several classes of flavonoid/ phenolic classes.

### UV and HPLC characteristics of acidic aqueous digests

The UV results are presented in Sections 4.16.1-4.16.2. The author was aware of the problems of comparing UV spectra of the potentially complex mixtures of the aqueous extracts; however there was a very

obvious difference in the spectra obtained. *P. robustissimum* samples had a different absorbance trace between 300-360nm, and did not show the peak/shoulder at ~325nm present in *P. quadrangulare* digests. There were also different responses to the shift reagents used.

The HPLC-DAD results are presented in Section 4.18.1. The results supported the basic UV conclusion. In addition the HPLC chromatograms indicated that there was one compound which was not detected in *P. robustissimum* Section 4.18.2 and present in large quantities in *P. quadrangulare* (Section 4.18.3). Subsequently the probable identity of this compound was determined (Section 4.19). The constituent was found to have the same HPLC retention time and DAD-UV spectra as the reference material 3-(3,4-dihydroxycinnamoyl)quinic acid,(chlorogenic acid). This was subsequently confirmed by LC-MS determination of the accurate mass (Section 4.19). Further examination of the mass spectra of other peaks eluting at different retention times suggested the presence of other isomeric forms of chlorogenic acid. These elute with different retention times. The spectra also indicated the presence of procyanidin dimers, trimers, p-coumaroylquinic acids and possibly an apigenin-7-O-glycoside. This is consistent with publications relating to other species of *Phoradendron*.

### Antibacterial activity

The results are presented in Sections 4.17.1-2 and 4.20.2. Antibacterial activity was undertaken as part of the screening as a proxy for biological activity. It was not possible to determine which bacterial or viral pathogens the *Ateles* may be exposed to. However opportunistically collected faecal samples were examined on behalf of the author for parasites. Parasite identification and pathophysiology are reported in Section 4.22 and 4.22.6 respectively.

Antibacterial activity of the digests was greatest against Gram +ve organisms but was different for the two *Phoradendron* species. None of the *P. robustissimum* samples exhibited activity against *S. aureus* and only weak activity against *B. subtilis*. *P. quadrangulare* had greater activity against *S. aureus* than *B. subtilis*.

5.1.2 **Conclusions relating to Specific Aim 2**-To determine whether differences in their phytochemistry influences the selection of the Phoradendron species consumed and if such a selection suggests a possible function for Phoradendron consumption.

There were differences in mineral content between the eaten and non-eaten *Phoradendron* species. The results are discussed in Section 4.21.1.1 in relation to dietary fruits and other mistletoe leaves. The small mass of mistletoe consumed by the *Ateles* was unlikely to make an important contribution to mineral or micronutrient intake. The consumption at a time of plentiful fruit is a situation where a general deficiency is less probable. It was not possible to support the mineral/micronutrient supplementation hypothesis. However *Phoradendron* consumption may be a further example of a facultative response as previously suggested by Best *et al*. (2013) for geophagy.

The results indicated that it was possible to differentiate both in simple screening tests and in more detailed analyses between the *P. quadrangulare* eaten mistletoe and the *P. robustissimum* non-eaten mistletoe. There were *Phoradendron* species differences in hydrolysable and condensed tannins and host tree induced differences in phytochemistry. A potential difference was the probable presence of 5-OH flavones/flavonols, 7-glycosides and phenylpropanoids in *P. quadrangulare* and the lack of evidence for

significant levels these compounds in *P. robustissimum* (from the shift reagent work).The single most obvious major difference was the presence of the chlorogenic acid.

There was no discernable pattern to the eating of the *P. quadrangulare* leaf based on the estimated chlorogenic acid. There was no discernable seasonal pattern to the chlorogenic acid content, however the levels may relate to mistletoe or host tree phenology. It was therefore not possible to link consumption to this single major difference in constituents.

The classes of compounds suggested as being present and those tentatively identified are regularly linked to antioxidant activity in the literature. The potential antioxidant properties were examined in the context of similar compounds present in dietary items (Section 4.22.4.1-4.22.4.2). It was concluded that there was a wide range of antioxidant activity in the dietary items but there may be seasonal variations related to fruit availability which may suggest mistletoe consumption is a facultative response. Many of theses classes of compounds have also been identified as having anti-infective properties (Sections 4.23.4.5 and 4.23.7.1-4.23.7.3). Chlorogenic acid has both antioxidant activity (Kono *et al.* 1998, Sato *et al.* 2011) and activity which may limit the establishment of infections (Ferrazzano *et al.* 2009, Lou *et al.* 2011, Karunanidhi *et al.* 2013, Kabir *et al.* 2014, Li *et al.* 2014).

In addition to direct anti-infective properties many of the compounds potentially identified have immuno-stimulant activity particularly the thionins and lectins. This is detailed in Sections 4.23.8-4.23.9. Chlorogenic acid also functions as an immune modulator (Graus *et al.* 2003). Alternative possible important physiological properties related to lectins and chlorogenic acid in relation to blood glucose and lipid levels are detailed in Section 4.24. Mistletoe preparations are used in herbal medicine in both Europe and South America for treating Type-2 diabetes.

In conclusion there were many possible complementary synergistic mechanisms which may limit establishment of infection, limit extent of infection load or eliminate infective organisms. This may also involve stimulation of an immune response. *Phoradendron* use may in this situation be a candidate for prophylactic medicinal use, in facilitating responses in the face of increased physiological challenge. That is a facultative response in a similar manner to that suggested for geophagy.

**Specific Aim 3** To determine whether there is any interaction when geophagy follows *Phoradendron* consumption.

**Objective 6.** To analyse the phytochemistry and biological activity of a combined Phoradendron and geophagy digest from the *Ateles* gastric model. The combined digests will be analysed using UV, RP-HPLC and subjected to microbiological testing.

Initial observations of *Phoradendron* consumption had been linked by the observers to geophagy. This observation together with the general perception of the toxicity of mistletoes lent itself to the detoxification hypothesis as a function for geophagy.

### UV analysis

Results of UV analysis are presented in Section 4.16.2. When samples of *P. quadrangulare* were digested with geophagy samples the UV spectra of the extracts had conflicting responses. Results of UV

analysis are presented in Section 4.16.2. There were instances of a both an increase and a decrease in UV absorbance at the $\lambda_{max}$ ~ 280nm suggesting there may be increases in constituents released from the leaf or adsorption. Results are presented in Section 4.16.2. An increased absorbance may be due to the abrasive effect of geophagy sample on the leaf. Any abrasive action may facilitate the release of compounds contributing to the absorbance. A reduction in UV absorbance $\lambda_{max}$ was observed for *P. robustissimum* sample suggesting adsorption of a constituent by the geophagy material.

HPLC analysis

In order to explore these potential changes examples of *P. quadrangulare* combined digests were analysed by HPLC. The results for Sample 152/153 Section 4.18.4, Table 4.34 showed an increase in mean peak area (detected at 280 and 325nm) for 4 major peaks at RT 13.459, 16.513, 19.007 and 23.178mins. In order to validate this response, leaf from Sample 178 was prepared and mixed then divided into 6 equal portions, to produce as homogeneous samples as possible. Three portions were digested alone and three with the geophagy sample. The mean peak areas were calculated from 5 samples injections for each extract. The results in Table 4.35 show similar and consistent increases for each of the 4 peak areas determined at 325nm measured.

Antibacterial activity

No direct antibacterial activity was detected for the geophagy samples or for geophagy sample leachate (Section 4.17.3). *Phoradendron* leaf was digested in the presence of geophagy material and the extract tested against the screening panel of organisms. Following the screening results extracts were tested further with *B. subtilis/ S. aureus.* Whilst there was some indication of increased ZI, the results were not statistically significant.

**5.1.3 Conclusions relating to Specific Aim 3** - To determine whether there is any interaction when geophagy follows *Phoradendron* consumption.

In conclusion combined digestion appears to have mixed effects on the release of UV absorbing compounds. The difference in response between the two *Phoradendron* spp. may be due to changes in the proportions and/or nature of the constituents in the different species. The compound/s released absorbing at 325nm may/may not have been responsible for the increased ZI. This release may have been insufficient to generate a statistically significant increase in the measured ZI.

The initial interlinking of *Phoradendron* consumption and geophagy is not confirmed by later observations of multiple instances of *Phoradendron* consumption without geophagy being observed. The results from the combined digests do not indicate detoxification of *Phoradendron* constituents as a specific beneficial function linking these behaviours.

## 5.2 Facultative responses

Environmental factors such as temperature, nutrient availability and the abundance of predators combined with the unpredictability of such factors affect life history strategies of an animal. Much of the *Ateles*

habitat is tropical rain forest, below 800m (Collins 2008). *Ateles geoffroyi* at Santa Rosa differ from the majority of populations in that they have a very seasonal habitat compared to the Amazonian tropical species and this has behavioural consequences (Gonzalez-Zamora *et al.* 2011).

Cannon (1929) coined the term 'homeostasis' to encompass the physiological processes required to maintain an organism at a 'steady state' and this was later expanded by Richter (Richter (1936) in Villalba *et al* 2007). Richter suggested that adaptations in behaviour may be a response to internal and external circumstances which cause deviation from 'steady state'. This has led to the idea that animals select from their environment substances which will aid or restore homeostasis.

*"Nutritional state is assessed through systemic nutrient-sensing mechanisms and hormonal feedbacks from body reserves. Integration of information about food composition and nutritional state occurs both at the periphery, by nutrient-specific modulation of taste receptors and more centrally as signals from systemic and peripheral sources converge onto the neural circuitry that controls feeding behaviour. Learning also plays a role, and in some cases animals are able to associate their current nutritional state with food cues previously associated with particular nutrients. Post ingestive regulatory responses assist in rebalancing an imbalanced nutrient intake*" (p. 353 in Simpson *et al*. 2012).

The Santa Rosa National Park established in 1971 is part of a regeneration area. Prior to establishment, areas had been used for cattle farming and planted with African grasses which were burnt annually (Fedigan *et al.* 2012). The park contains small forest areas and areas currently undergoing regeneration. This affects both the species present and their areas of distribution (Fedigan *et al*. 1998). Any differences in the variety of nutrients available due to the restricted diet may lead to unusual but local solutions to a dietary requirement. This may be the case with the geophagic behaviour and that of *Phoradendron* consumption.

A facultative response may also arise as the result of a change in bacterial/parasite load due to season or exposure. Gonzalez-Moreno *et al*. reported (2013) a higher number of faecal samples contained *Cryptosporidium* spp. during the rainy season. Zoonotic transmission of *Cryptosporidium parvum* (Nizeyi *et al.* 2002) and *Gardia duodenalis* (Graczyk *et al.* 2002) have been reported for park rangers and gorilla in Uganda.

Seasonality may also bring about other challenges. The incidence of parasite infections and infectious diseases is highly seasonal (Altizer *et al* .2006). Seasonal variations in rainfall may alter infection rates, during wetter periods there may be more breeding sites for vectors and infective stages of other parasites, such as intestinal parasites, survive longer outside of the host or vector (Altizer *et al*. 2007). Changes in host social interactions, such as aggregation at fruiting trees, will increase the potential for both intra and interspecies contact, which may influence infection rates. At Santa Rosa birth seasonality has been reported coinciding with the start of the wet season, during May and July (Chapman *et al* 1990). Births represent a new potential source of host for parasite colonisation.

The sensory system involves olfactory and taste receptors and visceral receptors which respond to nutrients/toxins and stimulate the central nervous system about the consequences of ingestion of the item. An example of such behavioural adaptation has been reported in butterflies who use self- medication to benefit their offspring (Lefevre *et al.* 2010) and also increased consumption of pyrrolizidine alkaloids PSM by caterpillars (Singer *et al.* 2009) in response to parasite infection. However the consumption of pyrrolizidine alkaloids whilst reducing parasite load also reduced growth rate, Singer suggests that *"this kind of fitness trade off can select for behavioural plasticity"* (Singer *et al.* 2009).

An example of a probable physiologically driven facultative response can be seen in the behaviour of two species of captive *Ateles*. There are two species of spider monkey at Twycross Zoo, both fed the same fresh fruit/vegetable diet and chow pellets. *Ateles paniscus* has been seen exhibiting geophagy whilst geophagy has not been seen with *Ateles fusciceps* (Personal communication (2014) Zak Showell, Research & Conservation Registrar, World Primate Centre, Twycross Zoo, Warwickshire, UK).

## 5.3 Future work related to geophagy

There are several research questions for which it is necessary to maintain observations at Santa Rosa. These include confirming that if, as it appears at present, geophagy is relatively uncommon and unlike observations in other populations of *Ateles* (Blake *et al.* 2010, Link *et al.* 2011a). Are there other potential sources e.g. wet/salido sites within the ranges of the *Ateles* present in the park where currently *Ateles* use has not been observed?

Is it possible to link geophagy to any other dietary item or change in diet as they become available? Also is there any obvious age/sex demographic bias? Both of these may suggest a facultative response.

More extensive analyses of a larger number of samples are required to confirm the particle size distribution. Further adsorption work should target compounds such as procyanidins, catechin, epicatechin and apigenin glycosides and also extend the combined digestion work. The analyses should be extended to include cation exchange capacity. Rather than attempt to measure antibacterial activity does the inclusion of geophagy material in liquid antibacterial cultures have any impact on growth rate? This may limit infection load to a tolerable level.

## 5.3 Future work related to *Phoradendron*

Observations should extend to the identification of *Phoradendron* plants on the appropriate hosts outside the National Park which could be sampled to provide sufficient material for extended examination. Extend if possible observations to include mature leaf material eaten by *Ateles* at sister site in Yucatan, Mexico. Include *Phoradendron* and other mistletoe species currently used or under investigation for clinical benefits e.g. diabetes. The collection of a single or multiple larger samples of *Phoradendron* would enable analyses to be extended. This would permit the preparation of aqueous *Phoradendron* digests for ICP-MS determination of minerals potentially released in the stomach and intestine. HPLC chromatography combined with Chemometric analysis should be attempted for both species of *Phoradendron* and other **mature** leaf consumed by *Ateles* identified at Santa Rosa or in Mexico. The larger sample size would permit confirmation of the presence of specific phoratoxins and mistletoe lectins using Polyacrylamide gel electrophoresis (PAGE). The investigation of antioxidant activity of digests could also be undertaken e.g. (ORAC) oxygen radical absorbance capacity and free radical scavenging ability e.g. DPPH free radical scavenging or ABTS free radical scavenging ability.

It may also be possible to investigate of the possibility of a local collaboration with a Veterinary parasitologist to test extracts against known *Ateles* GI parasites.

*Ateles* have high levels of taste discrimination for bitter tastants (Laska *et al*. 2009) and amino acids (Larsson *et al.* 2014). Further work relating to the taste modifying properties of chlorogenic acid in relation to bitter astringent compounds and the five amino acids rejected in (Larsson *et al.* 2014) may suggest a further hypothesis for both consumption of *Phoradendron* and the juxtaposition of geophagy.

# Appendices

# Appendix 1.1 Taxonomy of mistletoe and host trees

Table 1 Taxonomy of host trees and images of host leaf. (Missouri Botanical Garden. 01 Jan 2013 <http://www.tropicos.org).

| | *Tabebuia ochracea* (Cham.) Stand |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Lamiales Bromhead |
| Family | Bignoniaceae Juss. |
| Genus | Tabebuia Gomes ex DC. |

*Tabebuia ochracea* leaf

| | *Luehea speciosa* Willd. |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Malvales Juss. |
| Family | Malvaceae Juss. |
| Genus | Luehea Willd. |

*Luehea speciosa* leaf

| | *Manilkara chicle* (Pittier) Gilly |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Ericales Bercht. & J. Pres |
| Family | Sapotaceae Juss. |
| Genus | Manilkara Adans. |

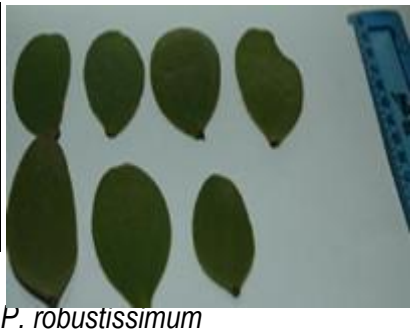
*Manilkara chicle* leaf

| | *Guazuma ulmifolia* Lam. |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Malvales Juss. |
| Family | Malvaceae Juss. |
| Genus | Guazuma Mill. |

*Guazuma ulmifolia* leaf

Table 1 (Cont.) Taxonomy of mistletoe species (Missouri Botanical Garden. 01 Jan 2013 <http://www.tropicos.org).Images of samples when collected.

| | *Phoradendron robustissimum* Eichler |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Santalales R. Br. |
| Family | Santalaceae R. Br. |
| Genus | Phoradendron Nutt. |



*P. robustissimum*
Collected from host tree *T. ochracea*

| | *Phoradendron robustissimum* Eichler |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Santalales R. Br. |
| Family | Santalaceae R. Br. |
| Genus | Phoradendron Nutt. |

*P. robustissimum*
Collected from host tree *L. speciosa*

| | *Phoradendron quadrangulare* (Kunth) Griseb |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Santalales R. Br. |
| Family | Santalaceae R. Br. |
| Genus | Phoradendron Nutt. |
| | |

*P. quadrangulare* collected
*from host tree M. chicle*

| | *Phoradendron quadrangulare* (Kunth) Griseb |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht. |
| Order | Santalales R. Br. |


*P. quadrangulare* collected from host
*G. ulmifolia.*

# Appendix 1.2 Geological terminology

Definitions from: McGraw-Hill Dictionary of Geology & Mineralogy, 2nd Edition[1], (2003). Dolphin reference Book, Dictionary of Geological Terms[2], (1962).

allocthonous[1] – of rocks whose primary constituents have not been formed in situ

andesite[2] - is a fine-grained, extrusive igneous rock composed mainly of plagioclase with other minerals such as hornblende, pyroxene and biotite. Andesite lava is of moderate viscosity and forms thick lava flows and domes.

autocthonous[2] - rocks of which dominant constituents formed in situ

dacite - is an felsic extrusive igneous rock, often light grey and composed of 63-68% SiO2, often found associated with andesite, and forms lava flows, dikes. The principle minerals are plagioclase, quartz, pyroxene or hornblende. (http://vulcan.wr.usgs.gov accessed 13 Oct 2014).

epiclastic[2] – mechanically deposited sediments (gravel, sand, mud) the weathered products of older rocks. Volcanic epiclasts are derived from erosion of volcanoes or ancient volcanic terrains.

felsic[2] - describes silicate minerals, magmas, and rocks which have a lower percentage of the heavier elements, and are high in the lighter elements, such as silicon, aluminium, and potassium. Common felsic minerals include quartz, muscovite mica, and the orthoclase feldspars. Granite is the most common felsic rock.

fluvio-lacustrine[2] – deposits laid down by lakes or rivers

hyaloclastic – the quenching and fragmentation of basaltic lavas due to contact with cool sea or lake waters. Hyaloclastites are fragments which are equant, angular shards of black glass, generally between 0.25 and 2.0 cm in diameter (http://www.geology.sdsu.edu accessed 13 Oct 2014).

Ignimbrite eruptions- Each ignimbrite flow unit has a basal layer finer grained than the body of the ignimbrite, with a ground surge deposit commonly underlying the ignimbrite, and a fine ash-fall deposit commonly overlying it. These two types of deposit, although not an integral part of the ignimbrite, are produced by the same eruptive act, and they and the ignimbrite constitute the several and varied products of a Peléan-phase eruption. Volcanic eruptions in which ignimbrite is generated show the following sequence of events so often that it may be regarded as the normal one: (1) a highly explosive, often Plinian, phase, producing a pumice-fall deposit; (2) a Peléan phase; and (3) an effusive phase, producing a lava flow. This sequence is believed to represent the tapping of progressively deeper levels in the magma chamber and the escape during the eruption of magma of progressively lower gas content.(Sparks *et al.* 1973).

ignimbrite - is a predominantly pumice pyroclastic flow deposit formed from the cooling of pyroclastic flow material ejected from an explosive volcanic eruption. As the pyroclastic material settles it can build up thick layers, and ignimbrites of reasonable thickness may be found 10-100 kilometres from the site of eruption (http://flexiblelearning.auckland.ac.nz/rocks_minerals accessed 13 Oct 2014).

island arcs - according to prevailing theory, island arcs are formed where two lithospheric plates (enormous rigid slabs that constitute segments of the Earth's surface) converge. Upon colliding, one of the plates—that bearing heavy, oceanic crust—buckles downward and is forced into the partially molten lower mantle beneath the second plate with lighter, continental crust. An island arc is built up from the surface of the overriding plate by the extrusion of basalts and andesites. The basalts are thought to be derived from the

semi-molten mantle, whereas the andesites are probably generated by the partial melting of the descending plate and the sediments that have accumulated on its surface. (www.britannica.com accessed Oct 2014).

lithic fragment The dense or crystalline components of a pyroclastic deposit. Three types are recognized. Cognate lithics are fragments of non-vesiculated, juvenile, magmatic material, e.g. dense, angular, glass fragments. Accessory lithics are fragments of country rocks ejected explosively during eruption. Accidental lithics are clasts picked up locally by pyroclastic flows and surges. Lithics range from blocks to ash-size fragments and are usually angular, but may be rounded by in-vent abrasion during eruption (Sparks et al. 1973)

mafic[2] – composed dominantly of the magnesian rock-forming silicates; said of some igneous rocks and their constituents. Crystallises from silicate minerals at relatively high temperatures; sometimes called basaltic since the class includes basalt and gabbro.

magmatic[2]- igneous rocks derived from solidified magma

nappe[2] - a large sheet-like body of rock that has been moved more than 1 mile from its original position, either by overthrusting or folding

pelagic[2]- related to sediment of the deep sea as distinct to that derived directly from the land.

phenocryst[2] – describes a relatively large and usually conspicuous crystal, distinctly larger than the grains of the rock groundmass of a igneous rock. Phenocrysts are formed either due to early growth within a magma or by post-emplacement recrystallization. They are common in felsite and andesite rocks.

porphyritic[1] – describes igneous rocks where there are distinct differences in size of crystals present at least one group of crystals obviously larger than another group.

pumice - is a light-colored, low density, vesicular igneous rock. It forms through very rapid solidification of a melt. The vesicular texture is a result of gas trapped in the melt at the time of solidification, (http://geology.com. accessed 13 Oct 2014).

pyroclastic flow[2] – a fluidized mixture of solid to semi-solid fragments and hot, expanding gases that flows down the side of a volcano depositing once cooled, fragments of volcanic origin, as agglomerate, tuff, and certain other rocks

thrust fault[2] – a reverse fault that is characterised by a low angle of inclination with reference to the horizontal plane.

tuff[2] - a rock formed of compacted volcanic fragments, generally < 4mm in size. Tuffs commonly are composed of much shattered volcanic rock glass-chilled magma blown into the air and then deposited. If volcanic particles fall to the ground at a very high temperature, they may fuse together, forming a welded tuff

ultramafic[2] - some igneous and most varieties of meteorites containing rocks less than 45% silica; containing no quartz or feldspar and composed essentially of ferromagnesian silicates, metallic oxides and sulphides, and native metals or all three. Generally >18% MgO, high FeO, low potassium, and are composed of usually greater than 90% mafic minerals

volcanoclastic sediment - is composed primarily of undisturbed deposits of volcanic material. This material may be pyroclastic (fragments derived from explosive volcanism) or hyaloclastic (fragments formed by thermal shock when hot lava comes in contact with cool sea or lake water). (http://www.springerreference.com accessed 13 Oct 2014).

# Appendix 1.3 Glossary

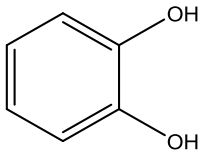
aglycone - the non sugar component of a glycoside molecule that results from hydrolysis and loss of the sugar group

Arzneimittelrichtline, (AMR) - Directive on the regulation of medicines in the doctor care provision, produced by the Federal Joint Committee on the Regulation of Medicines.

bioaccessiblit .- the amount of a pharmaceutical/nutrient that is released into solution from an ingested material and available for absorption.

bioavailability - describes the proportion of the bioaccessible fraction that reaches the systemic circulation and exerts an effect.

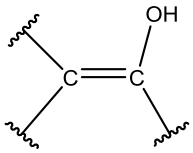
catechol - two hydroxyl groups attached to benzene ring




CFU - olony forming units i.e. viable organisms

chemometrics - he chemical discipline that uses mathematical and statistical methods, (a) to design or selelct optimal measurement procedures and experiments, and (b) to provide maximum chemical information by analysing chemical data. The techniques used in analysis of plant extracts includes: Computer-aided-similarity-evaluation (CASE); Principal component analysis (PCA), projection Pursuit (PP), Cluster Analysis and Hierarchical Cluster Analysis. There are commercial software applications available to undertake this type of data handling.

enol group - an hydroxyl group (OH) group directly bonded to an alkene (C=C).




frit - is a porous element at either end of a column that serves to contain the column packing. It is placed at the very ends of the column tube. Frits are made from inert metal or plastic, such as porous PTFE or polypropylene. Their function is to prevent unwanted particles from entering the LC system. Particles may come from the sample, the solvent or debris generated by the LC system itself (i.e. pump or injector).

folivore - a folivore is a herbivore that specializes in eating leaves

frugivore - a folivore is a bird or animal that specializes in eating fruits

FTIR - Fourier transform infrared spectroscopy (FTIR)

glycoside - a compound containing a sugar group bonded via a glycosidic link

goniometer - a goniometer is an instrument that either measures an angle or allows an object to be rotated to a precise angular position

herbivore - A herbivore is an animal that gets its energy from eating only plants material

IgA - immunoglobulin A is a class of antibodies found in saliva, sweat, tears primarily involved in preventing attachment to epithelial surfaces of pathogens e.g. virus or bacteria

IgG - immunoglobulin G is a class of antibodies found in blood serum and lymph, active against bacteria fungi and viruses and foreign particles

immunomodulation - acting upon the immune system as either stimulant or suppressant of immune response

isomers - An isomer is a chemical species with the same number and types of atoms as another chemical species, but possessing different chemical and physical properties. There are structural isomers, geometric isomers, optical isomers and stereoisomers.

LOB - Limit of the Blank is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested (Armbruster *et al*. 2008).

LOD - Limit of detection is the lowest analyte concentration likely to be reliably distinguished from the LOB and at which detection is feasible (Armbruster *et al*. 2008).

LOQ Limit of quantification is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LOQ may be equivalent to the LOD or it could be at a much higher concentration (Armbruster *et al*. 2008).

lyophilisation - freeze drying

mEq - meq a milliequivalent is an expression of concentration of substance it represents amount in milligrams, of a solute equal to 1/1000 of its gram equivalent weight taking into account the valence the ions

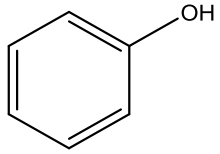
mEq = (mg x valence)/atomic, molecular or formula weight

To calculate mg/ml=(mEq/ml x atomic, molecular or formula weight)/valence

$MS^1$ and $MS^{2-}$ Ions from the first round of fragmentation $MS^1$ are then fragmented further by a second round of fragmentation i.e. Tandem mass spectrometry (MS/MS) producing $MS^2$ ions

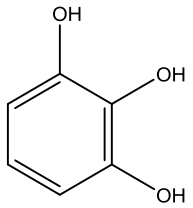
plant secondary metabolites (PSM) - chemicals synthesised by plants that are not required for its immediate survival but increase the fitness of the plant to survive by allowing it to interact with its environment, including resistance to microbial pathogens, insect attack, herbivory and protection against UV damage

phenolic group - a hydroxyl group (OH) group bonded to a benzene ring




ICP- plasma - an argon plasma which serves as the ion source of the ICP-MS. Samples are introduced into an argon plasma as aerosol droplets. The plasma dries the aerosol, dissociates the molecules and removes an electron from the components forming singly-charged ions. (Perkin Elmer - The 30-Minute Guide to ICP-MS http://www.perkinelmer.co.uk/ accessed 16 October 2014).

pyrogallol - three OH groups attached to a benzene ring




salado/salido - mineral-licks

schistosome - a type of parasitic flatworm

siderophore - Low-molecular-mass molecules that have a high specificity for chelating or binding iron. Siderophores are produced by many microorganisms, including bacteria, yeast, and fungi, to obtain iron from the environment (Leong 1986).

thionins - Type III containing 45-46 amino acids have been isolated from stems and leaves of mistletoe species such as *Viscum album* (viscotoxins A1, A2, A3, B,B2, 1-PS, U-PS, C1), *Phoradendron tomentosum* (phoratoxins A, B), *Dendrophtora clavata* (denclatoxin B) and *Phoradendron liga* (ligatoxin A).

# Appendix 1.4 Geophagy sites and collection data

Table 1.2 Coordinates and elevation of geophagy sample sites

| Site identity | GPS Co-ordinates | Elevation (m) |
|---|---|---|
| Site 1 Control Site | 10 $^0$ 50.320'N 85 $^0$36.629'W | 323 |
| Site 2 Ateles eating Site | 10 $^0$ 50.326'N 85 $^0$36.628'W | 316 |
| Site 3 Control Site | 10 $^0$ 50.320'N 85 $^0$36.626'W | 308 |
| Site 4 Control Site | 10 $^0$ 50.331'N 85 $^0$36.630'W | 326 |
| Site 5 Boulder | 10 $^0$ 50.309'N 85$^0$36.632'W | 310 |
| Site 6 Control Site | 10 $^0$ 50.346'N 85 $^0$36.636'W | 337 |
| Site 7 Control Site | 10 $^0$ 50.347'N 85 $^0$36.633'W | 318 |
| Site 8 *Capuchin* eating Site | 10 $^0$ 50.352'N 85 $^0$36.650'W | 344 |
| Site 9 *Ateles* eating Site | 10 $^0$ 50.291'N 85 $^0$36.685'W | 322 |
| Site 10 Ateles eating Site | 10 $^0$ 50.298'N 85$^0$36.675'W | 335 |

Table 1.3 Description of sample situations/positions

| Site | Sample Number | Sample position |
|---|---|---|
| 1 | 1 | 5-10cm below surface |
| | 2 | 12cm below surface |
| | 3 | 5cm below surface |
| | 4 | 9cm below surface |
| | 5 | 5-10cm below surface |
| | 6 | solid from 9cm |
| | 7 | 7-8cm below surface |
| | 8 | 3cm below surface |
| | 9 | 7.5cm below surface |
| | 10 | 1cm below surface |
| 2 | 12 | lower front edge - known eating site |
| | 13 | white area on overhang above site |
| | 14 | overhang above site |
| | 15 | upper surface of 'cave' |
| 3 | 17 | Block which separated from surface when surface hit with hammer |
| | 18 | Block which separated from surface when surface hit with hammer |

Table 1.3 (cont) Description of sample situations/positions

| Site | Sample Number | Sample position |
|---|---|---|
| 4 | 19 | 3-4cm below surface |
| | 20 | 4.5-6cm below surface |
| | 21 | surface material around 'claw marks' |
| | 22 | 5-8cm below surface |
| | 23 | hard stony fragments |
| | 24 | surface material around 'claw marks' |
| | 25 | surface material |
| | 26 | surface material from overhang |
| | 27 | 5-7.5cm from surface |
| | 28 | pumice'? |
| | 29 | pumice'? |
| | 30 | hard fragments from area around 'claw marks' |
| | 31 | surface material around 'claw marks' |
| | 32 | solid fragment |
| 6 | 39 | large block divided into sub samples |
| | 40 | |
| | 41 | |
| | 42 | |
| | 43 | 4-6cm below surface |
| | 44 | dark brown area adjacent to site 43 |
| | 45 | 4-6cm below surface |
| | 46 | lower front edge where possible 'clay/teeth' marks |
| | 47 | surface material with distinct bright yellow colouration |
| | 48 | surface material with distinct brown colouration |
| | 49 | solid fragment |
| | 50 | solid fragment |
| | 51 | 1cm below surface 'dark-green' coloured material |
| | 52 | 4cm below surface purple/grey colour |
| 7A | 53 | all were chosen to reflect the different distinct bands of colouration visible at this site |
| | 54 | |
| | 55 | |
| | 56 | |
| | 57 | |
| | 58 | |
| | 59 | |
| | 60 | |
| | 61 | |
| 7B | 62 | all were chosen to reflect the different characteristics visible at this site |
| | 63 | |
| | 64 | |
| | 65 | |
| | 66 | |
| | 67 | |
| | 68 | |
| | 69 | |
| 8 | 70 | material with a 'soil' appearance eaten by capuchin monkeys |
| | 71 | |
| | 72 | |
| 9 | 74 | above the shallow 'cave' |
| | 75 | front edge - monkeys gnaw here |
| | 76 | samples from 'roof' of shallow 'cave' |
| | 77 | |
| 10 | 100 | Unseen |

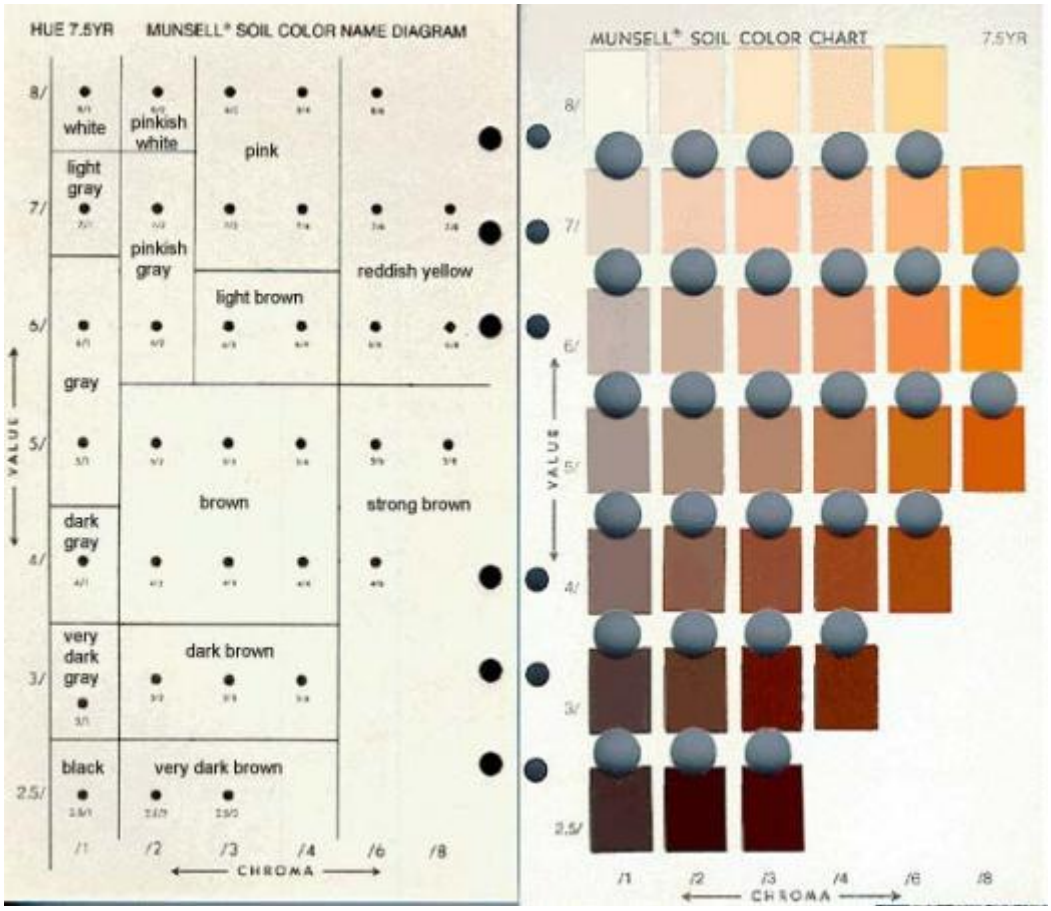



Figure 1 A typical page of the Munsell© Soil Chart showing the 7.5YR series.

# Appendix 1.5 Adsorption calibration plots

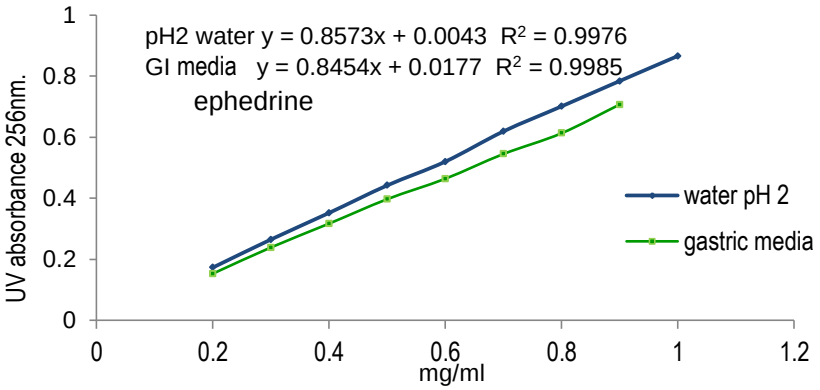



Figure 2 Calibration plot ephedrine HCl

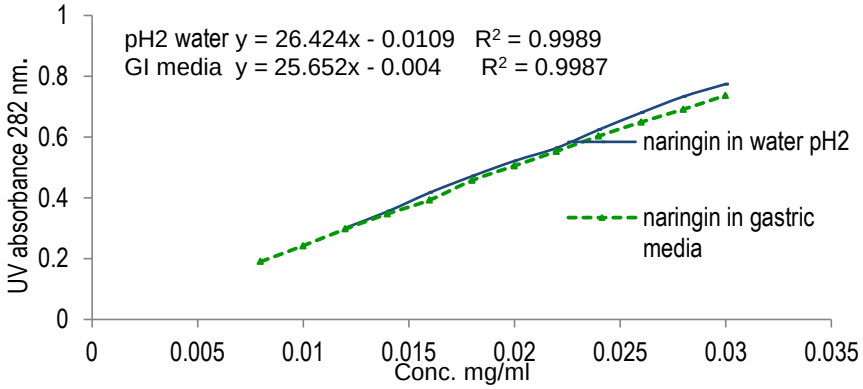



Figure 3 Calibration plot naringin

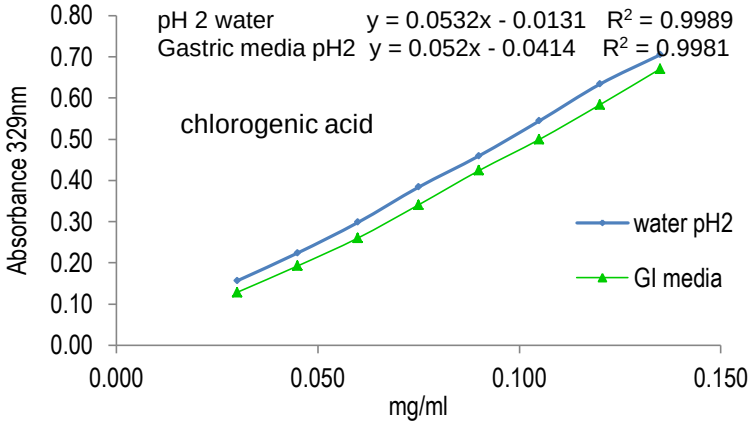



Figure 4 Calibration plot chlorogenic acid

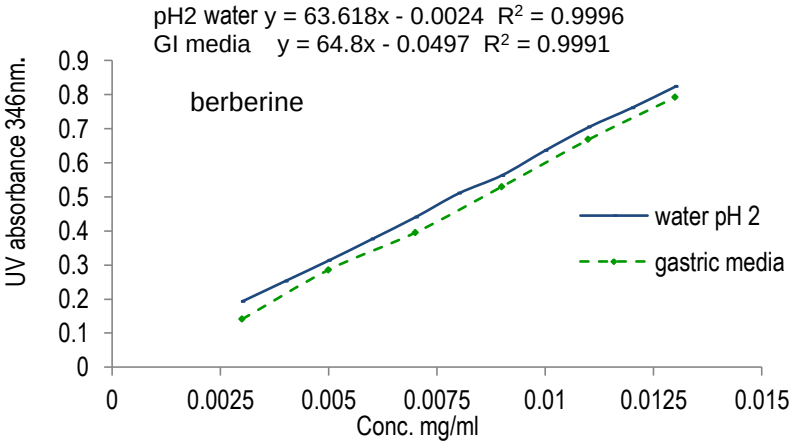



Figure 5 Calibration plot berberine

# Appendix 1.6 ICP-MS Calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of the blank (LOB). LOB is estimated by measuring replicates of a blank sample and then calculating the mean result and the standard deviation (SD). LOB is defined as the highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested. This takes into account noise and the possibility that there may be a small amount of the element detected in the sample.

Limit of detection LOD. LOD is based on the assumption is that if analyte is present, it will produce a signal greater than the analytical noise in the absence of analyte i.e. > LOB.

Limit of Quantification LOQ. LOQ is defined as the lowest concentration of an element where the RSD of a single value is within ±20%.

Calibration equation for the element and the LOB values for the element determination

Table 1.5 Limit of Detection (LOD) and Limit of Quantification (LOQ), ICP-MS analysis

| Mass ion | Equation | $R^2$ | Mean+ 3 SD blank | Mean+ 10SD blank | LOD ppb | LOQ ppb |
|---|---|---|---|---|---|---|
| 11 Bo | y=0.0106x+0.0092 | 1.0000 | 0.0347 | 0.0444 | 2.403 | 3.321 |
| 23 Na | y=0.0411x+0.4162 | 0.9994 | 0.4940 | 0.7740 | 1.893 | 8.706 |
| 24 Mg | y=0.0265x-0.0268 | 0.9999 | 0.0339 | 0.0744 | 2.289 | 3.819 |
| 27 Al | y=0.0354x+0.0827 | 0.9998 | 0.0844 | 0.0939 | 0.047 | 0.316 |
| 31 P | y=0.0013x+0.0588 | 0.9999 | 0.0587 | 0.0604 | 0.078 | 1.249 |
| 39 K | y=0.0394x+1.1737 | 0.9998 | 1.1769 | 1.4637 | 0.081 | 7.360 |
| 42 Ca | y=0.0004x+0.1336 | 0.9982 | 0.1413 | 0.1467 | 19.297 | 32.823 |
| 44 Ca | y=0.0015x+0.01 | 0.9990 | 0.0299 | 0.0399 | 13.260 | 19.933 |
| 52 Cr | y=0.0377x-0.0496 | 0.9999 | 0.0085 | 0.0098 | 1.542 | 1.574 |
| 55 Mn | y=0.0749x-0.0478 | 0.9999 | 0.0075 | 0.0172 | 0.738 | 0.868 |
| 58 Fe | y=0.0453x-0.0859 | 0.9999 | 0.0107 | 0.0122 | 0.859 | 0.933 |
| 59 Co | y=0.0004x+0.0005 | 0.9999 | 0.0023 | 0.0064 | 1.947 | 2.038 |
| 60 Ni | y=0.0749x-0.0478 | 0.9999 | 0.0027 | 0.0039 | 5.550 | 8.525 |
| 62 Ni | y=0.0012x+0.0023 | 0.9999 | 0.0021 | 0.0043 | 0.167 | 1.700 |
| 63 Cu | y=0.0218x+0.0046 | 0.9999 | 0.0044 | 0.0055 | 0.415 | 0.461 |
| 68 Zn | y=0.0061x+0.0034 | 1.0000 | 0.0051 | 0.0070 | 0.279 | 0.585 |
| 76 Se | y=0.0007x+0.0372 | 0.9999 | 0.0382 | 0.0425 | 1.360 | 7.600 |
| 95 Mo | y=0.0061x+0.0034 | 1.0000 | 0.0031 | 0.0040 | 1.019 | 1.084 |
| 96 Mo | y=0.0156x-0.0197 | 1.0000 | 0.0014 | 0.0021 | 1.355 | 1.397 |
| 98 Mo | y=0.0242x-0.0269 | 1.0000 | 0.0021 | 0.0031 | 1.196 | 1.241 |
| 116 Sn | y=0.0182x-0.0032 | 1.0000 | 0.0352 | 0.0975 | 2.108 | 5.531 |
| 127 I | y=0.0002x+0.0128 | 0.9999 | 0.0200 | 0.0222 | 36.241 | 46.835 |
| 138Ba | y=0.1558x-0.467 | 0.9999 | 0.0025 | 0.0037 | 3.014 | 3.021 |

# Appendix 1.7 LC-MS Phoratoxin analysis data from RBG Kew

Table 2.1 HPLC-MS Results *Phoradendron* analyses at RBG, KEW Courtesy G. Kite/F. Aureli.

| Batch | Sample No | Area of phoratoxin chromatographic peak (arbitary units) Phoratoxin-m/z1607 | Phoratoxin-m/z1593 | Phoratoxin-m/z1603 | Phoratoxin-m/z1610 | Phoratoxin-m/z1561 | Host tree species | Monkeys seen eating Phora | Collection month (rainy season May-Dec) | Drying method |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 3341 [8913*] | + | - | - | - | M.c | Y | June | Silica then oven |
| 1 | 2 | 5746 [5729*] | + | - | - | - | M.c | Y | July | Silica then oven |
| 1 | 3 | 8953 | + | - | - | - | M.c | Y | July | Silica then oven |
| 1 | 4 | 4424 | + | + | - | - | M.c | Y | Aug | Silica then oven |
| 1 | 5 | + | (+) | - | - | - | L.s | Y | Aug | Silica then oven |
| 1 | 6 | 3200 | - | - | - | - | M.c | Y | Aug | oven dried |
| 1 | 7 | + | - | - | 2765 | 1361 | G.u | N | Sept | oven dried |
| 1 | 8 | 925 | (+) | - | - | - | M.c | Y | Sept | oven dried |
| 1 | 9 | 955 | (+) | (+) | - | - | M.c | Y | Sept | oven dried |
| 1 | 10 | - | - | - | - | - | L.s | N | Sept | oven dried |
| 1 | 11 | 3481 | + | - | - | - | M.c | N | Sept | oven dried |
| 1 | 12 | + | - | - | - | - | L.s | N | Sept | oven dried |
| 1 | 13 | - | - | - | - | - | T.o | N | Sept | oven dried |
| 1 | 14 | 1476 | 1213 | + | - | - | M.c | Y | Sept | oven dried |
| 1 | 15 | 1502 (correction) | + | + | - | - | M.c | N | Sept | oven dried |
| 1 | 16 | 2956 | + | - | - | - | M.c | Y | Sept | oven dried |
| 1 | 17 | 5717 | + | - | - | - | M.c | N | Sept | oven dried |
| 1 | 18 | + | - | - | - | - | C.a | N | Sept | oven dried |
| 1 | 19 | - | - | - | - | - | T.o | Y | Sept | oven dried |
| 1 | 20 | - | - | - | - | - | L.s | ? | Sept | oven dried |
| 1 | 21 | 1574 | - | + | + | - | M.c | ? | Sept | oven dried |
| 1 | 22 | 1743 | - | + | - | - | M.c | Y | Sept | oven dried |
| 2 | 23 | 2215 | + | - | - | - | M.c | Y | Sept | Silica only |
| 2 | 24 | 9648 | 1884 | - | - | - | M.c | Y | Oct | Silica only |
| 2 | 25 | 6905 | 1321 | - | - | - | M.c | Y | Dec | Silica only |
| 2 | 26 | + | 5470 | - | - | - | M.c | Y | Jan | Silica only |
| 2 | 31 | 7795 | 2164 | - | - | - | M.c | Y | Feb | Silica only |
| 2 | 32a | 7303 | 1256 | - | - | - | M.c | Y | Mar | Silica only |
| 2 | 32b | 1447 | + | - | - | - | M.c | Y | Mar | oven dried |
| 2 | 69 | 6271 | 1240 | - | - | - | M.c | Y | Apr | Silica only |
| 2 | 73 | 2493 | 0 | - | - | - | M.c | Y | May | Silica only |
| 2 | 27 | 0 | 0 | - | 1285 | + | G.u | N | Jan | Silica only |
| 2 | 28 | 0 | 0 | - | - | - | L.s | Y | Jan | Silica only |
| 2 | 29 | 0 | 0 | - | - | - | L.s | Y | Jan | Silica only |
| 2 | 30 | 0 | 2467 | - | - | - | C.v. | N | Jan | Silica only |

+ = less than 1000

* area from analysis of re-extraction done at same time as batch 2

sample from the same tree

sample from the same tree

# Bibliography


National Nutritional Database for Standard Reference, US. Dept. of. Agriculture, 2012.

Abrahams P. W., Davies T. C., Solomon A. O., Trow A. J. & Wragg J. 2013. Human Geophagia, Calabash Chalk and Undongo: Mineral Element Nutritional Implications. Page e53304. Plos One.

Abrahams P. W., Follansbee M. H., Hunt A., Smith B. & Wragg J. 2006. Iron nutrition and possible lead toxicity: An appraisal of geophagy undertaken by pregnant women of UK Asian communities. Applied Geochemistry 21:98-108.

Abreu M. T.,Fukata M. & Arditi M. 2005. TLR signaling in the gut in health and disease. The Journal of Immunology 174:4453-60.

Adams D. B.,Rehg J. & Watsa M. 2011. Observations of termitarium geophagy by Bald-faced Saki monkeys (*Pithecia irrorata)* in Madre Dios, Peru American Journal of Primatology 73:60-60.

Adaramoye O., Amanlou M., Habibi-Rezaei M., Pasalar P. & Ali M. M. 2012. Methanolic extract of African mistletoe (*Viscum album*) improves carbohydrate metabolism and hyperlipidemia in streptozotocin-induced diabetic rats. Asian Pac J Trop Med 5:427-33.

Adelman J. S. & Martin L. B. 2009. Vertebrate sickness behaviors: Adaptive and integrated neuroendocrine immune responses. Integrative and Comparative Biology 49:202-14.

Aerts R. 1996. Nutrient resorption from senescing leaves of perennials: Are there general patterns? Journal of Ecology 84:597-608.

Ali M. A.,Sayeed M. A. & Absar N. 2004. Purification and characterization of three lectins extracted from *Cassia fistula* seeds and effect of various physical and chemical agents on their stability. Journal of the Chinese Chemical Society 51:647-54.

Altizer S., Dobson A., Hosseini P., Hudson P., Pascual M. & Rohani P. 2006. Seasonality and the dynamics of infectious diseases. Ecology Letters 9:467-84.

Altizer S.,Nunn C. L. & Lindenfors P. 2007. Do threatened hosts have fewer parasites? A comparative study in primates. Journal of Animal Ecology 76:304-14.

Alvarado G. E., Vega E., Chaves J. & Vásquez M. 2004. Los grandes deslizamientos (volcánicos y no volcánicos) de tipo debris avalanche en Costa Rica. Revista Geológica de América Central.

Amat A., Clementi C., De Angelis F., Sgamellotti A. & Fantacci S. 2009. Absorption and Emission of the Apigenin and Luteolin Flavonoids: A TDDFT Investigation. The Journal of Physical Chemistry A 113:15118-26.

Amer B., Juvik O. J., Dupont F., Francis G. W. & Fossen T. 2012. Novel aminoalkaloids from European mistletoe (*Viscum album* L.). Phytochemistry Letters 5:677-81.

Andersen O. M. & Markham K. R. 2010. Flavonoids: Chemistry, Biochemistry and Applications. Taylor & Francis.

Andersson D. E. & Nygren A. 1978. Four cases of long-standing diarrhoea and colic pains cured by fructose-free diet--a pathogenetic discussion. Acta Med Scand 203:87-92.

Andjelković M., Van Camp J., De Meulenaer B., Depaemelaere G., Socaciu C., Verloo M. & Verhe R. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. Food Chemistry 98:23-31.

Andrade-Cetto A. & Heinrich M. 2005. Mexican plants with hypoglycaemic effect used in the treatment of diabetes. Journal of Ethnopharmacology 99:325-48.

Andrade-Cetto A. & Vazquez R. C. 2010. Gluconeogenesis inhibition and phytochemical composition of two *Cecropia* species. J Ethnopharmacol 130:93-7.

Anjos M. J., Lopes R. T., Jesus E. F. O., Assis J. T., Cesareo R., Barroso R. C. & Barradas C. A. A. 2002. Elemental concentration analysis in soil contaminated with recyclable urban garbage by tube-excited energy-dispersive X-ray fluorescence. Radiation Physics and Chemistry 65:495-500.

Arda N., Onay E., Koz O. & Kirmizigul S. 2003. Monosaccharides and polyols from mistletoes (*Viscum album* L.) growing on two different host species. Biologia 58:1037-41.

Arellano-Cardenas S., Gallardo-Velazquez T., Poumian-Gamboa G. V., Osorio-Revilla G., Lopez-Cortez S. & Rivera-Espinoza Y. 2012. Sorption of Naringin from Aqueous Solution by Modified Clay. Clays and Clay Minerals 60:153-61.

Armbruster D. A. & Pry T. 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29:S49-S52.

Arnett E., Lehrer R. I., Pratikhya P., Lu W. & Seveau S. 2011. Defensins enable macrophages to inhibit the intracellular proliferation of *Listeria monocytogenes*. Cellular microbiology 13:635-51.

Aron P. M. & Kennedy J. A. 2008. Flavan-3-ols: Nature, occurrence and biological activity. Molecular Nutrition & Food Research 52:79-104.

Arrigo-Nelson S. J., Baden A. L., Salisbury R. L., Wright P. C. & McGee E. M. 2010. The Adaptive Significance of geophagy in the Milne-Edwards' sifaka (*Propithecus edwardsi*) at Ranomafana National Park Madagaskar. International Primatological Society XXIII Congress. Primate Society Kyoto.

Arruda F. V., Melo A. A., Vasconcelos M. A., Carneiro R. F., Barroso-Neto I. L., Silva S. R., Pereira-Junior F. N., Nagano C. S., Nascimento K. S., Teixeira E. H., Saker-Sampaio S., Sousa Cavada B. & Sampaio A. H. 2013. Toxicity and binding profile of lectins from the Genus canavalia on brine shrimp. Biomed Res Int 2013:154542.

Arruda R., Fadini R. F., Carvalho L. N., Del-Claro K., Mourão F. A., Jacobi C. M., Teodoro G. S., Berg E. v. d., Caires C. S. & Dettke G. A. 2012. Ecology of neotropical mistletoes: an important canopy-dwelling component of Brazilian ecosystems. Acta Botanica Brasilica 26:264-74.

Artis D. & Grencis R. 2008. The intestinal epithelium: sensors to effectors in nematode infection. Mucosal immunology 1:252-64.

Asai M., Lee J.-W., Itakura Y., Choi B.-K., Yonezawa T., Teruya T., Cha B.-Y., Ohnishi M., Yamaguchi A. & Woo J.-T. 2012. Effects of veraguensin and galgravin on osteoclast differentiation and function. Cytotechnology 64:315-22.

Asensio N.,Korstjens A. H. & Aureli F. 2009. Fissioning minimizes ranging costs in spider monkeys: a multiple-level approach. Behav Ecol Sociobiol 63:649–59.

Asensio N., Lusseau D., Schaffner C. M. & Aureli F. 2012a. Spider monkeys use high-quality core areas in a tropical dry forest Journal of Zoology 287:250-58.

Asensio N.,Schaffner C. M. & Aureli F. 2012b. Variability in core areas of spider monkeys (*Ateles geoffroyi*) in a tropical dry forest in Costa Rica Primates 53:147-56.

Athanasiadou S. & Kyriazakis I. 2004. Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. Proceedings of the Nutrition Society 63:631-39.

Athanasiadou S., Kyriazakis I., Jackson F. & Coop R. L. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. Veterinary Parasitology 99:205-19.

Atsalis S. 2008. A natural history of the Brown mouse lemur. Prentice Hall, Upper Saddle River (N.J.).

Aufreiter S., Mahaney W. C., Milner M. W., Huffman M. A., Hancock R. G. V., Wink M. & Reich M. 2001. Mineralogical and chemical interactions of soils eaten by chimpanzees of the Mahale Mountains and Gombe Stream National Parks, Tanzania. Journal of Chemical Ecology 27:285-311.

Aukema J. E. 2003. Vectors, viscin, and Viscaceae: mistletoes as parasites, mutualists, and resources. Frontiers in Ecology and the Environment 1:212-19.

Aureli F. & Schaffner C. M. 2008. Social interactions, social relationships and the social system of spider monkeys. Pages 236-65 *in* C. J. Campbell., Editor. Spider Monkeys: Behavior, Ecology and Evolution of the Genus *Ateles.* Cambridge University Press.

Ayensu E. S. 1981. Medicinal Plants of the West Indies. Reference Publications, Inc, Algonac, Michigan.

Ayotte J. B., Parker K. L., Arocena J. M. & Gillingham M. P. 2006. Chemical composition of lick soils: Functions of soil ingestion by four ungulate species. Journal of Mammalogy 87:878-88.

Azen E. A.,Leutenegger W. & Peters E. H. 1978. Evolutionary and dietary aspects of salivary basic (Pb) and post Pb (PPb) proteins in anthropod primates. Nature 273:775-78.

Babula P., Adam V., Havel L. & Kizek R. 2009. Noteworthy Secondary Metabolites Naphthoquinones - their Occurrence, Pharmacological Properties and Analysis. Current Pharmaceutical Analysis 5:47-68.

Baeshad I. 1955. Absortive and swelling properties of clay-water system. Bull. Calif. Dept. Nat. Resources, Div. Mines. 169.

Bagetta G., Cosentino M., Corasaniti M. T. & Sakurada S. 2011. Herbal Medicines: Development and Validation of Plant-derived Medicines for Human Health. Taylor & Francis.

Balls A. K. & Hale W. S. 1940. A sulphur-bearing constituent of the petroleum-ether extract of wheat flour (preliminary report). Cereal Chemistry 17:243-45.

Bansil R.,Stanley E. & LaMont J. T. 1995. Mucin Biophysics. Annual Review of Physiology 57:635-57.

Bansil. R.,S. B. & Turner A. G. 2006. Mucin structure, aggregation, physiological functions and biomedical applications. Current Opinion in Colloid & Interface Science 11:164-70.

Bar-Sel G. 2011. White-Berry mistletoe (*Viscum album* L.) as complimentary treatment in cancer: Does it help? European Journal of Integrative medicine 3:55-62.

Bar-Sela G., Wollner M., Hammer L., Agbarya A., Dudnik E. & Haim N. 2013. Mistletoe as complementary treatment in patients with advanced non-small-cell lung cancer treated with carboplatin-based combinations: a randomised phase II study. European Journal of Cancer 49:1058-64.

Barbosa Pelegrini P., Del Sarto R. P., Silva O. N., Franco O. L. & Grossi-de-Sa M. F. 2011. Antibacterial peptides from plants: what they are and how they probably work. Biochemistry Research International 2011.

Bassoli B. K., Cassolla P., Borba-Murad G. R., Constantin J., Salgueiro-Pagadigorria C. L., Bazotte R. B., da Silva R. S. & de Souza H. M. 2008. Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: effects on hepatic glucose release and glycaemia. Cell Biochem Funct 26:320-8.

Battaglia S.,Leoni L. & Sartori F. 2006. A method for determining the CEC and chemical composition of clays via XRF. Clay Minerals 41:717-25.

Bavington C. & Page C. 2005. Stopping Bacterial Adhesion: A Novel Approach to Treating Infections. Respiration 72:335-44.

Beaune D., Bretagnolle F., Bollache L., Bourson C., Hohmann G. & Fruth B. 2013. Ecological services performed by the bonobo (*Pan paniscus*): seed dispersal effectiveness in tropical forest. Journal of Tropical Ecology 29:367-80.

Behera P. R., Nayak P., Barik D. P., Rautray T. R., Thirunavoukkarasu M. & Chand P. K. 2010. ED-XRF spectrometric analysis of comparative elemental composition of in vivo and in vitro roots of Andrographis paniculata (Burm.f.) Wall. ex Nees--a multi-medicinal herb. Appl Radiat Isot 68:2229-36.

Behie A. M. & Pavelka M. S. M. 2012. The Role of Minerals in Food Selection in a Black Howler Monkey (*Alouatta pigra*) Population in Belize Following a Major Hurricane. American Journal of Primatology 74:1054-63.

Bell E. A. & Charlwood B. V., Editors. 1980. Secondary Plant Products. Springer Verlag, Berlin, Berlin, Heidelberg, New York

Bennek A. 2002. Interaction of Plant Polyphenols with Salivary Proteins. Critical Reviews in Oral Biology & Medicine. Sage.

Bergaya F. & Lagaly G. 2006a. Chapter 1 General Introduction: Clays, Clay Minerals, and Clay Science. Pages 1-18 *in* B. K. G. T. Faïza Bergaya and Gerhard L., Editors. Developments in Clay Science. Elsevier.

Bergaya F.,Theng B. K. G. & Lagaly G. 2006b. Chapter 11 Clays, Environment and Health. Pages 623 -743 *in* F. Bergaya, B. K. G. Theng, and G. Lagaly, Editors. Developments in Clay Science. Elsevier.

Berrocal-Lobo M., Molina A., Rodriguez-Palenzuela P., Garcia-Olmedo F. & Rivas L. 2009. *Leishmania donovani:* thionins, plant antimicrobial peptides with leishmanicidal activity. Exp Parasitol 122:247-9.
Best E. C.,Joseph J. & Goldizen A. W. 2013. Facultative geophagy at natural licks in an Australian marsupial. Journal of Mammalogy 94:1237-47.
Bhaskar K. R., Gong D. H., Bansil R., Pajevic S., Hamilton J. A., Turner B. S. & LaMont J. T. 1991. Profound increase in viscosity and aggregation of pig gastric mucin at low pH. Am J Physiol Gastrointest Liver Physiol 261:G827-G32.
Bicca-Marques J. C. & Calegaro-Marques C. 1994. A case of geophagy in the Black howler monkey *Alouatta caraya.* Neotropical Primates 2:7-9.
Blake J. G., Guerra J., Mosquera D., Torres R., Loiselle B. A. & Romo D. 2010. Use of Mineral Licks by White-bellied Spider monkeys (*Ateles belzebuth*) and Red howler monkeys (*Alouatta seniculus*) in Eastern Ecuador. International Journal of Primatology 31:471-83.
Blake J. G., Mosquera D., Guerra J., Loiselle B. A., Romo D. & Swing K. 2011. Mineral licks as diversity hotspots in lowland forest of eastern Ecuador. Diversity 3:217-34.
Blake J. G.,Mosquera D. & Salvador J. 2013. Use of mineral licks by mammals and birds in hunted and non-hunted areas of Yasuni National Park, Ecuador. Animal Conservation 16:430-37.
Blaschek W.,Gramann B. & Classen B. 2011. Localisation of mistletoe lectins ML I-III in *Viscum album* L. by immunofluorescent and immunogold labeling. 5th Mistletoe Symposium: Mistletoe in Cancer Therapy – Basic Research and Clinical Practice, November 10-12, 2011. Phytomedicine (Elsevier-Verlag), European Academy of Otzenhausen, Nonnweiler, Saarland.
Boettner D. R.,Huston C. & Petri W. A. 2002. Galactose/N-acetylgalactosamine lectin: the coordinator of host cell killing. Journal of Biosciences 27:553-57.
Bohlmann H. & Apel K. 1991. Thionins. Annual Review of Plant Physiology and Plant Molecular Biology 42:227-40.
Bohm B. A. 1999. Introduction to Flavonoids. Taylor & Francis.
Bohn T. 2014. Dietary factors affecting polyphenol bioavailability. Nutrition reviews.
Bolton K. A.,Campbell V. M. & Burton F. D. 1998. Chemical analysis of soils of Kowloon (Hong Kong) eaten by hybrid macaques. Journal of Chemical Ecology 24:195-205.
Boneberg E.-M. & Hartung T. 2001. Mistletoe Lectin-1 Increases Tumor Necrosis Factor-α Release in Lipopolysaccharide-Stimulated Whole Blood via Inhibition of Interleukin-10 Production. Journal of Pharmacology and Experimental Therapeutics 298:996-1000.
Bonita J. S., Mandarano M., Shuta D. & Vinson J. 2007. Coffee and cardiovascular disease: *in vitro*, cellular, animal, and human studies. Pharmacol Res 55:187-98.
Bourjot M., Apel C., Martin M.-T., Grellier P., Nguyen V. H., Guéritte F. & Litaudon M. 2010. Antiplasmodial, antitrypanosomal and cytotoxic activities of prenylated flavonoids isolated from the stem bark of *Artocarpus styracifolius*. Planta Med 76:1600,04.
Brandon E., Oomen A., Rompelberg C., Versantvoort C., van Engelen J. & Sips A. 2006. Consumer products *in vitro* digestion model: Bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology 44:161-71.
Bravo A.,Harms K. E. & Emmons L. H. 2012. Keystone resource *(Ficus*) chemistry explains lick visitation by frugivorous bats. Journal of Mammalogy 93:1099-109.
Bravo A., Harms K. E., Stevens R. D. & Emmons L. H. 2008. Collpas: Activity hotspots for frugivorous bats (*Phyllostomidae*) in the Peruvian Amazon. Biotropica 40:203-10.
Brayer G. D.,Luo Y. & Withers S. G. 1995. The structure of human pancreatic alpha-amylase at 1.8 A resolution and comparisons with related enzymes. Protein Sci. 4:1730-4.
Breitmaier E. 2006. Terpenes: Importance, General Structure, and Biosynthesis. Pages 1-9 Terpenes. Wiley-VCH Verlag GmbH & Co. KGaA.
Brightsmith D. & Munoz-Najar R. 2004. Avian geophagy and soil characteristics in south-eastern Peru. Biotropica 36:534-43.
Brightsmith D.,Taylor J. & Phillips T. 2008. The Roles of Soil Characteristics and Toxin Adsorption in Avian Geophagy. Biotropica 40:766-74.
Brindley G. W., Kao C. C., Harrison J. L., Lipsicas M. & Raythatha R. 1986. Relationship between structural disorder and other characteristics of kaolinites and dickites. Clays and Clay Minerals 34:239-49.
Broekaert W. F., Cammue B. P. A., DeBolle M. F. C., Thevissen K., DeSamblanx G. W. & Osborn R. W. 1997. Antimicrobial peptides from plants. Critical Reviews in Plant Sciences 16:297-323.
Brooks F. P., Ridley P., Attinger F., Bjovedt G. & Neff K. 1963. Twenty-four hour collections of gastric content in Spider monkeys. American Journal of Physiology 205:1096-98.
Brouillard M.-Y. & Rateau J.-G. 1989. Adsorption potency of two clays, smectite and kaolin on bacterial endotoxin. Gastroenterol. Clin. Biol. 13:18-24.
Broussalis A. M., Goransson U., Coussio J. D., Ferraro G., Martino V. & Claeson P. 2001. First cyclotide from Hybanthus (Violaceae). Phytochemistry 58:47-51.
Brunet S. & Hoste H. 2006. Monomers of condensed tannins affect the larval exsheathment of parasitic nematodes of ruminants. Journal of Agricultural and Food Chemistry 54:7481-87.
Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. Journal of Experimental Botany 48:181-99.
Bullen J. J.,Rogers H. J. & Griffiths E. 1978. Role of iron in bacterial infection. Curr Top Microbiol Immunol 80:1-35.
Buondonno A.,Fellaca D. & Violante A. 1989. Properties of organo-mineral complexes formed by differetn addition sequences of hydroxy-A1, montmorillonite and tannic acid. Clays and Clay Minerals 37:235-42.
Büssing A. 2000. Biological and Pharmacological properties of *Viscum album* L. *in* A. Büssing, Editor. Mistletoe: The

Genus Viscum. Harwood Academic Publishers, Amsterdam.
Bussing A. & Schietzel M. 1999a. Apoptosis-inducing properties of *Viscum album* L. extracts from different host trees, correlate with their content of toxic mistletoe lectins. Anticancer Research 19:23-28.
Bussing A., Stein G. M., Wagner M., Wagner B., Schaller G., Pfuller U. & Schietzel M. 1999b. Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes induced by thionins from *Viscum album* L. European Journal of Biochemistry 262:79-87.
Buurman P.,de Boer K. & Pape T. 1997. Laser diffraction grain-size characterisation of Andisols in perhumid Costa Rica: the aggregate size of allophane. Geoderma 78:71-91.
Buzzini P., Arapitsas P., Goretti M., Branda E., Turchetti B., Pinelli P., Ieri F. & Romani A. 2008. Antimicrobial and Antiviral activity of hydrolysable tannins. Mini-Reviews in Medicinal Chemistry 8:1179-87.
Bystrom L. M., Lewis B. A., Brown D. L., Rodriguez E. & Obendorf R. L. 2008. Characterisation of phenolics by LC–UV/Vis, LC–MS/MS and sugars by GC in *Melicoccus bijugatus* Jacq. 'Montgomery' fruits. Food Chemistry 111:1017-24.
Caerovic Z. G., Samson G., Morales F., N.Tremblay N. & Maoya I. 1999. Ultraviolet-induced fluorescence for plant monitoring: present state and prospects. Agronomie 19:543-78.
Calder M. 1983. The biology of mistletoes. Page 384 *in* P. Bernhardt, Editor. Academic Press, Sydney.
Callemien D. & Collin S. 2008. Use of RP-HPLC-ESI (-)-MS/MS to differentiate various proanthocyanidin isomers in lager beer extracts. J. Am. Soc. Brew. Chem 66:109-15.
Calzado-Flores C., Hurtado-Ramirez M. B., Flores-Villanueva Z., Verde-Star M. J., Segura-Luna J., Lozano-Garza G. & Aguilar-Cuestas G. 2002. Preliminary chronic toxicological study of aqueous extract of *Phoradendron tormentosum.* Proceedings Western Pharmacology Society 45:162-63.
Campbell C. J., Aureli F., Chapman C. A., Ramos-Fernandez G., Matthews K., Russo S. E., Suarez S. & Vick L. 2003. Terrestrial behavior of Spider monkeys (*Ateles* spp.): A comparative study. American Journal of Physical Anthropology:74-74.
Campbell C. J., Aureli F., Chapman C. A., Ramos-Fernandez G., Matthews K., Russo S. E., Suarez S. & Vick L. 2005. Terrestrial behavior of *Ateles* spp. International Journal of Primatology 26:1039-51.
Campos N. V., Pereira T. A. R., Machado M. F., Guerra M. B. B., Tolentino G. S., Araujo J. S., Rezende M. Q., da Silva M. & Schaefer C. 2014. Evaluation of micro-energy dispersive X-ray fluorescence and histochemical tests for aluminium detection in plants from High Altitude Rocky Complexes, Southeast Brazil. Anais Da Academia Brasileira De Ciencias 86:285-96.
Cant J. 1990. Feeding ecology of Spider monkeys (*Ateles geoffroyi* ) at Tikal, Guatemala. Human Evolution 5:269-81.
Cannon W. B. 1929. Bodily changes in pain, hunger, fear and rage. 2nd Edition. D.Appleton And Company., New York.
Cantrell C. L., Klun J. A., Bryson C. T., Kobaisy M. & Duke S. O. 2005. Isolation and identification of mosquito bite deterrent terpenoids from leaves of American *(Callicarpa americana*) and Japanese (*Callicarpa japonica)* beautyberry. J Agric Food Chem 53:5948-53.
Careaga-Olivares J., Aguilar-Cuestas G., Verde-Star M. J., Cerda-Flores R. M., Lozano-Garza G., Echávarri-Guzmán M. Á. & Calzado-Flores C. 2006. Hypoglycemic Activity of *Phoradendron tomentosum* in Streptozotocin-diabetic Rats. Proc West Pharmacol Soc 49:80-82.
Carlini C. R. & Grossi-de-Sa M. F. 2002. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon 40:1515-39.
Carrai V., Borgognini-Tarli S. M., Huffman M. A. & Bardi M. 2003. Increase in tannin consumption by sifaka (*Propithecus verreauxi verreauxi*) females during the birth season: a case for self-medication in prosimians. Primates 44:61-66.
Carrasco L., Vazquez D., Hernandezlucas C., Carbonero P. & Garciaolmedo F. 1981. Thionins - Plant peptides that modify membrane-permeability in cultured mammalian cells European Journal of Biochemistry 116:185-89.
Caspi R., Altman T., Dale J. M., Dreher K., Fulcher C. A., Gilham F., Kaipa P., Karthikeyan A. S., Kothari A., Krummenacker M., Latendresse M., Mueller L. A., Paley S., Popescu L., Pujar A., Shearer A. G., Zhang P. & Karp P. D. 2010. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Research 38:D473-D79.
Casteel S. W., Cowart R., Weis C. P., Henningsen G. M., Hoffman E., Brattin W. J., Guzamn R. J., Starost M. F., Payne J. T., Stockham S. L., Becker S. V., Drexler J. W. & Turk J. R. 1997. Bioavailability of lead to juvenile swine dosed with soil from the Smuggler Mountain NPL site, Colorado. Fund. Appl. Toxicol. 36:177-187. 36:177-87.
Casteel S. W., Weis C. P., Henningsen G. M. & Brattin W. J. 2006. Estimation of relative bioavailability of lead in soil and soil-like materials using young swine. Environ Health Perspect. 114:1162-71.
Castellanos H. G. & Chanin P. 1996. Seasonal differences in food choice and patch preference of long-haired Spider monkeys *Ateles belzebuth*. Page 555 *in* M. A. Norconck, Rosenberger A. L., and Garber P. A., Editors. Adaptive Radiations of Neotropical Primates. Plenum Press, New York.
Castro M. S. & Fontes W. 2005. Plant defense and antimicrobial peptides. Protein and Peptide Letters 12:13-18.
Castro O. C. 1993. Chemical and Biological Extractives of Lauraceae Species in Costa Rican Tropical Forests. Pages 65-87 *in* K. Downum, Romeo J., and Stafford H., Editors. Phytochemical Potential of Tropical Plants. Springer US.
Cattadori I. M., Boag B., Bjornstad O. N., Cornell S. J. & Hudson P. J. 2005. Peak shift and epidemiology in a seasonal host-nematode system. Proceedings of the Royal Society B-Biological Sciences 272:1163-69.
Cazarolli L. H., Zanatta L., Alberton E. H., Reis Bonorino Figueiredo M. S., Folador P., Damazio R. G., Pizzolatti M. G. & Mena Barreto Silva F. R. 2008. Flavonoids: cellular and molecular mechanism of action in glucose homeostasis. Mini reviews in medicinal chemistry 8:1032-38.
Cazetta E.,Schaefer H. M. & Galetti M. 2008. Does attraction to frugivores or defense against pathogens shape fruit pulp

composition? Oecologia 155:277-86.
Ćetković G. S., Đilas S. M., Čanadanović-Brunet J. M. & Tumbas V. T. 2003. Thin-layer chromatography analysis and scavenging activity of marigold (*Calendula officinalis* L.) extracts. Acta periodica technologica:93-102.
Chandrika U. G., Basnayake B. M., Athukorala I., Colombagama P. W. & Goonetilleke A. 2010. Carotenoid content and *in vitro* bioaccessibility of lutein in some leafy vegetables popular in Sri Lanka. J Nutr Sci Vitaminol (Tokyo) 56:203-7.
Chapman C. 1987. Flexibility in diets of three species of Costa Rican primates. Folia Primatologica 49:90-105.
Chapman C. 1988. Patterns of foraging and range use by three species of neotropical primates. Primates 29:177.
Chapman C. A. & Chapman L. J. 1990. Reproductive Biology of Captive and Free-Ranging Spider Monkeys. Zoo Biology 9:1-9.
Chapman C. A. & Chapman L. J. 1991. The foraging itinerary of Spider monkeys - When to eat leaves. Folia Primatologica 56:162-66.
Chapman C. A. & Chapman L. J. 2002. Foraging challenges of Red colobus monkeys: influence of nutrients and secondary compounds. Comparative Biochemistry And Physiology A-Molecular And Integrative Physiology 133:861-75.
Chapman C. A.,Gillespie T. R. & Goldberg T. L. 2005. Primates and the Ecology of their Infectious Diseases: How will Anthropogenic Change Affect Host-Parasite Interactions? Evolutionary Anthropology: Issues, News, and Reviews 14:134-44.
Charles-Dominique P. 1991. Feeding Strategy and Activity Budget of the Frugivorous Bat *Carollia perspicillata* (Chiroptera: Phyllostomidae) in French Guiana. Journal of Tropical Ecology 9:243-56.
Chase M. W. & Hills H. H. 1991. Silica-Gel - an ideal material for field preservation of leaf samples for DNA studies. Taxon 40:215-20.
Chaverri C. & Cicció J. F. 2005. Essential oil of trees of the genus *Ocotea* (Lauraceae) in Costa Rica. I. *Ocotea brenesii.* Revista de Biología Tropical 53:431-36.
Chevallier A. 2000. Encyclopedia of herbal medicine. Dorling Kindersley Publishing, Incorporated.
Chew Y. L., Chan E. W., Tan P. L., Lim Y. Y., Stanslas J. & Goh J. K. 2011. Assessment of phytochemical content, polyphenolic composition, antioxidant and antibacterial activities of Leguminosae medicinal plants in Peninsular Malaysia. BMC Complement Altern Med 11:12.
Chiesa S. 1991. El Flujo de pomez biotica del Rio Liberia, Guanacaste, Costa Rica, America Central. Rev. Geol. Amer. Central. 13:73-84.
Chiesa S. 1993. Rilevamento geologico del parco nazionale de Santa Rosa, ICE ( Instituto Costarriciense de Electricidad).
Chiesa S., Civelli G., Gillot P. Y., Mora O. & Alvarado G. E. 1992. Rocas piroclastica associadas con la formacion de la caldera de Guayabo, Cordillera de Guanacste, Costa Rica. Rev. Geol. Amer. Central. 14:59-75.
Chiesa S.,Corella M. & Mora O. 1987. Geologia de la meseta ignimbritica de Santa Rosa, Guanacaste, Costa Rica. .
Chipera S. J. & Bish D. L. 2001. Baseline studies of The Clay Minerals Society Source Clays: Powder X-ray diffraction analyses. Clays and Clay Minerals 49:398-409.
Chirumbolo S. 2011. Plant polyphenolic compounds as potential antimicrobial drugs. Journal of Medical Microbiology 60:1562-63.
Chivers D. J. & Hladik C. M. 1980. Morphology of the Gastrointestinal tract in Primates: Comparisons with other mammals in relation to diet. Jounal of Morphology 166:337 - 86.
Choi O., Yahiro K., Morinaga N., Miyazaki M. & Noda M. 2007. Inhibitory effects of various plant polyphenols on the toxicity of Staphylococcal toxin. Microbial pathogenesis 42:215-24.
Churchman G. J., Gates W. P., Theng B. K. G. & Yuan G. 2006. Chapter 11.1 Clays and Clay Minerals for Pollution Control. Pages 625-75 *in* B. K. G. T. Faïza Bergaya and Gerhard L., Editors. Developments in Clay Science. Elsevier.
Cioffi G., Escobar L. M., Braca A. & De Tommasi N. 2003. Antioxidant Chalcone Glycosides and Flavanones from *Maclura* (Chlorophora) tinctoria. Journal of Natural Products 66:1061-64.
Claeson P., Goransson U., Johansson S., Luijendijk T. & Bohlin L. 1998. Fractionation Protocol for the Isolation of Polypeptides from Plant Biomass. J Nat Prod 61:77-81.
Clifford M. N., Johnston K. L., Knight S. & Kuhnert N. 2003. Hierarchical Scheme for LC-MSn Identification of Chlorogenic Acids. Journal of Agricultural and Food Chemistry 51:2900-11.
Coe F. G. & Anderson G. J. 1996. Screening of medicinal plants used by the Garifuna of eastern Nicaragua for bioactive compounds. J Ethnopharmacol 53:29-50.
Coe F. G., Parikh D. M., Johnson C. A. & Anderson G. J. 2012. The good and the bad: alkaloid screening and brineshrimp bioassays of aqueous extracts of 31 medicinal plants of eastern Nicaragua. Pharm Biol 50:384-92.
Cole A. M., Hong T., Boo L. M., Nguyen T., Zhao C., Bristol G., Zack J. A., Waring A. J., Yang O. O. & Lehrer R. I. 2002. Retrocyclin: a primate peptide that protects cells from infection by T-and M-tropic strains of HIV-1. Proceedings of the National Academy of Sciences 99:1813-18.
Cole H. B., Ezzell J. W., Keller K. F. & Doyle R. J. 1984. Differentiation of *Bacillus anthracis* and other *Bacillus* species by lectins. Journal of Clinical Microbiology 19:48-53.
Collin F.,Karkare S. & Maxwell A. 2011. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. Appl Microbiol Biotechnol 92:479-97.
Collins A. C. 2008. The taxonomic status of the Spider monkey in the twenty-first centuary.*in* C. J. Campbell, Editor. Spider Monkeys. Behaviour, Ecology and Evolution of the Genus Ateles. Cambridge University Press, Cambridge.
Collins R. A., Ng T. B., Fong W. P., Wan C. C. & Yeung H. W. 1998. Removal of polyphenolic compounds from aqueous

plant extracts using polyamide minicolumns. Biochemistry and Molecular Biology International 45:791 - 96.
Cook N. C. & Samman S. 1996. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. The Journal of Nutritional Biochemistry 7:66-76.
Cooke J. & Leishman M. R. 2011. Silicon concentration and leaf longevity: is silicon a player in the leaf dry mass spectrum? Functional Ecology 25:1181-88.
Coop R. L. & Holmes P. H. 1996. Nutrition and parasite interaction. International Journal for Parasitology 26:951-62.
Costa R. M. P. B., Vaz A. F. M., Oliva M. L. V., Coelho L. C. B. B., Correia M. T. S. & Carneiro-da-Cunha M. G. 2010. A new mistletoe *Phthirusa pyrifolia* leaf lectin with antimicrobial properties. Process Biochemistry 45:526-33.
Cowlishaw G. 1997. Trade-offs between foraging and predation risk determine habitat use in a desert baboon population. Animal Behaviour 53:667-86.
Crossley N. S. & Djerassi C. 1962. Naturally occurring oxygen heterocyclics. Part XI. Veraguensin. Journal of the Chemical Society:1459-62.
Crozier A., Burns J., Aziz A. A., Stewart A. J., Rabiasz H. S., Jenkins G. I., Edwards C. A. & Lean M. E. J. 2000. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. Biological Research 33:79-88.
Crozier A.,Clifford M. N. & Ashihara H. 2008. Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet. Wiley.
Cunningham T. M., Koehl J. L., Summers J. S. & Haydel S. E. 2010. pH-Dependent Metal Ion Toxicity Influences the Antibacterial Activity of Two Natural Mineral Mixtures. Plos One 5:e9456.
Cushnie T. P. & Lamb A. J. 2005. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 6:343-56.
Cushnie T. P. T. & Lamb A. J. 2011. Recent advances in understanding the antibacterial properties of flavonoids. International Journal of Antimicrobial Agents 38:99-107.
Cuyckens F., Rozenberg R., de Hoffmann E. & Claeys M. 2001. Structure characterization of flavonoid O-diglycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. Journal of mass spectrometry 36:1203-10.
D'Mello J. P. F.,Duffus C. M. & Duffus J. H. 1991. Toxic Substances in Crop Plants. Elsevier Science.
da Silva V. C., de Carvalho M. G., Borba H. R. & Silva S. L. C. 2008. Anthelmintic activity of flavonoids isolated from roots of *Andira anthelmia* (Leguminosae). Revista Brasileira de Farmacognosia 18:573-76.
Dai J. & Mumper R. J. 2010. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. Molecules 15:7313-52.
Davies A. G. & Baillie I. C. 1988. Soil-eating by Red leaf monkeys (*Presbytis rubicunda*) in Sabah, Northern Borneo. Biotropica 20:252-58.
Davies T. J. & Pedersen A. B. 2008. Phylogeny and geography predict pathogen community similarity in wild primates and humans. Proceedings of the Royal Society B: Biological Sciences 275:1695-701.
de Cassia da Silveira E. S. R., Andrade L. N., Dos Reis Barreto de Oliveira R. & de Sousa D. P. 2014. A review on anti-inflammatory activity of phenylpropanoids found in essential oils. Molecules 19:1459-80.
de Mejia E. G. & Prisecaru V. I. 2005. Lectins as bioactive plant proteins: A potential in cancer treatment. Critical Reviews in Food Science and Nutrition 45:425-45.
de Menezes J. E. S. A., Lemos T. L. G., Silveira E. R., Pessoa O. D. L., Santiago G. M. P. & Nascimento R. F. 2006. Chemical Composition and Larvicidal Activity of the Essential Oil From Leaves of *Cordia globosa* (Jacq.) H.B.K. from Northeastern Brazil. Journal of Essential Oil Research 18:253-55.
de Oliveira Oyama S., de Souza L. A., Baldoqui D. C., Sarragiotto M. H. & Silva A. A. 2013. Prenylated flavonoids from *Maclura tinctoria* fruits. Química Nova 36:800-02.
de Rijke E., Out P., Niessen W. M. A., Ariese F., Gooijer C. & Brinkman U. A. T. 2006. Analytical separation and detection methods for flavonoids. Journal of Chromatography A 1112:31-63.
de Roode J. C.,Lefèvre T. & Hunter M. D. 2013. Self-Medication in Animals. Science 340:150-51.
de Souza Cândido E., e Silva Cardoso M. H., Sousa D. A., Viana J. C., de Oliveira-Júnior N. G., Miranda V. & Franco O. L. 2014. The use of versatile plant antimicrobial peptides in agribusiness and human health. Peptides 55:65-78.
de Souza L. L., Ferrari S. F., da Costa M. L. & Kern D. C. 2002. Geophagy as a correlate of folivory in Red handed Howler monkeys *(Alouatta belzebul)* from Eastern Brazilian Amazonia Journal of Chemical Ecology 28:1613-21.
Dean W. E. 1974. Determination of Carbonate and Organic Matter in Calcareous Sediments and Sedimentary Rocks by Loss on Ignition: Comparison With Other Methods. Journal of Sedimentary Petrology 44 242-48.
Dearing M. D.,Foley W. J. & McLean S. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Pages 169-89 Annual Review of Ecology Evolution and Systematics.
deBoer G. B., deWeerd C., Thoenes D. & Goossens H. W. 1987. Laser diffraction spectrometry: Fraunhofer versus Mie scattering. Part.Charact. 4:14-19.
Deeni Y. Y. & Sadiq N. M. 2002. Antimicrobial properties and phytochemical constituents of the leaves of African mistletoe (*Tapinanthus dodoneifolius* (DC) Danser) (Loranthaceae): an ethnomedicinal plant of Hausaland, Northern Nigeria. Journal of Ethnopharmacology 83:235-40.
Deer W. A. 2001. Rock-forming minerals. Geological Society of London Pub. House, Bath.
Delehanty J. B., Johnson B. J., Hickey T. E., Pons T. & Ligler F. S. 2007. Binding and neutralization of lipopolysaccharides by plant proanthocyanidins. Journal of Natural Products 70:1718-24.
Delgado-Vargas F.,Jiménez A. R. & Paredes-López O. 2000. Natural Pigments: Carotenoids, Anthocyanins, and Betalains — Characteristics, Biosynthesis,Processing, and Stability. Critical Reviews in Food Science and Nutrition 40:173-289.
Deliorman D., Çaliş I., Ergun F. & Tamer U. 1999. The comparative studies on phenylpropanoid glycosides of *Viscum*

*album* subspecies by High Performance Liquid Chromatography Journal of Liquid Chromatography & Related Technologies 22:3101-14.
Deliorman D. & Ergun F. 2000. High Performance Liquid Chromatography determination of Syringin in *Viscum album* L.ssp. collected from different host plants. Journal of Liquid Chromatography and Related Technologies 23:3033-42.
Déprez S., Brezillon C., Rabot S., Philippe C., Mila I., Lapierre C. & Scalbert A. 2000. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. The Journal of Nutrition 130:2733-38.
Dew J. L. 2002. How specialized are ripe-fruit specialists?: dietary selection in the face of sympatric competitors and shifting fruit abundance. American Journal of Physical Anthropology:63-63.
Dew J. L. 2005. Foraging, food choice, and food processing by sympatric ripe-fruit specialists: *Lagothrix lagotricha poeppigii* and *Ateles belzebuth belzebuth*. International Journal of Primatology 26:1107-35.
Di Fiore A.,Link A. & Dew J. L. 2008. Diets of wild spider monkeys *in* C. J. Campbell, Editor. Spider Monkeys. Behaviour, Ecology and Evolution of the genus *Ateles*. Cambridge University Press, Cambridge.
Didry N., Seidel V., Dubreuil L., Tillequin F. & Bailleul F. 1999. Isolation and antibacterial activity of phenylpropanoid derivatives from *Ballota nigra*. Journal of Ethnopharmacology 67:197-202.
Diez M. T., del Moral P. G., Resines J. A. & Arin M. J. 2008. Determination of phenolic compounds derived from hydrolysable tannins in biological matrices by RP-HPLC. Journal of Separation Science 31:2797-803.
DIN. 2004. DIN 179738-07.Bodenbeschaffenheit - Resorptionsverfügbarkeit von organischen und anorganischen Schadstoffen aus kontaminiertem (Soil quality - Absorption availability of organic and inorganic pollutants from contaminated soil material).Bodenmaterial Beuth Verlag GmbH, Berlin.
Ding X. L. & Henrichs S. M. 2002. Adsorption and desorption of proteins and polyamino acids by clay minerals and marine sediments. Marine Chemistry 77:225-37.
Dixon R. A. & Pasinetti G. M. 2010. Flavonoids and Isoflavonoids: From Plant Biology to Agriculture and Neuroscience. Plant Physiology 154:453-57.
Domingo J. L.,Gomez M. & Colomina M. T. 2011. Oral silicon supplementation: an effective therapy for preventing oral aluminum absorption and retention in mammals. Nutr Rev 69:41-51.
Dominy N. J.,Davoust E. & Minekus M. 2004. Adaptive function of soil consumption: an *in vitro* study modeling the human stomach and small intestine. Journal of Experimental Biology 207:319-24.
Donnelly R., Best A., White A. & Boots M. 2013. Seasonality selects for more acutely virulent parasites when virulence is density dependent. Proceedings of the Royal Society B-Biological Sciences 280.
Doss M., White M. R., Tecle T., Gantz D., Crouch E. C., Jung G., Ruchala P., Waring A. J., Lehrer R. I. & Hartshorn K. L. 2009. Interactions of α-, β-, and θ-defensins with influenza A virus and surfactant protein D. The Journal of Immunology 182:7878-87.
Dossaji S. F.,Becker H. & Exner J. 1983. Flavone C-glycosides of *Phoradendron tomentosum* from different host trees. Phytochemistry 22:311-12.
Doyle R. & Keller K. 1984. Lectins in Diagnostic Microbiology. European Journal of Clinical Microbiology & Infectious Diseases 3:4-9.
Duke J. & Wain K. 1981. Medicinal Plants of the world, Computer index with more than 85.000 entries In: Handbook of Medicinal Herbs. CRC press, Boca Raton, Florida.
Duke J. A. 2008. Duke's Handbook of Medicinal Plants of Latin America. Taylor & Francis.
Egorov T. A. & Odintsova T. 2012. Defense peptides of plant immunity. Russian Journal of Bioorganic Chemistry 38:1-9.
Ehleringer J. R.,Cook C. S. & Tieszen L. L. 1986a. Comparative water use and nitrogen relationships in a mistletoe and its host Oecologia 68:279-84.
Ehleringer J. R. & Schulze E. D. 1985a. Mineral concentrations in an autoparasitic *Phoradendron californicum* growing on a parasitic *P. californicum* and its host *Cercidium floridum* American Journal of Botany 72:568-71.
Ehleringer J. R., Schulze E. D., Ziegler H., Lange O. L., Farquhar G. D. & Cowar I. R. 1985b. Xylem tapping mistletoes - Water or nutrient parasites. Science 227:1479-81.
Ehleringer J. R., Ullmann I., Lange O. L., Farquhar G. D., Cowan I. R., Schulze E. D. & Ziegler H. 1986b. Mistletoes: a hypothesis concerning morphological and chemical avoidance of herbivory. Oecologia 70:234-37.
Eichinger D. 2001. Encystation in parasitic protozoa. Curr Opin Microbiol 4:421-6.
Eifler R., Pfüller K., Pfüller U. & Gockeritz W. 1993. Improved procedures for isolation of mistletoe lectins and their subunits: Lectin pattern of the european mistletoe. Pages 144-51 *in* J. Basu, Kundu M., and Chakrabarti P., Editors. Lectins: Biology, Biochemistry, Clinical Biochemistry Wiley Eastern Ltd. (India).
Ekinci N., Ekinci R., Polat P. & Budak G. 2004. Analysis of trace elements in medicinal plants with energy dispersive x -ray fluorescence. Journal of Radioanalytical and Nuclear Chemistry 260:127-31.
El-Ghorab A. H.,Mansour A. F. & El-massry K. F. 2004. Effect of extraction methods on the chemical composition and antioxidant activity of Egyptian marjoram (*Majorana hortensis* Moench). Flavour and Fragrance Journal 19:54-61.
El-Sherbini G. T. & Osman S. M. 2013. Anthelmintic activity of unripe *Mangifera indica* L.(Mango) against *Strongyloides stercoralis*. Int. J. Curr. Microbiol. App. Sci 2:401-09.
El Sohly H. N., Joshi A. S., Nimrod A. C., Walker L. A. & Clark A. M. 2001. Antifungal chalcones from *Maclura tinctoria*. Planta Med 67:87-9.
Elluru S., Van Huyen J. P. D., Delignat S., Prost F., Bayry J., Kazatchkine M. D. & Kaveri S. V. 2006. Molecular mechanisms underlying the immunomodulatory effects of mistletoe (*Viscum album* L.) extracts Iscador - Review. Arzneimittel-Forschung-Drug Research 56:461-66.

Eloff J. N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med 64:711-3.
Endo Y., Oka T., Tsurugi K. & Franz H. 1989. The mechanism of action of the cytotoxic lectin from *Phoradendron californicum*: the RNA N-glycosidase activity of the protein. FEBS Lett. 248:115-8.
Endo Y.,Tsurugi K. & Franz H. 1988. The site of action of the A-chain of mistletoe lectin I on eukaryotic ribosomes. The RNA N-glycosidase activity of the protein. FEBS Lett 231:378–80.
Enesel M. B., Acalovschi I., Grosu V., Sbarcea A., Rusu C., Dobre A., Weiss T. & Zarkovic N. 2005. Perioperative application of the *Viscum album* extract Isorel in digestive tract cancer patients. Anticancer Research 25:4583-90.
Eno A. E., Ofem O. E., Nku C. O., Ani E. J. & Itam E. H. 2008. Stimulation of insulin secretion by *Viscum album* (mistletoe) leaf extract in streptozotocin-induced diabetic rats. Afr J Med Med Sci 37:141-7.
Escher P., Eiblmeier M., Hetzger I. & Rennenberg H. 2004a. Seasonal and spatial variation of carbohydrates in mistletoes (*Viscum album*) and the xylem sap of its hosts (*Populus x euamericanna and Abies alba*). Physiologia Plantarum 120:212-19.
Escher P., Eiblmeier M., Hetzger I. & Rennenberg H. 2004b. Spatial and seasonal variation in amino compounds in the xylem sap of a mistletoe (*Viscum album*) and its hosts (*Populus* spp. and *Abies alba*). Tree Physiology 24:639-50.
Eshel G., Levy G. J., Mingelgrin U. & Singer M. J. 2004. Critical evaluation of the use of Laser Diffraction for Particle-Size Distribution Analysis. Soil Sci Soc Am J 68:736-43.
Evangelista V. 2005. From Cells to Proteins: Imaging Nature across Dimensions: Proceedings of the NATO Advanced Study Institute, Held in Pisa, Italy, 12-23 September 2004. Springer.
Evans W. C. 2009a. Alkaloids. Pages 353-415 Trease and Evans' Pharmacognosy. Saunders Elsevier, London.
Evans W. C., Editor. 2009b. Trease and Evans Pharmacognosy 16th edition. Saunders, Elsevier, London.
Fashing P. J.,Dierenfeld E. S. & Mowry C. B. 2007. Influence of Plant and Soil Chemistry on Food Selection, Ranging Patterns, and Biomass of *Colobus guereza* in Kakamega Forest, Kenya. International Journal of Primatology 28:673-703.
Favaro G., Clementi C., Romani A. & Vickackaite V. 2007. Acidichromism and Ionochromism of Luteolin and Apigenin, the Main Components of the Naturally Occurring Yellow Weld: A Spectrophotometric and Fluorimetric Study. Journal of Fluorescence 17:707-14.
Favreaua A., Baumonta R., Ferreirab G., Dumonta B. & Ginanea G. 2010. Do sheep use umami and bitter tastes as cues of post-ingestive consequences when selecting their diet? Applied Animal Behaviour Science 125:115–23.
Fawole O. A., Ndhlala A. R., Amoo S. O., Finnie J. W. & Van Staden J. 2009. Anti-inflammatory and phytochemical properties of twelve medicinal plants used for treating gastro-intestinal ailments in South Africa. Journal of Ethnopharmacology 123:237-43.
Fedigan L. M.,Fedigan L. & Champman C. 1985. A census of *Alouatta palliata* and *Cebus capucinus* monkeys in Santa Rosa National Park, Costa Rica. Brenesia 23:309-22.
Fedigan, L. M., L. M. Rose, and R. M. Avila. 1998. Growth of Mantled Howler Groups in a Regenerating Costa Rican Dry Forest. International Journal of Primatology 19.
Fedigan L. M. & Jack K. M. 2012. Tracking Neotropical Monkeys in Santa Rosa:Lessons from a Regenerating Costa Rican Dry Forest. Page 460 in Peter M. Kappeler and Watts D. P., editors. Long-Term Field Studies of Primates. Springer.
Feleke S. 2013. Site factor on nutritional content of *Arundinaria alpina* and *Oxytenanthera abyssinica* bamboo shoots in Ethiopia. Journal of Horticulture and Forestry 5:115-21.
Felton A. M., Felton A., Wood J. T. & Lindenmayer D. B. 2008. Diet and feeding ecology of *Ateles chamek* in a Bolivian semi-humid forest: The importance of *Ficus* as a staple food resource. International Journal of Primatology 29:379-403.
Ferdous A.,Imam M. Z. & Ahmed T. 2010. Antioxidant, Antimicrobial and Cytotoxic Activities of Samanea saman (Jacq.) Merr. Stamford Journal of Pharmaceutical Sciences 3:11-17.
Fernandez T., Cerda Zolezzi P., Aulicino P., Caldas Lopes E., Wagner M., Ricco R., Hajos S., Gurni A. & Alvarez E. 2003. Immunobiological features of the galactoside lectin L-Lc isolated from the Argentine mistletoe *Ligaria cuneifolia*. J Ethnopharmacol 85:81-92.
Fernandez T., Wagner M. L., Varela B. G., Ricco R. A., Hajos S. E., Gurni A. A. & Alvare E. 1998. Study of an Argentine mistletoe, the hemiparasite *Ligaria cuneifolia* (R. et P.) Tiegh. (Loranthaceae). Journal of Ethnopharmacology 62:25-34.
Ferrari S. F.,Veiga L. A. & Urbani B. 2008. Geophagy in New World Monkeys (*Platyrrhini*): Ecological and Geographic Patterns. Folia Primatologica 79:402–15.
Ferrazzano G. F., Amato I., Ingenito A., De Natale A. & Pollio A. 2009. Anti-cariogenic effects of polyphenols from plant stimulant beverages (cocoa, coffee, tea). Fitoterapia 80:255-62.
Ferreres F., Pereira D. M., Valentao P., Andrade P. B., Seabra R. M. & Sottomayor M. 2008. New phenolic compounds and antioxidant potential of *Catharanthus roseus*. J Agric Food Chem 56:9967-74.
Finley-Jones H. J.,Molloyx J. L. & Holcombe J. A. 2008. Choosing internal standards based on a multivariate analysis approach with ICP(TOF)MS. Journal of Analytical Atomic Spectrometry 23:1214–22.
Fioramonti J.,Droy-Lefaix M. T. & Buéno L. 1987a. Changes in Gastro-Intestinal Motility Induced by Cholera Toxin and Experimental Osmotic Diarrhoea in Dogs: Effects of Treatment with an Argillaceous Compound. Digestion 36:230-37.
Fioramonti J.,Fargeas M. J. & Bueno L. 1987b. Action of t-2 toxin on gastrointestinal transit in mice: Protective effect of an

argillaceous compound. Toxicology Letters 36:227-32.
Firon N.,Ofek I. & Sharon N. 1983. Carbohydrate specificity of the surface lectins of *Escherichia coli, Klebsiella pneumoniae,and Salmonella typhimurium*. Carbohydrate Research 120:235-49.
Fischer U., Jaksch A., Carle R. & Kammerer D. 2013. Influence of origin source, different fruit tissue and juice extraction methods on anthocyanin, phenolic acid, hydrolysable tannin and isolariciresinol contents of pomegranate (*Punica granatum* L.) fruits and juices. European Food Research and Technology 237:209-21.
Florack D. E. & Stiekema W. J. 1994. Thionins: properties, possible biological roles and mechanisms of action. Plant Mol Biol 26:25-37.
Fogg F. J. J., Hutton D. A., Jumel K., Pearson J. P., Harding S. E. & Allen A. 1996. Characterization of pig colonic mucins. Biochem. J. 316:937-42.
Fontes M. P. F. & Carvalho I. A. 2005. Color Attributes and Mineralogical Characteristics, Evaluated by Radiometry, of Highly Weathered Tropical Soils. Soil Sci. Soc. Am. J. 69:1162-72.
Foo L. Y. & Porter L. J. 1980. The phytochemistry of proanthocyanidin polymers. Phytochemistry 19:1747-54.
Fowler A.,Koutsioni Y. & Sommer V. 2007. Leaf-swallowing in Nigerian chimpanzees: evidence for assumed self-medication. Primates 48:73–76.
Fragaszy D. M. & Perry S., Editors. 2003. The Biology of Traditions: Models and Evidence. Cambridge University Press, Cambridge.
Fragaszy D. M.,Visalbergi E. & Fedigan L. M. 2005. The Complete Capuchin - The Biology of the Genus *Cebus*. Cambridge University Press.
Frank O.,Zehentbauer G. & Hofmann T. 2006. Screening and identification of bitter compounds in roasted coffee brew by taste dilution analysis. Pages 165-68 *in* L. P. B. Wender and Mikael Agerlin P., Editors. Developments in Food Science. Elsevier.
Franz H. 1986. Mistletoe lectins and their A and B chains. Oncology 43 Suppl 1:23-34.
Franz H.,Ziska P. & Kindt A. 1981. Isolation and properties of 3 lectins from mistletoe (*Viscum album* L.). Biochemical Journal 195:481-84.
Franz M., Vollmer S., Wacker R., Jager S., Scheer R., Stoeva S. & Voelter W. 2004. Isolation and quantification of chitin-binding mistletoe lectin from mistletoe extracts and validation of this method. Arzneimittel-Forschung-Drug Research 54:230-39.
Freeland W. J. & Janzen D. H. 1974. Strategies in Herbivory by Mammals: The Role of Plant Secondary Compounds. The American Naturalist 108:269-89.
Frohnmeyer H. & Staiger D. 2003. Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection. Plant Physiology 133:1420-28
Gaballa A. & Helmann J. D. 2010. Iron uptake in Gram-positive (*Bacillus*) *in* P. Cornelis and Andrews S. C., Editors. Iron Uptake and Homeostasis in Microorganisms. Caister Academic Press.
Gad H. A., El-Ahmady S. H., Abou-Shoer M. I. & Al-Azizi M. M. 2013. Application of chemometrics in authentication of herbal medicines: a review. Phytochemical Analysis 24:1-24.
Gaidamashvili M. & van Staden J. 2002. Interaction of lectin-like proteins of South African medicinal plants with *Staphylococcus aureus* and *Bacillus subtilis*. Journal of Ethnopharmacology 80:131-35.
Galán E. 2006. Chapter 14 Genesis of Clay Minerals. Pages 1129-62 *in* B. K. G. T. Faïza Bergaya and Gerhard L., Editors. Developments in Clay Science. Elsevier.
Gallina S. 1988. Importance of mistletoe (*Phoradendron Spp*.) in Deer Diet. Southwestern Naturalist 33:21-25.
Gallo S. A., Wang W., Rawat S. S., Jung G., Waring A. J., Cole A. M., Lu H., Yan X., Daly N. L. & Craik D. J. 2006. θ-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. Journal of Biological Chemistry 281:18787-92.
Gálvez M.,Martín-Cordero C. & Ayuso M. J. 2006. Pharmacological activities of phenylpropanoids glycosides. Pages 675-718 *in* R. Atta-ur, Editor. Studies in Natural Products Chemistry. Elsevier.
Gan F. & Ye R. 2006. New approach on similarity analysis of chromatographic fingerprint of herbal medicine. Journal of Chromatography A 1104:100-05.
Ganzhorn J. U. 1987. Soil consumption of two groups of semi-free ranging lemurs (*Lemur catta and Lemur fulvus)* Ethology 74:146-54.
García-Olmedo F. 1999. Thionins. Pages 709-26 *in* P. Shewry and Casey R., Editors. Seed Proteins. Springer Netherlands.
Garciá-Olmedo F., Rodríguez-Palenzuéla P., Hernández-Lucas C., Ponz F., Maraña C., Carmona M. J., Lopez-Fando J., Fernandez J. A. & Carbonero P. 1989. The thionins: a protein family that includes purothionins, viscotoxins and crambins. Oxford Surveys of Plant Molecular & Cell Biology 6:31-60.
Garcia A. E., Ösapay G., Tran P. A., Yuan J. & Selsted M. E. 2008. Isolation, Synthesis, and Antimicrobial Activities of Naturally Occurring θ-Defensin Isoforms from Baboon Leukocytes. Infection and Immunity 76:5883-91.
Gautam M. K., Gangwar M., Nath G., Rao C. V. & Goel R. K. 2012. *In–vitro* antibacterial activity on human pathogens and total phenolic, flavonoid contents of *Murraya paniculata* Linn. leaves. Asian Pacific Journal of Tropical Biomedicine 2:S1660-S63.
Gazel E.,Denyer P. & Baumgartner P. O. 2006. Magmatic and geotectonic significance of Santa Elena Peninsula, Costa Rica Geologicaacta 4:193-202.
Gazzani G.,Daglia M. & Papetti A. 2012. Food components with anticaries activity. Current Opinion in Biotechnology 23:153-59.
Geissler P. W., Mwaniki D., Thiong F. & Friis H. 1998. Geophagy as a risk factor for geohelminth infections: a longitudinal study of Kenyan primary schoolchildren. Trans R Soc Trop Med Hyg 92:7-11.

Ghanem S. J., Ruppert H., Kunz T. H. & Voigt C. C. 2013. Frugivorous bats drink nutrient- and clay-enriched water in the Amazon rain forest: support for a dual function of mineral-lick visits. Journal of Tropical Ecology 29:1-10.
Gilardi J. D., Duffey S. S., Munn C. A. & Tell L. A. 1999. Biochemical functions of geophagy in parrots: Detoxification of dietary toxins and cytoprotective effects. Journal of Chemical Ecology 25:897-922.
Gillespie T. R. & Chapman C. A. 2006. Prediction of parasite infection dynamics in primate metapopulations based on attributes of forest fragmentation. Conservation Biology 20:441-48.
Gillespie T. R. & Chapman C. A. 2008a. Forest fragmentation, the decline of an endangered primate, and changes in host–parasite interactions relative to an unfragmented forest. American Journal of Primatology 70:222-30.
Gillespie T. R.,Nunn C. L. & Leendert F. L. 2008b. Integrative Approaches to the Study of Primate Infectious Disease: Implications for Biodiversity Conservation and Global Health. Yearbook of Physical Anthropology 51:53-69.
Gimenez M. C., Hidalgo M., Petenatti E., Del Vitto L., Marchevsky E. & Pellerano R. 2012. Caracterización multivariable de "muérdago criollo", *Ligaria cuneifolia* (Loranthaceae) de acuerdo a su composición mineral. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 11.
Gioacchini A. M., Roda A., Galletti G. C., Bocchini P., Manetta A. C. & Baraldini M. 1996. High-performance liquid chromatographic-electrospray mass spectrometric analysis of phenolic acids and aldehydes. Journal of Chromatography A 730:31-37.
Giudici A. M., Regente M. C., Villalain J., Pfuller K., Pfuller U. & de la Canal L. 2004. Mistletoe viscotoxins induce membrane permeabilization and spore death in phytopathogenic fungi. Physiologia Plantarum 121:2-7.
Giudici M., Pascual R., de la Canal L., Pfuller K., Pfuller U. & Villalain J. 2003. Interaction of viscotoxins A(3) and B with membrane model systems: Implications to their mechanism of action. Biophysical Journal 85:971-81.
Glander K. E. 1977. Secondary compounds and feeding behavior of leaf eating primates. American Journal of Physical Anthropology 47:133-33.
Glander K. E. 1982. The Impact of Plant Secondary Compounds on Primate Feeding Behavior. Yearbook of Physical Anthropology 25:1-18.
Glander K. E. 1994. Non-human primate Self-medication with wild plant foods. Pages 227-39 *in* N. L. Etkin, Editor. Eating on the Wildside. University of Arizona Press, Tuscon & London.
Glanfield A., McManus D. P., Anderson G. J. & Jones M. K. 2007. Pumping iron: a potential target for novel therapeutics against schistosomes. Trends in Parasitology 23:583-88.
Glatzel G. 1983. Mineral Nutrition and Water Relations of Hemiparasitic Mistletoes: A Question of Partitioning. Experiments with *Loranthus europaeus* on *Quercus petraea* and *Quercus robur*. Oecologia 56:193-201.
Glatzel G. & Geils B. W. 2009. Mistletoe ecophysiology: host-parasite interactions. Botany-Botanique 87:10-15.
Goh T. B. & Huang P. M. 1986. Influence of citric and tannic acids on hydroxy-Al interlayering in montmorillonite. Clays and Clay Minerals, 34:37-44.
Gonzalez-Moreno O., Hernandez-Aguilar R. A., Piel A., Stewart F., Gracenea M. & Moore J. 2013. Prevalence and climatic associated factors of *Cryptosporidium* sp. infections in savanna chimpanzees from Ugalla, Western Tanzania. Parasitology Research 112:393-99.
Gonzalez-Zamora A., Arroyo-Rodriguez V., Chaves O. M., Sanchez-Lopez S., Aureli F. & Stoner K. E. 2011. Influence of climatic variables, forest type and condition on activity patterns of Geoffroyi's Spider monkeys throughout Mesoamerica. American Journal of Primatology 73:1189-98.
Goodarzi M.,Russell P. J. & Vander Heyden Y. 2013. Similarity analyses of chromatographic herbal fingerprints: A review. Analytica Chimica Acta 804:16-28.
Gould K.,Davies K. M. & Winefield C. 2008. Anthocyanins: Biosynthesis, Functions, and Applications. Springer.
Graczyk T. K., Bosco-Nizeyi J., Ssebide B., Thompson R. C. A., Read C. & Cranfield M. R. 2002. Anthropozoonotic *Gardia duodenalis* genotype (assemblage) A infections in habitats of free ranging human-habituated gorillas, Uganda. Journal of Parasitology 88:905-09.
Graus Y. M. F., Smit H. F., Osterhaus A. D. M. E. & Hageman R. J. J. 2003. Chlorogenic acid and an analogue thereof for immune system stimulation.*in* U. S. patent, Editor. Nutricia N.V., Zoetermeer (NL).
Guaní-Guerra E., Santos-Mendoza T., Lugo-Reyes S. O. & Terán L. M. 2010. Antimicrobial peptides: General overview and clinical implications in human health and disease. Clinical Immunology 135:1-11.
Guerra-Silveira F. & Abad-Franch F. 2013. Sex bias in infectious disease epidemiology: patterns and processes. Plos One 8:e62390.
Guerra M. B. B., Schaefer C., de Carvalho G. G. A., de Souza P. F., Santos D., Nunes L. C. & Krug F. J. 2013. Evaluation of micro-energy dispersive X-ray fluorescence spectrometry for the analysis of plant materials. Journal of Analytical Atomic Spectrometry 28:1096-101.
Hagerman A. E. & Butler L. G. 1978. Protein Precipitaion Method for the Quantitative Determination of Tannins. J.Agric.Food Chem. 26:809-12.
Hagerman A. E. & Butler L. G. 1981. The specificity of proanthocyanidin-protein interactions. Journal of Biological Chemistry 256:4494-97.
Hagerman A. E. 2002. The Tannin Handbook. Miami University, Oxford OH 45056.
Hannah R. S., Vogel T. A., Patino L. C., Alvarado G. E., Perez W. & Smith D. R. 2002. Origin of silicic volcanic rocks in Central Costa Rica: a study of a chemically variable ash-flow sheet in the Tiribi Tuff. Bulletin of Volcanology 64:117-33.
Hajto T., Berki T., Boldizsar F. & Nemeth P. 2003. Galactoside-specific plant lectin, *Viscum album* agglutinin-I induces enhanced proliferation and apoptosis of murine thymocytes *in vivo.* Immunology Letters 86:23-27.
Hajto T., Hostanska K., Frei K., Rordorf C. & Gabius H.-J. 1990. Increased Secretion of Tumor Necrosis Factor α, Interleukin 1, and Interleukin 6 by Human Mononuclear Cells Exposed to β-Galactoside-specific Lectin from

Clinically Applied Mistletoe Extract. Cancer Research 50:3322-26.
Hajto T.,Hostanska K. & Gabius H. J. 1989. Modulatory potency of the β-galactoside-specific lectin from mistletoe extract (Iscador) on the host defence system *in vivo* rabbits and patients. Cancer Research 49:4803-08.
Hall A. H.,Spoerke D. G. & Rumack B. H. 1986. Assessing Mistletoe Toxicity. Annals of Emergency Medicine 15:1320-23.
Hall L. J., Murphy C. T., Hurley G., Quinlan A., Shanahan F., Nally K. & Melgar S. 2013. Natural Killer Cells Protect against Mucosal and Systemic Infection with the Enteric Pathogen *Citrobacter rodentium*. Infection and Immunity 81:460-69.
Hall N. T., Smoot J. M., Knight R. J. & Nagy S. 1980. Protein and amino acid compositions of ten tropical fruits by gas-liquid chromatography. Journal of Agricultural and Food Chemistry 28:1217-21.
Hallberg L. 1974. The pool concept in food iron absorption and some of its implications. Proc Nutr Soc 33:285-91.
Hallberg L.,Brune M. & Rossander L. 1986. Effect of ascorbic acid on iron absorption from different types of meals. Studies with ascorbic-acid-rich foods and synthetic ascorbic acid given in different amounts with different meals. Hum Nutr Appl Nutr 40:97-113.
Hancock R. E. W. & Lehrer R. 1998. Cationic peptides: a new source of antibiotics. Trends in Biotechnology 16:82-88.
Hanhineva K., Törrönen R., Bondia-Pons I., Pekkinen J., Kolehmainen M., Mykkänen H. & Poutanen K. 2010. Impact of dietary polyphenols on carbohydrate metabolism. International Journal of Molecular Sciences 11:1365-402.
Hannah R. S., Vogel T. A., Patino L. C., Alvarado G. E., Perez W. & Smith D. R. 2002. Origin of silicic volcanic rocks in Central Costa Rica: a study of a chemically variable ash-flow sheet in the Tiribi Tuff. Bulletin of Volcanology 64:117-33.
Harborne J. B. 1989a. General procedures and measurement of Total Phenolics. Pages 1-28 *in* P. M. Dey and Harborne J. B., Editors. Methods in Plant Biochemistry. Academic Press Ltd, London.
Harborne J. B., Editor. 1989b. Methods in Plant Biochemistry. Academic press Limited, London.
Harborne J. B. 1998a. Phytochemical Methods A Guide to Modern Techniques of Plant Analysis. Springer.
Harborne J. B. 1998b. The Terpenoids. Pages 89-131 Phytochemical Methods A Guide to Modern Techniques of Plant Analysis. Chapman & Hall, London.
Harnly J. M.,Bhagwat S. & Lin L. Z. 2007. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. Anal Bioanal Chem 389:47-61.
Harris T. R.,Chapman C. A. & Monfort S. L. 2010. Small folivorous primate groups exhibit behavioral and physiological effects of food scarcity. Behavioral Ecology 21:46-56.
Hart B. L. 2005. The evolution of herbal medicine: behavioural perspectives. Animal Behaviour 70:975-89.
Hart B. L. 2011. Behavioural defences in animals against pathogens and parasites: parallels with the pillars of medicine in humans. Philosophical Transactions of the Royal Society B: Biological Sciences 366:3406-17.
Harvala E.,Exner J. & Becker H. 1984. Flavonoids of *Loranthus europaeus*. Journal of Natural Products 47:1054-55.
Haslam E. 2007. Vegetable tannins – Lessons of a phytochemical lifetime. Phytochemistry 68:2713-21.
Hatano T., Kusuda M., Inada K., Ogawa T. O., Shiota S., Tsuchiya T. & Yoshida T. 2005. Effects of tannins and related polyphenols on methicillin-resistant *Staphylococcus aureus*. Phytochemistry 66:2047-55.
Haydel S. E.,Remenih C. M. & Williams L. B. 2008. Broad-spectrum *in vitro* antibacterial activities of clay minerals against antibiotic-susceptible and antibiotic-resistant bacterial pathogens. J Antimicrob Chemother 61:353-61.
Hazrati E., Galen B., Lu W., Wang W., Ouyang Y., Keller M. J., Lehrer R. I. & Herold B. C. 2006. Human α-and β-defensins block multiple steps in herpes simplex virus infection. The Journal of Immunology 177:8658-66.
Hedges J. I. & Hare P. E. 1987. Amino acid adsorption by Clay Minerals in distilled water. Geochimica et Cosmochimica Acta 51:255-59.
Heim K. E.,Tagliaferro A. R. & Bobilya D. J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry 13:572-84.
Helixor®. 2011. Integrative Mistletoe Therapy with Helixor® - Basics of scientific approval Helixor Heilmittel GmbH & Co. KG., Rosenfeld.
Hemaiswarya S., Rohini S., Narasumani M. L. & Doble M. 2011. Phenylpropanoids inhibit protofilament formation of *Escherichia coli* cell division protein FtsZ. Journal of Medical Microbiology 60:1317-25.
Hemmerle H., Burger H.-J., Below P., Schubert G., Rippel R., Schindler P. W., Paulus E. & Herling A. W. 1997. Chlorogenic acid and synthetic chlorogenic acid derivatives: novel inhibitors of hepatic glucose-6-phosphate translocase. Journal of Medicinal Chemistry 40:137-45.
Hemmes R. B.,Alvarado A. & Hart B. L. 2002. Use of California bay foliage by wood rats for possible fumigation of nest-borne ectoparasites. Behavioral Ecology 13:381-85.
Henderson C. L. 2010. Birds of Costa Rica: A Field Guide. University of Texas Press.
Hendrich A. B. 2006. Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. Acta Pharmacol Sin 27:27-40.
Hendricks S. B. 1941. Base Exchange of the Clay Mineral Montmorillonite for Organic Cations and its Dependence upon Adsorption due to van der Waals Forces. The Journal of Physical Chemistry 45:65-81.
Henry J. & Kwong A. M. 2003. Why is geophagy treated like dirt? Deviant Behavior 24:353-71.
Henry T. A. 1949. The Plant Alkaloids. 4th edition. J. & A. Churchill Ltd., London.
Hernández-Orduño G., Torres-Acosta J. F. J., Sandoval-Castro C. A., Aguilar-Caballero A. J., Capetillo-Leal C. M. & Alonso-Díaz M. A. 2012. In cafeteria trials with tannin rich plants, tannins do not modify foliage preference of goats with browsing experience. Ethology Ecology & Evolution 24:332-43.
Hernandez V., Malafronte N., Mora F., Pesca M. S., Aquino R. P. & Mencherini T. 2014. Antioxidant and antiangiogenic activity of *Astronium graveolens* Jacq. leaves. Nat Prod Res.
Hernández V., Mora F., Araque M., Montijo de S., Rojas L., Meléndez P. & Tommasi de N. 2013. Chemical composition

and antibacterial activity of *Astronium graveolens* Jacq. essential oil. Revista Latinoamericana de Química 41:89-94.
Herrera C. M. 1981. Are tropical fruits more rewarding to dispersers than temperate ones? . Am. Nat. 118:896-907.
Hespanhol H., Fabón G., Monforte L., Martínez-Abaigar J. & Núñez-Olivera E. 2014. Among- and within-genus variability of the UV-absorption capacity in saxicolous mosses. The Bryologist 117:1-9.
Heymann E. & Hartmann G. 1991. Geophagy in moustached tamarins, *Saguinus mystax* (platyrrhini: Callitrichidae), at the Río Blanco, Peruvian Amazonia Primates 32:533-37.
Hiramatsu C., Melin A. D., Aureli F., Schaffner C. M., Vorobyev M., Matsumoto Y. & Kawamura S. 2008. Importance of Achromatic Contrast in Short-Range Fruit Foraging of Primates. Plos One 3.
Hiramatsu C., Tsutsui T., Matsumoto Y., Aureli F., Feddigan L. M. & Kawamura S. 2005. Color vision polymorphism in wild capuchins (*Cebus capuchinus*) and Spider Monkeys (*Ateles geoffroyi*) in Costa Rica. American Journal of Primatology 67:447-61.
Hladik C. M. 1977. Chimpanzees of Gabon and chimpanzees of Gombe: some comparative data on diet. Pages 481-501 *in* T. H. Clutton-Brock, Editor. Primate Ecology: Studies on Feeding and Ranging Behaviour in Lemurs, Monkeys and Apes. Academic press, London.
Ho L., Varghese M., Wang J., Zhao W., Chen F., Knable L. A., Ferruzzi M. & Pasinetti G. M. 2012. Dietary supplementation with decaffeinated green coffee improves diet-induced insulin resistance and brain energy metabolism in mice. Nutritional Neuroscience 15:37-45.
Hoessli D. C. & Ishtiaq A. 2008. Mistletoe Lectins: Carbohydrate-Specific Apoptosis Inducers and Immunomodulators. Current Organic Chemistry 12:918-25.
Holdo R. M.,Dudley J. P. & McDowell L. R. 2002. Geophagy in the African elephant in relation to availability of dietary sodium. Journal of Mammalogy 83:652-64.
Holdo R. M. & McDowell L. R. 2004. Termite mounds as nutrient-rich food patches for elephants. Biotropica 36:231-39.
Holt R. R., Lazarus S. A., Sullards M. C., Zhu Q. Y., Schramm D. D., Hammerstone J. F., Fraga C. G., Schmitz H. H. & Keen C. L. 2002. Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. The American Journal of Clinical Nutrition 76:798-804.
Hosseini P. R.,Dhondt A. A. & Dobson A. 2004. Seasonality and wildlife disease: how seasonal birth, aggregation and variation in immunity affect the dynamics of *Mycoplasma gallisepticum* in house finches. Proc. R. Soc. Lond. B 271:2569-77.
Hoste H., Jackson F., Athanasiadou S., Thamsborg S. M. & Hoskin S. O. 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. Trends in Parasitology 22:253-61.
Hostettmann K.,Marston A. & Hostettmann M. 1998. Preparative Chromatography Techniques: Applications in Natural Product Isolation. Springer.
Hsiung C.-S., Andrade J. D., Costa R. & Owen Ash K. 1997. Minimizing interferences in the quantitative multielement analysis of trace elements in biological fluids by inductively coupled plasma mass spectrometry Clinical Chemistry 43.
Huang P. M. 1995. The Role of short range ordered Mineral Colloids in Abiotic Transformation of organic components in the environment.*in* J. B. P. M. Huang, Jean-Marc Bollag, William B. McGill, Editor. Environmental Impacts of Soil Component Interactions: Land Quality, Natural and Anthropogenic Organics. CRC Press, Taylor & Francis Inc, Bosa Roca/US.
Hubbell S. P. 1979. Tree Dispersion, Abundance, and Diversity in a Tropical Dry Forest. Science, New Series, 203:1299-309.
Huber R., Claßen K., Werner M. & Klein R. 2006. *In vitro* Immunoreactivity towards Lectin-rich or Viscotoxin-rich Mistletoe (Viscum album L.) Extracts Iscador Applied to Healthy Individuals. Arzneimittelforschung 56:447-56.
Hucker H. B. 1970. Species Differences in Drug Metabolism. Annual Review of Pharmacology 10:99-118.
Huffman M. A. 1997. Current Evidence for Self-Medication in Primates: A Multidisciplinary Perspective. Yearbook of Physical Anthropology 40:171-200.
Huffman M. A. 2003. Animal self-medication and ethno-medicine: exploration and exploitation of the medicinal properties of plants. Proceedings of the Nutrition Society 62:371-81.
Huffman M. A. 2005. The Study of Primate Self-Medication - A collection of multidisciplinary research work by members of The C.H.I.M.P.P. Group* (the first 18 years, 1987 – 2005). Primate Research Institute, Kyoto University, Kyoto.
Huffman M. A., Gotoh S., Turner L. A., Hamai M. & Yoshida K. 1997. Seasonal trends in intestinal nematode infection and medicinal plant use among chimpanzees in the Mahale Mountains, Tanzania. Primates 38:111-25.
Huffman M. A. & Seifu M. 1989. Observations on the illness and consumption of a possibly medicinal plant *Vernonia amygdalina* (DEL), by a wild chimpanzee in the Mahale Mountains National Park, Tanzania. Primates 30:51-63.
Hughes A. L., Green J. A., Piontkivska H. & Roberts R. M. 2003. Aspartic Proteinase Phylogeny and the Origin of Pregnancy-Associated Glycoproteins. Molecular Biology and Evolution 20:1940-45.
Hund-Rinke K. & Kordel W. 2003. Underlying issues in bioaccessibility and bioavailability: experimental methods. Ecotoxicology and Environmental Safety 56:52-62.
Hunt J. W., Dean A. P., Webster R. E., Johnson G. N. & Ennos A. R. 2008. A novel mechanism by which silica defends grasses against herbivory. Ann Bot 102:653-6.
Hunter J. M. 1973. Geophagy in Africa and in the United States: A Culture-Nutrition Hypothesis. Geographical Review 63:170-95.
Hunter J. M. 1984. Geophagy in Central Ameria. Geographical Review 74:157-69.
Hutchings M. R., Athanasiadou S., Kyriazakis I. & Gordon I. J. 2003. Can animals use foraging behaviour to combat parasites? Proceedings of the Nutrition Society 62:361-70.

Hwang K. Y., Song H. K., Chang C., Lee J. S., Lee S. Y., Kim K. K., Choe S., Sweet R. M. & Suh S. W. 1997. Crystal structure of thermostable alpha-amylase from *Bacillus licheniformis* refined at 1.7 A resolution. Mol Cells. 30:251-8.

Iason G. R. & Villalba J. J. 2006. Behavioral strategies of mammal herbivores against plant secondary metabolites: The avoidance-tolerance continuum. Journal of Chemical Ecology 32:1115-32.

Ibrahim R. & Barron D. 1989. Phenylpropanoids. Pages 75-111 *in* A. J. Harborne, Editor. Plant Phenolics. Academic Press, London.

Ikhsan J., Johnson B. B., Wells J. D. & Angove M. J. 2004. Adsorption of aspartic acid on kaolinite. Journal of Colloid and Interface Science 273:1-5.

Iqbal Z., Sarwar M., Jabbar A., Ahmed S., Nisa M., Sajid M. S., Khan M. N., Mufti K. A. & Yaseen M. 2007. Direct and indirect anthelmintic effects of condensed tannins in sheep. Veterinary Parasitology 144:125-31.

Irwin M. T. 2008. Feeding ecology of *Propithecus didema* in forest fragments and continuous forest. Int J Primatol 29:95-115.

Islam R., Khurshid Alam A. H. M., Hossain M. A., Mosaddik M. A. & Sadik G. 2004. Biological screening of Bangladeshi mango mistletoe bark extracts. Fitoterapia 75:405-08.

Itoigawa M., Ito C., Tokuda H., Enjo F., Nishino H. & Furukawa H. 2004. Cancer chemopreventive activity of phenylpropanoids and phytoquinoids from Illicium plants. Cancer Letters 214:165-69.

The IUCN Red List of Threatened Species. Version 2014.2. <www.iucnredlist.org>. Downloaded on 06 October 2014.

Izawa K. 1993. Soil-Eating by *Alouatta* and *Ateles spp.* International Journal of Primatology 14:229-42.

Izhaki I. 1998. Essential Amino Acid Composition of Fleshy Fruits Versus Maintenance Requirements of Passerine Birds. Journal of Chemical Ecology 24:1333-45.

Jacobs A. & Greenman D. A. 1969. Availability of Food Iron. BMJ 1:673-76.

Jacobs G. H. 2007. New World Monkeys and Color. International Journal of Primatology 28:729-59.

Jacobs M., Laws S., Willett K., Schmieder P., Odum J. & Bovee T. F. 2013. *In vitro* metabolism and bioavailability tests for endocrine active substances: What is needed next for regulatory purposes? Altex 30:331 – 51.

Jaiswal R., Sovdat T., Vivan F. & Kuhnert N. 2010. Profiling and Characterization by LC-MSn of the Chlorogenic Acids and Hydroxycinnamoylshikimate Esters in Maté *(Ilex paraguariensis*). Journal of Agricultural and Food Chemistry 58:5471-84.

Jamkhande P. G., Barde S. R., Patwekar S. L. & Tidke P. S. 2013. Plant profile, phytochemistry and pharmacology of *Cordia dichotoma* (Indian cherry): A review. Asian Pacific Journal of Tropical Biomedicine 3:1009–12.

Jansen M. A.,Gaba V. & Greenberg B. M. 1998. Higher plants and UV-B radiation: balancing damage, repair and acclimation. Trends in Plant Science 3:131-35.

Jander G., Norris S. R., Joshi V., Fraga M., Rugg A., Yu S., Li L. & Last R. L. 2004. Applicaton of a high-throughput HPLC-MS/MS assay to *Arabidopsis* mutant screening: evidence that threonine aldolase plays a role in seed nutritional quality. The Plant Journal 39:465-75.

Janzen D. H. 1967. Synchronization of sexual reproduction of trees within the dry season in Central America. Evolution:620-37.

Janzen D. H. 1978. Complications in interpreting the chemical defenses of trees against tropical arboreal vertebrates. The Ecology of Arboreal Folivores:73–94.

Janzen D. H. 1979. How to be a fig. Annual Review Of Ecology And Systematics 10:13-51.

Janzen D. H. & Waterman P. G. 1984. A seasonal census of phenolics, fibre and alkaloids in foliage of forest trees in Costa Rica: some factors influencing their distribution and relation to host selection by Sphingidae and Saturniidae. Biological Journal of the Linnean Society 21:439-54.

Jaynes W. F., Zartman R. E., Green C. J., San Francisco M. J. & Zak J. C. 2005. Castor oil toxin adsorption to clay minerals Clays and Clay Minerals 53:268-77.

Jeannoda V., Rakotonirina O., Randrianarivo H., Rakoto D., Wright P. & Hladik C. M. 2003. The toxic principle of the bamboo eaten by *Hapalemur aureus* is not neutralized by soil consumption. Revue D Ecologie-La Terre Et La Vie 58:151-53.

Jimenez-Estrada M., Velazquez-Contreras C., Garibay-Escobar A., Sierras-Canchola D., Lapizco-Vazquez R., Ortiz-Sandoval C., Burgos-Hernandez A. & Robles-Zepeda R. E. 2013. *In vitro* antioxidant and antiproliferative activities of plants of the ethnopharmacopeia from northwest of Mexico. BMC Complement Altern Med 13:12.

Jisaka M., Kawanaka M., Sugiyama H., K.Takegawa K., Huffman M. A., Ohigashi H. & Koshimizu K. 1992. Antischistosomal Activities of Sesquiterpene Lactones and Steroid Glucosides from *Vernonia amygdalina*, Possibly Used by Wild Chimpanzees against Parasite-related Diseases. Bioscience, biotechnology, and biochemistry Vol. 56 845-46

Johansson S., Gullbo J., Lindholm P., Ek B., Thunberg E., Samuelsson G., Larsson R., Bohlin L. & Claeson P. 2003. Small, novel proteins from the mistletoe *Phoradendron tomentosum* exhibit highly, selective cytotoxicity to human breast cancer cells. Cellular and Molecular Life Sciences 60:165-75.

Johns T. 1986. Detoxification function of geophagy and the domestication of the potato. J Chemical Ecology 12:635-46.

Johns T. & Duquette M. 1991. Detoxification and mineral supplementation as functions of geophagy. American Journal Of Clinical Nutrition 53:448-56.

Johnson L. 2008. Iron and siderophores in fungal-host interactions. Mycological Research 112:170.

Johnston K. L.,Clifford M. N. & Morgan L. M. 2003. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: glycemic effects of chlorogenic acid and caffeine. Am J Clin Nutr 78:728-33.

Jordano P. 1983. Fig-Seed Predation and Dispersal by Birds. Biotropica 15:38-41.

José A.,Sáenz R. & M. Nassar. 1971. Phytochemical screening of Costa Rican plants: Alkaloid analysis. IV. Rev. Biol.

Trop. 18.
Judd W. J. 1980. The role of lectins in blood group serology. Crit Rev Clin Lab Sci 12:171-214.
Jung U. J., Matthews B. W., Kim H. J., Lee J. S., Lee M., Kim O. H., Park E. J., Kim H. K., Jeon T. S. & Choi M. S. 2003. Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects. Clin Nutr 22:561-8.
Jürgens A., Dötterl S., Liede-Schumann S. & Meve U. 2010. Floral scent composition in early diverging taxa of Asclepiadoideae, and Secamonoideae (Apocynaceae). South African Journal of Botany 76:749 - 61.
Kabir F., Katayama S., Tanji N. & Nakamura S. 2014. Antimicrobial effects of chlorogenic acid and related compounds. Journal of the Korean Society for Applied Biological Chemistry 57:359-65.
Kalko E. K. V.,Herre E. A. & Handley Jr. C. O. 1996. Relation of fig fruit characteristics to fruit-eating bats in the New and Old World Tropics. Journal of Biogeography 23:565-76.
Karagoz A., Onay E., Arda N. & Kuru A. 2003. Antiviral potency of mistletoe *Viscum album* extracts against human parainfluenza virus type 2 in Vero cells. Phytotherapy Research 17:560-62.
Kararli T. T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharmaceutics & drug disposition 16:351-80.
Karunanidhi A., Thomas R., van Belkum A. & Neela V. 2013. *In vitro* Antibacterial and Antibiofilm Activities of Chlorogenic Acid against Clinical Isolates of *Stenotrophomonas maltophilia* including the Trimethoprim/Sulfamethoxazole Resistant Strain. BioMed Research International 2013:7.
Katalinic V., Milos M., Kulisic T. & Jukic M. 2006. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chemistry 94:550-57.
Kaur T., Singh J., Tong S., Humphrey C., Clevenger D., Tan W., Szekely B., Wang Y., Li Y. & Alex Muse E. 2008. Descriptive epidemiology of fatal respiratory outbreaks and detection of a human-related metapneumovirus in wild chimpanzees (*Pan troglodytes*) at Mahale Mountains National Park, Western Tanzania. American Journal of Primatology 70:755-65.
Kawai K., Saathoff E., Antelman G., Msamanga G. & Fawzi W. W. 2009. Geophagy (Soil-eating) in relation to Anemia and Helminth infection among HIV-infected pregnant women in Tanzania. Am J Trop Med Hyg 80:36-43.
Kawasaki I., Jeong M. H., Oh B. K. & Shim Y. H. 2010. Apigenin inhibits larval growth of *Caenorhabditis elegans* through DAF-16 activation. FEBS Letters 584:3587-91.
Keen C. L., Uriu-Adams J. Y., Ensunsa J. L. & Gershwin M. E. 2004. Trace Elements/Minerals and Immunity.*in* M. E. Gershwin, Nestel P., and Keen C. L., Editors. Handbook of Nutrition and Immunity. Humana Press Inc., Totowa, New Jersey
Kelly D. & Conway S. 2005. Bacterial modulation of mucosal innate immunity. Molecular immunology 42:895-901.
Kelsall A., Fitzgerald A. J., Howard C. V., Evans R. C., Singh R., Rhodes J. M. & Goodlad R. A. 2002. Dietary lectins can stimulate pancreatic growth in the rat. International Journal of Experimental Pathology 83:203-08.
Kempter K. A. 1997. Geologic Evolution of the Rincon de la Vieja volcanic complex, N.W Costa Rica. University of Texas, Austin.
Kerboeuf D.,Riou M. & Guegnard F. 2008. Flavonoids and related compounds in parasitic disease control. Mini Rev Med Chem 8:116-28.
Kernan M. R., Amarquaye A., Chen J. L., Chan J., Sesin D. F., Parkinson N., Ye Z., Barrett M., Bales C., Stoddart C. A., Sloan B., Blanc P., Limbach C., Mrisho S. & Rozhon E. J. 1998. Antiviral phenylpropanoid glycosides from the medicinal plant *Markhamia lutea*. J Nat Prod 61:564-70.
Ketch L., Malloch D., Mahaney W. & Huffman M. 2001. Comparative microbial analysis and clay mineralogy of soils eaten by chimpanzees (*Pan troglodytes schweinfurthii)* in Tanzania. Soil Biology and Biochemistry 33:199-203.
Khan D. H. 1970. Studies on Mg, B, and Zn deficiencies in acid 'Wilder' soil of California. Plant and Soil 32:177-88.
Khoddami A.,Wilkes M. A. & Roberts T. H. 2013. Techniques for Analysis of Plant Phenolic Compounds. Molecules 18:328-2375.
Khokhar S. & Owusu Apenten R. K. 2003. Iron binding characteristics of phenolic compounds: some tentative structure–activity relations. Food Chemistry 81:133-40.
Khuder A., Sawan M. K., Karjou J. & Razouk A. K. 2009. Determination of trace elements in Syrian medicinal plants and their infusions by energy dispersive X-ray fluorescence and total reflection X-ray fluorescence spectrometry. Spectrochimica Acta Part B: Atomic Spectroscopy 64:721-25.
Khwaja T. A.,Dias C. B. & Pentecost S. 1986. Recent studies on the anticancer activities of mistletoe *(Viscum album*) and its alkaloids. Oncology 43 Suppl 1:42-50.
Khwaja T. A., Varven J. C., Pentecost S. & Pande H. 1980. Isolation of biologically active alkaloids from Korean mistletoe, *Viscum album, coloratum*. Experientia 36:599-600.
Kikouama J. R. O., Konan K. L., Katty A., Bonnet J. P., Balde L. & Yagoubi N. 2009a. Physicochemical characterization of edible clays and release of trace elements. Applied Clay Science 43:135-41.
Kikouama J. R. O., Le Cornec F., Bouttier S., Launay A., Balde L. & Yagoubi N. 2009b. Evaluation of trace elements released by edible clays in physicochemically simulated physiological media. International Journal Of Food Sciences And Nutrition 60:130-42.
Kim W.-H., Park W. B., Gao B. & Jung M. H. 2004. Critical Role of Reactive Oxygen Species and Mitochondrial Membrane Potential in Korean Mistletoe Lectin-Induced Apoptosis in Human Hepatocarcinoma Cells. Molecular Pharmacology 66:1383-96.
Klancnik A., Piskernik S., Jergek B. & Mozina S. S. 2010. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods 81:121-26.
Klaus G. & Schmid G. B. 1998. Geophagy at natural licks and mammal ecology: a review. Mammalia 62:482–98.

Klein N.,Frohlich F. & Krief S. 2008. Geophagy: soil consumption enhances the bioactivities of plants eaten by chimpanzees. Naturwissenschaften 95:325-31.
Klein R., Classen K., Fischer S., Errenst M., Scheffler A., Stein G., Scheer R. & von Laue H. 2002. Induction of antibodies to viscotoxins A1, A2, A3, and B in tumour patients during therapy with an aqueous mistletoe extract. European Journal of Medical Research 7:359-67.
Kloos H. & McCullough F. S. 1982. Plant molluscicides. Planta Med 46:195-209.
Knezevich M. 1998. Geophagy as a therapeutic mediator of endoparasitism in a free-ranging group of Rhesus macaques (*Macaca mulatta*). American Journal of Primatology 44:71-82.
Koch P. E., Bonness M. S., Lu H. & Mabry T. J. 1996. Protoplasts from *Phytolacca dodecandra* L'Herit (endod) and *P. americana* L. (pokeweed). Plant Cell Reports 15:824-28.
Kodama H.,Kotlyar L. S. & Ripmeester J. A. 1989. Quantification of Crystalline and Noncrystalline material in Ground kaolinite by X-Ray-Powder Diffraction, Infra-red, Solid-State Nuclear Magnetic-Resonance, and Chemical-Dissolution Analyses. Clays and Clay Minerals 37:364-70.
Kodama H. & Oinuma K. 1963. Identification of kaolin minerals in the presence of chlorite by X-ray diffraction and Infra-red absorption spectra. Pages 236-49 *in* Clays and Clay Minerals,Proc. 11th Natl. Conf.,. Pergamon Press, New York, Ottawa, Ontario, Canada.
Köndgen S., Kühl H., N'Goran P. K., Walsh P. D., Schenk S., Ernst N., Biek R., Formenty P., Mätz-Rensing K. & Schweiger B. 2008. Pandemic human viruses cause decline of endangered great apes. Current Biology 18:260-64.
Kono Y., Kashine S., Yoneyama T., Sakamoto Y., Matsui Y. & Shibata H. 1998. Iron chelation by chlorogenic acid as a natural antioxidant. Bioscience, biotechnology, and biochemistry 62:22-27.
Korkina L., Kostyuk V., De Luca C. & Pastore S. 2011. Plant phenylpropanoids as emerging anti-inflammatory agents. Mini Rev Med Chem 11:823-35.
Korkina L. G. 2007. Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health Cellular and Molecular Biology 53:15-25.
Kourmanova A. G., Soudarkina O. J., Olsnes S. & Kozlov J. V. 2004. Cloning and characterization of the genes encoding toxic lectins in mistletoe (*Viscum album* L). European Journal of Biochemistry 271:2350-60.
Kovacs E.,Hajto T. & Hostanska K. 1991. Improvement of DNA repair in lymphocytes of breast cancer patients treated with *Viscum album* extract (Iscador). European Journal of Cancer and Clinical Oncology 27:1672-76.
Krenzelok E. P.,Jacobsen T. & Aronis J. 1997. American mistletoe exposures. The American journal of emergency medicine 15:516-20.
Krief S., Huffman M. A., Sevenet T., Hladik C.-M., Grellier P., Loiseau P. M. & Wrangham R. W. 2006. Bioactive properties of plant species ingested by chimpanzees (*Pan troglodytes schweinfurthii*) in the Kibale National Park, Uganda. American Journal of Primatology 68:51-71.
Krishnamani R. & Mahaney W. C. 2000. Geophagy among primates: adaptive significance and ecological consequences. Animal Behaviour 59:899-915.
Kumar M. A., Timm D. E., Neet K. E., Owen W. G., Peumans W. J. & Rao A. G. 1993. Characterization of the lectin from the bulbs of *Eranthis hyemalis* (winter aconite) as an inhibitor of protein synthesis. J Biol Chem 268:25176-83.
Kyriazakis I.,Tolkamp B. J. & Hutchings M. R. 1998. Towards a functional explanation for the occurrence of anorexia during parasitic infections. Animal Behaviour 56:265-74.
Lacy E. R. & Ito S. 1982. Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. Gastroenterology 83:619-25.
Lambert J. E. 1998. Primate digestion: Interactions among anatomy, physiology, and feeding ecology. Evolutionary Anthropology 7:8-20.
Lambert J. E. 2002. Digestive Retention Times in Forest Guenons (*Cercopithecus* spp.) with Reference to Chimpanzees (*Pan troglodytes*). International Journal of Primatology, 23.
Lamont B. B. 1983. Mineral nutrition of Mistletoes. The Biology of Mistletoes. Academic Press, New York.
Lamont B. B. & Southall K. J. 1982. Distribution of mineral nutrients between the mistletoe *Amyema preisii* and its host, *Acacia acuminata.* . Annals of Botany 49:721-25.
Larsson J., Maitz A., Hernandez Salazar L. T. & Laska M. 2014. Gustatory responsiveness to the 20 proteinogenic amino acids in the spider monkey (Ateles geoffroyi). Physiol Behav 127:20-6.
Larsson S. 2007. Mistletoes and Thionins. University of Uppsala, Uppsala.
Laska M., Salazar L. H., Luna E. R. & Hudson R. 2000. Gustatory responsiveness to food-associated acids in the Spider monkey (*Ateles geoffroyi*). Primates 41:213-21.
Laska M.,Rivas Bautista R. M. & Salazar L. T. H. 2009. Gustatory Responsiveness to Six Bitter Tastants in Three Species of Nonhuman Primates. Journal of Chemical Ecology 35:560-71.
Laskin J. D.,Heck D. E. & Laskin D. L. 2002. The ribotoxic stress response as a potential mechanism for MAP kinase activation in xenobiotic toxicity. Toxicological Sciences 69:289-91.
Lavastre V., Cavalli H., Ratthe C. & Girard D. 2004. Anti-inflammatory effect of *Viscum album* agglutinin-I (VAA-I): induction of apoptosis in activated neutrophils and inhibition of lipopolysaccharide-induced neutrophilic inflammation *in vivo*. Clinical and Experimental Immunology 137:272-78.
Lavelle E. C., Grant G., Pusztai A., Pfuller U. & O'Hagan D. T. 2000. Mucosal immunogenicity of plant lectins in mice. Immunology 99:30-37.
Lavie S. & Stotzky G. 1986a. Adhesion of the clay minerals montmorillonite, kaolinite, and attapulgite reduces respiration of *Histoplasma capsulatum.* Appl Environ Microbiol. 51:65-73.
Lavie S. & Stotzky G. 1986b. Interactions between Clay minerals and Siderophores affect the respiration of *Histoplasma*

*capsulatum.* Applied and Environmental Microbiology 51:74-79.
Le Bourvellec C. & Renard C. M. G. C. 2012. Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms. Critical Reviews in Food Science and Nutrition 52:213-48.
Le J. 2012. Drug Absorption and Drug Bioavailability. The Merck Index. Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., Whitehouse Station, N.J., U.S.
Ledochowski M., Widner B., Bair H., Probst T. & Fuchs D. 2010. Fructose- and Sorbitol-reduced Diet Improves Mood and Gastrointestinal Disturbances in Fructose Malabsorbers. Scand J Gastroenterol 10:1048-52.
Lee H. C., Jenner A. M., Low C. S. & Lee Y. K. 2006. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. Res Microbiol 157:876-84.
Lee H. S., Kimb Y. S., Kim S. B., Choi B. E., Woo B. H. & Lee K. C. 1999. Isolation and characterization of biologically active lectin from Korean mistletoe, *Viscum album* var. Coloratum. Cellular and Molecular Life Sciences 55:679-82.
Lee R. T.,Gabius H. J. & Lee Y. C. 1992. Ligand-binding characteristics of the major mistletoe Lectin. Journal of Biological Chemistry 267:23722-27.
Lee S. S., Kennedy S., Tolonen A. C. & Ruvkun G. 2003. DAF-16 Target Genes That Control *C. elegans* Life-Span and Metabolism. Science 300:644-47.
Lee W. M., De la Barca A. M. C., Drake D. & Doyle R. J. 1998. Epidemiology of infection - Lectin-oral *streptococci* interactions. Journal of Medical Microbiology 47:29-37.
Leendertz F. H., Ellerbrok H., Boesch C., Couacy-Hymann E., Matz-Rensing M., Hakenbeck R., Bergmann C., Abaza P., Junglen S., Moebius Y., Vigilant L., Formenty P. & Pauli G. 2004. Anthrax kills wild chimpanzees in a tropical rainforest. Nature 430:451-52.
Lefevre T., Oliver L., Hunter M. D. & de Roode J. C. 2010. Evidence for trans-generational medication in nature. Ecology Letters 13:1485-93.
Legault J. & Pichette A. 2007. Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. J Pharm Pharmacol 59:1643-7.
Lentz D. L., Clark A. M., Hufford C. D., Meurer-Grimes B., Passreiter C. M., Cordero J., Olbrahimi O. & Okunade A. L. 1998. Antimicrobial properties of Honduran medicinal plants. Journal of Ethnopharmacology 63:253–63.
Leonard A.,Droylefaix M. T. & Allen A. 1994. Pepsin hydrolysis of the adherent mucus barrier and subsequent gastric-mucosal damage in the rat. Effect diosmectite and 16,16-dimethyl prostaglandin. Gastroenterologie Clinique Et Biologique 18:609-16.
Leong J. 1986. Siderophores - Their biochemistry and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology 24:187-209.
Leonti M.,Sticher O. & Heinrich M. 2003. Antiquity of medicinal plant usage in two Macro-Mayan ethnic groups (Mexico). Journal of Ethnopharmacology 88:119-24.
Leroy E. M., Epelboin A., Mondonge V., Pourrut X., Gonzalez J. P., Muyembe-Tamfum J. J. & Formenty P. 2009. Human Ebola outbreak resulting from direct exposure to fruit bats in Luebo, Democratic Republic of Congo, 2007. Vector Borne Zoonotic Dis 9:723-8.
Leroy E. M., Rouquet P., Formenty P., Souquiere S., Kilbourne A., Froment J. M., Bermejo M., Smit S., Karesh W., Swanepoel R., Zaki S. R. & Rollin P. E. 2004. Multiple Ebola virus transmission events and rapid decline of central African wildlife. Science 303:387-90.
Leung M. C. K., Williams P. L., Benedetto A., Au C., Helmcke K. J., Aschner M. & Meyer J. N. 2008. *Caenorhabditis elegans:* An Emerging Model in Biomedical and Environmental Toxicology. Toxicological Sciences 106:5-28.
Lev E.,Ephraim M. & Ben-Arye E. 2011. European and Oriental mistletoe: From mythology to contemporary integrative cancer care. European Journal of Integrative medicine 3:133-37.
Levey D.,Bissell H. & O'Keefe S. 2000. Conversion of Nitrogen to Protein and Amino Acids in Wild Fruits. Journal of Chemical Ecology 26:1749-63.
Levin D. A. 1976. Alkaloid-Bearing Plants: An Ecogeographic Perspective. The American Naturalist 110:261-84.
Li D., Zhang L., Yin H., Xu H., Trask J., Smith D., Li Y., Yang M. & Zhu Q. 2014a. Evolution of primate α and θ defensins revealed by analysis of genomes. Molecular Biology Reports 41:3859-66.
Li G., Qiao M., Guo Y., Wang X., Xu Y. & Xia X. 2014b. Effect of Subinhibitory Concentrations of Chlorogenic Acid on Reducing the Virulence Factor Production by *Staphylococcus aureus*. Foodborne Pathog Dis.
Li S. S., Gullbo J., Lindholm P., Larsson R., Thunberg E., Samuelsson G., Bohlin L. & Claeson P. 2002. Ligatoxin B, a new cytotoxic protein with a novel helix-turn-helix DNA-binding domain from the mistletoe *Phoradendron liga*. Biochemical Journal 366:405-13.
Lima H. D., Rodríguez N., Barrios M. A., Ávila Á., Cañizales I. & Gutiérrez S. 2008. Isolation and molecular identification of *Leishmania chagasi* from a bat (*Carollia perspicillata*) in northeastern Venezuela. Memórias do Instituto Oswaldo Cruz 103:412-14.
Lin J. H. 1995. Species similarities and differences in pharmacokinetics. Drug Metabolism and Disposition 23:1008-21.
Lin L.-Z. & Harnley J. M. 2007. A screening method for the identification of glycosylated flavonoids and other phenolic compounds using a standard analytical approach for all plant materials Journal of Agricultural and Food Chemistry 55:1084-96
Link A., de Luna A. G., Arango R. & Diaz M. C. 2011a. Geophagy in Brown Spider monkeys (*Ateles hybridus*) in a lowland tropical rainforest in Colombia. Folia Primatologica 82:25-32.
Link A., Galvis N., Fleming E. & Di Fiore A. 2011b. Patterns of mineral lick visitation by Spider monkeys and Howler monkeys in Amazonia: Are licks perceived as risky areas? American Journal of Primatology 73:386-96.
Link A., Palma A. C., Velez A. & de Luna A. G. 2006. Costs of twins in free-ranging White-bellied Spider monkeys (*Ateles*

*belzebuth belzebuth*) at Tinigua National Park, Colombia. Primates 47:131-39.
Linthicum K. J., Anyamba A., Tucker C. J., Kelley P. W., Myers M. F. & Peters C. J. 1999. Climate and Satellite Indicators to Forecast Rift Valley Fever Epidemics in Kenya. Science 285:397-400.
Liu B.,Bian H.-j. & Bao J.-k. 2010. Plant lectins: Potential antineoplastic drugs from bench to clinic. Cancer Letters 287:1-12.
Llovo J., Lopez A., Fabregas J. & Munoz A. 1993. Interaction of Lectins with *Cryptosporidium parvum*. Journal of Infectious Diseases 167:1477-80.
Lobo V., Patil A., Phatak A. & Chandra N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 4:118–26.
Lock S. & Bender A. E. 1980. Measurement of chemically-available iron in foods by incubation with human gastric juice *in vitro* Measurement of chemically-available iron in foods by incubation with human gastric juice *in vitro*. British Journal of Nutrition 43:413-20.
Longhi A., Reif M., Mariani E. & Ferrari S. 2014. A Randomized Study on Postrelapse Disease-Free Survival with Adjuvant Mistletoe versus Oral Etoposide in Osteosarcoma Patients. Evidence-Based Complementary and Alternative Medicine 2014.
López-Martínez S., Navarrete-Vázquez G., Estrada-Soto S., León-Rivera I. & Rios M. Y. 2012. Chemical constituents of the hemiparasitic plant *Phoradendron brachystachyum* DC Nutt (Viscaceae). Natural Product Research:1-7.
López J. A., Barillas W., Gomez-Laurito J., Lin F.-T., Al-Rehaily A. J., Sharaf M. H. & Schiff Jr P. L. 1996. Aporphine alkaloids of *Ocotea brenesii*. Pharmaceutical biology 34:145-47.
Lorimer S. D., Perry N. B., Foster L. M., Burgess E. J., Douch P. G. C., Hamilton M. C., Donaghy M. J. & McGregor R. A. 1996. A Nematode larval motility inhibition assay for screening plant extracts and natural products. Journal of Agricultural and Food Chemistry 44:2842.
Lou Z., Wang H., Zhu S., Ma C. & Wang Z. 2011. Antibacterial activity and mechanism of action of chlorogenic acid. J Food Sci 76:M398-403.
Lozano G. A. 1998. Parasitic stress and Self-medication in wild animals. Academic Press, London.
Luczkiewicz M., Cisoski W., Kaiser P., Ochocka R. & Piotrowski A. 2001. Comparative analysis of phenolic acids in mistletoe plants from various hosts. Acta Polonia Pharmaceutica - Drug Research 58:373-79.
Luther P., Theise H., Chatterjee B., Karduck D. & Uhlenbruck G. 1980. The lectin from *Viscum album* L.--isolation, characterization, properties and structure. Int J Biochem 11:429-35.
Lyu S.-Y. & Park W. 2006. Mistletoe Lectin (*Viscum album coloratum*) Modulates Proliferation and Cytokine Expressions in Murine Splenocytes. Journal of Biochemistry and Molecular Biology 39:662-70.
Ma J. F. & N. Y. 2006. Silicon uptake and accumulation in higher plants. Trends in Plant Science 11.
MacIntosh A. J. J.,Hernandez A. D. & Huffman M. A. 2010. Host age, sex, and reproductive seasonality affect nematode parasitism in wild Japanese macaques. Primates 51:353-64.
Mack A. L. 1990. Is Frugivory Limited by Secondary Compounds in Fruits? Oikos 57:135-38.
Madejova J. 2003. FTIR techniques in clay mineral studies. Vibrational Spectroscopy 31:1-10.
Madejova J. & Komadel P. 2001. Baseline studies of The Clay Minerals Society Source Clays: Infrared methods. Clays and Clay Minerals 49:410-32.
Magnusson G.,Hultgren S. J. & Kihlberg J. 1995. Specificity mapping of bacterial lectins by inhibition of hemagglutination using Deoxy and Deoxyfluoro analogs of Receptor-active saccharides. Pages 105-14 Adhesion of Microbial Pathogens.
Mahaney W. C.,Aufreiter S. & Hancock R. G. V. 1995a. Mountain gorilla geophagy: a possible seasonal behavior for dealing with the effects of dietary changes International Journal of Primatology 16:475-88.
Mahaney W. C., Hancock R. G., Aufreiter S. & Huffman M. A. 1996. Geochemistry and clay mineralogy of termite mound soil and the role of geophagy in chimpanzees of the Mahale Mountains, Tanzania Primates 37:121-34.
Mahaney W. C.,Hancock R. G. V. & Aufreiter R. S. 1995b. Mountain gorilla geophagy: a possible strategy for dealing with intestinal problems. Int. J. Primatology 16:475- 88.
Mahaney W. C. & Krishnamani R. 2003. Understanding geophagy in animals: Standard procedures for sampling soils. Journal of Chemical Ecology 29:1503-23.
Mahaney W. C., Milner M. M., Aufreiter S., Hancock R. G. V., Wrangham R. & Campbell S. 2005. Soils consumed by chimpanzees of the Kanyawara community in the Kibale Forest, Uganda. International Journal of Primatology 26:1375-98.
Mahaney W. C., Milner M. W., Hs M., Hancock R. G. V., Aufreiter S., Reich M. & Wink M. 2000. Mineral and chemical analyses of soils eaten by humans in Indonesia. Int J Environ Health Res 10:93-109.
Mahaney W. C., Milner M. W., Sanmugadas K., Hancock R. G. V., Aufreiter S., Wrangham R. & Pier H. W. 1997. Analysis of geophagy soils in Kibale Forest, Uganda Primates 38:159-76.
Mahaney W. C., Stambolic A., Knezevich M., Hancock R. G. V., Aufreiter S., Sanmugdas K., Kessler M. J. & Grymnas M. D. 1995c. Geophagy amongst *Rhesus macaques* on Cayo-Santiago, Puerto-Rico. Primates 36:323-33.
Mahaney W. C.,Watts D. P. & Hancock R. G. V. 1990. Geophagia by mountain gorillas *(Gorilla gorilla beringei*) in the Virunga Mountains, Rwanda Primates 31:113-20.
Mahaney W. C., Zippin J., Milner M. W., Sanmugadas K., Hancock R. G. V., Aufreiter S., Campbell S., Huffman M. A., Wink M., Malloch D. & Kalm V. 1999. Chemistry, mineralogy and microbiology of termite mound soil eaten by the chimpanzees of the Mahle Mountains, western Tanzania. Journal of Tropical Ecology 15:565-88.
Majid A., Sarmani S., Yusoff N., Wei Y. & Hamzah F. 1995. Trace elements in Malaysian medicinal plants. Journal of Radioanalytical and Nuclear Chemistry 195:173-83.
Makkar H. P. S.,Siddhuraju P. & Becker K. 2007. Alkaloids. Pages 107-11 Plant Secondary Metabolites. Humana Press.

Malešev D. & Kunti V. 2007. Investigation of metal–flavonoid chelates and the determination of flavonoids via metal–flavonoid complexing reactions. J. Serb. Chem. Soc. 72:921-39.
Marçal R. M.,Almeida D. S. & Carvalho A. C. S. 2007. Pharmacological evidence of the mechanisms of action of *Phoradendron piperoides* Kunth (Viscaceae) aqueous extract. Planta Med 73:P_494.
March W. A. & Watson D. M. 2010. The contribution of mistletoes to nutrient returns: Evidence for a critical role in nutrient cycling. Austral Ecology 35:713-21.
Marguí E.,Queralt I. & Hidalgo M. 2009. Application of X-ray fluorescence spectrometry to determination and quantitation of metals in vegetal material. Trends in Analytical Chemistry 28:362-72.
Marin C., Ramirez-Macias I., Lopez-Cespedes A., Olmo F., Villegas N., Diaz J. G., Rosales M. J., Gutierrez-Sanchez R. & Sanchez-Moreno M. 2011. *In vitro* and *in vivo* trypanocidal activity of flavonoids from *Delphinium staphisagria* against Chagas disease. Journal of Natural Products 74:744-50.
Markham K. R. 1989. Flavones, Flavonols and their Glycosides. Pages 197 - 235 *in* P. M. Dey and Harborne J. B.,Editors. Methods in Plant Biochemistry. Academic Press Ltd, London.
Markman O., Rao U., Lewis K., Heffron G., Stec B. & Teeter M. 1993. Mode of Phospholipid Binding to the Membrane Active Plant Toxin Phoratoxin-A. Pages 263-74 *in* K. W. A. Wirtz, Packer L., Gustafsson J. Å., Evangelopoulos A. E., and Changeux J. P., Editors. New Developments in Lipid-Protein Interactions and Receptor Function. Springer US.
Marshall A. J., Boyko C. M., Feilen K. L., Boyko R. H. & Leighton M. 2009. Defining fallback foods and assessing their importance in primate ecology and evolution. American Journal of Physical Anthropology 140:603-14.
Martignoni M.,Groothuis G. M. & de Kanter R. 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opinion on Drug Metabolism & Toxicology 2:875-94.
Martin F. W. 1970. The ultraviolet absorption profile of stigmatic extracts. New Phytologist 69:425-30.
Martin L. B.,Weil Z. M. & Nelson R. J. 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Phil. Trans. R. Soc. B 363:321-39.
Martínez-Esquivel L. 2010. Comparison of dietary habits and their relation to parasitic infections in howler monkeys *(Alouatta palliata*) of Chomes and Palo Verde, Costa Rica. Ciudad Universitaria Rodrigo Facio, San José.
Martinez M., Amidon G., Clarke L., Jones W. W., Mitra A. & Riviere J. 2002. Applying the biopharmaceutics classification system to veterinary pharmaceutical products: Part II. Physiological considerations. Advanced Drug Delivery Reviews 54:825-50.
Massey F. P. & Hartley S. E. 2006. Experimental demonstration of the antiherbivore effects of silica in grasses: impacts on foliage digestibility and vole growth rates. Proc Biol Sci. 273:2299-304.
Mathew A. G. & Lakshminarayana S. 1969. Polyphenols of immature sapota fruit. Phytochemistry 8:507-09.
Matsubayashi H., Ahmad A. H., Wakamatsu N., Nakazono E., Takyu M., Majalap N., Langan P. & Sukor J. R. A. 2011. Natural-lick use by orangutans and conservation of their habitats in a Bornean Tropical production forest. The Raffles Bulletin of Zoology 59:109-15.
Matsubayashi H., Lagan P., Majalap N., Tangah J., Sukor J. R. A. & Kitayama K. 2007. Importance of natural licks for the mammals in Bornean inland tropical rain forests. Ecological Research 22:742-48.
Mattson D. J.,Green G. I. & Swalley R. 1999. Geophagy by Yellowstone grizzly bears. Ursus 11.
Maurice P.,Haack E. & Mishra B. 2009. Siderophore sorption to clays. Biometals 22:649.
Maury C., Sarni-Manchado P., Lefebvre S., Cheynier V. & Moutounet M. 2001. Influence of fining with different molecular weight gelatins on proanthocyanidin composition and perception of wines. American journal of enology and viticulture 52:140-45.
McArthur C.,Sanson G. D. & Beal A. M. 1995. Salivary proline rich proteins in mammals: roles in oral homeostasis and counteracting dietary tannins. Journal of Chemical Ecology, 21:663-91.
McCarty M. F. 2005. A chlorogenic acid-induced increase in GLP-1 production may mediate the impact of heavy coffee consumption on diabetes risk. Medical Hypotheses 64:848-53.
McClure S. J. 2008. How minerals may influence the development and expression of immunity to endoparasites in livestock. Parasite Immunology 30:89-100.
McGraw-Hill. 2003. Dictionary of Geology & Mineralogy. McGraw-Hill Professional.
Mellstrand S. T. 1974. Phoratoxin, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. macrophyllum (Loranthaceae). Acta Pharmaceutica Suecica 11:410-12.
Mellstrand S. T. & Samuelsson G. 1973. Phoratoxin, a Toxic Protein from mistletoe *Phoradendron tomentosum* Subsp. Macrophyllum (Loranthaceae) - Improvements in isolation procedure and further studies on properties. European Journal of Biochemistry 32:143-47.
Menzel C., Fowler A., Tennie C. & Call J. 2013. Leaf Surface Roughness Elicits Leaf Swallowing Behavior in Captive Chimpanzees (*Pan troglodytes*) and Bonobos (*P. paniscus*), but not in Gorillas (*Gorilla gorilla*) or Orangutans (*Pongo abelii*). International Journal of Primatology 34:533-53.
Mermut A. R. & Cato A. F. 2001. Baseline studies of the Clay Mineral Society Source Clays: Chemical analyses of major elements. Clays and Clay Minerals 49:381-86.
Meyer B. N., Ferrigni N. R., Putnam J. E., Jacobsen L. B., Nichols D. E. & McLaughlin J. L. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 45:31-4.
Miah M. Y. & Chino M. 1999. Observation of element distribution in potassium deficient barley (*Hordeum vulgare* L.) leaf by energy dispersive X-ray fluorescence analysis. Bot. Bull. Acad. Sin. 40.
Miethke M. & Marahiel M. A. 2007. Siderophore based iron acquisition and pathogen control. Microbiol. Mol. Biol. Rev. 71:413-51.

Mijangos F.,Varona F. & Villota N. 2006. Changes in solution color during phenol oxidation by Fenton reagent. Environ Sci Technol 40:5538-43.
Miller D. D., Schricker B. R., Rasmussen R. R. & Van Campen D. 1981. An *in vitro* method for estimation of iron availability from meals. The American Journal of Clinical Nutrition 34:2248-56.
Miller R. O. & Kissel D. E. 2010. Comparison of Soil pH Methods on Soils of North America. Soil Science Society of America 74:310-16.
Mills A. & Milewski A. 2007. Geophagy and nutrient supplementation in the Ngorongoro Conservation Area, Tanzania, with particular reference to selenium, cobalt and molybdenum. Journal of Zoology 271:110-18.
Milton K. 1981. Food choice and digestive strategies of two sympatric primate species. The American Naturalist 117:496-505.
Milton K. 1999. Nutritional characteristics of wild primate foods: Do the diets of our closest living relatives have lessons for us? Nutrition 15:488-98.
Min B. R., Barry T. N., Attwood G. T. & McNabb W. C. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Animal Feed Science and Technology 106:3-19.
Mohammed R. S., Abou Zeid A. H., El Hawary S. S., Sleem A. A. & Ashour W. E. 2014. Flavonoid constituents, cytotoxic and antioxidant activities of *Gleditsia triacanthos* L. leaves. Saudi Journal of Biological Sciences.
Molan A. L.,Waghorn G. C. & McNabb W. C. 2002. Effect of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis in vitro*. Veterinary Record 150:65-69.
Molan A. L., Waghorn G. C., Min B. R. & McNabb W. C. 2000. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro*. Folia Parasitologica 47:39-44.
Molina E.,Leon T. E. & Armenteras D. 2014. Characteristics of natural salt licks located in the Colombian Amazon foothills. Environ Geochem Health 36:117-29.
Molina González E. 2010. Salt licks: keystone resources for the Inga culture, useful for its territorial management, the traditional practices development and the biological conservation. Universidad Nacional de Colombia.
Monsen E. R., Hallberg L., Layrisse M., Hegsted D. M., Cook J. D., Mertz W. & Finch C. A. 1978. Estimation of available dietary iron. Am J Clin Nutr 31:134-41.
Montenegro O. L. 2004. Natural licks as keystone resources for wildlife and people in Amazonia. Doctoral Dissertation, University of Florida.
Mora O. 1988. Estudio geologico- petrologico de las piroclastitas en los alrededores de Bagaces, Province de Guanacaste, Costa Rica. Universidad de Costa rica, San Jose.
Morel I., Lescoat G., Cillard P. & Cillard J. 1994. Role of flavonoids and iron chelation in antioxidant action. Methods in Enzymology 234:437-43.
Mowery C., McCann C., Lessnau R. & Dierenfeld E. 1997. Secondary compounds in foods selected by free-ranging primates on St. Catherines Island, GA. *in* Proceedings of the American Zoo and Aquarium Association (AZA) Nutrition Advisory Group, Forth Worth, Texas.
Mubarak A., Bondonno C. P., Liu A. H., Considine M. J., Rich L., Mas E., Croft K. D. & Hodgson J. M. 2012. Acute effects of chlorogenic acid on nitric oxide status, endothelial function, and blood pressure in healthy volunteers: a randomized trial. J Agric Food Chem 60:9130-6.
Mueller-Harvey I. 2001. Analysis of hydrolysable tannins. Animal Feed Science and Technology 91:3-20.
Mueller-Harvey I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. Journal of Science of Food and Agriculture 86:2010-37.
Müller K.-H.,Ahl C. & Hartmann G. 1997. Geophagy in Masked titi monkeys (*Callicebus personatus melanochir*) in S Brazil Primates 38:69-77.
Nair V. D. P. & Kanfer I. 2008. Development of Dissolution Tests for the Quality Control of Complementary/Alternate and Traditional Medicines: Application to African Potato Products. J Pharm Pharmaceut Sci 11:35-44.
Narasinga Rao B. S. & Prabhavathi T. 1978. An *in vitro* method for predicting the bioavailability of iron from foods. The American Journal of Clinical Nutrition 31:169-75.
Natural Resources Conservation Service 2004. Use of Reaction (pH) in Soil Taxonomy. Measuring Soil pH for Soil Taxonomy.
Naumann H. D., Muira J. P., Lambert B. D., Tedeschi L. O. & Kothmanne M. M. 2013. Condensed Tannins In The Ruminant Environment: A Perspective On Biological Activity. Journal of Agricultural Sciences 1:8-20.
Negre A., Tarnaud L., Roblot J. F., Gantier J. C. & Guillot J. 2006. Plants consumed by *Eulemur fulvus* in Comoros Islands (Mayotte) and potential effects on intestinal parasites. International Journal of Primatology 27:1495-517.
Nehmann N., Schade U. M., Pfueller U., Schachner M. & Schumacher U. 2009. Mistletoe lectin binds to multidrug resistance-associated protein MRP5. Anticancer Research 29:4941-48.
Nelson A. C. & Kursar T. A. 1999. Interactions among plant defense compounds: a method for analysis. Chemoecology 2:81-92.
Nelson R. J. & Demas G. E. 1996. Seasonal changes in immune function. Q Rev Biol 71:511-48.
Nevitt T. & Thiele D. J. 2011. Host iron withholding demands siderophore utilization for *Candida glabrata* to survive macrophage killing. Plos Pathogens 7.
Ngole V. M., Ekosse G. E., de Jager L. & Songca S. P. 2010. Physicochemical characteristics of geophagic clayey soils from South Africa and Swaziland. African Journal of Biotechnology 9:5929-37.
Nguyen T. X.,Cole A. M. & Lehrer R. I. 2003. Evolution of primate θ-defensins: a serpentine path to a sweet tooth. Peptides 24:1647-54.
Nicasio P., Aguilar-Santamaria L., Aranda E., Ortiz S. & Gonzalez M. 2005. Hypoglycemic effect and chlorogenic acid content in two *Cecropia* species. Phytother Res 19:661-4.

Nicholas W. Lerche. 2011. Parasitic diseases of nonhuman primates. www.merckmanuals.com.
Nickrent D. L. 2011. Santalales (including Mistletoes). Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd., Chichester.
Nieuwenhuyse A.,Verburg P. S. J. & Jongmans A. G. 2000. Mineralogy of a soil chronosequence on andesitic lava in humid tropical Costa Rica. Geoderma 98:61-82.
Nir S., El Nahhal Y., Undabeytia T., Rytwo G., Polubesova T., Mishael Y., Rabinovitz U. & Rubin B. 2006. Chapter 11.2 Clays and Pesticides. Pages 677-91 *in* B. K. G. T. Faïza Bergaya and Gerhard L., Editors. Developments in Clay Science. Elsevier.
Nizeyi J. B., Sebunya D., Dasilva A. J., Cranfield M. R., Pieniazek N. J. & Graczyk T. K. 2002. Cryptosporidiosis in people sharing habitats with free-ranging mountain gorillas (*Gorilla gorilla beringei*), Uganda. The American Journal of Tropical Medicine and Hygiene 66:442-44.
Nobuko Y.,Tyler K. & Llewellyn M. S. 2011. Invasion mechanisms among emerging food-borne protozoan parasites. Trends in Parasitology 27:459-66.
Nollet L. M. L. & Toldra F. 2012. Handbook of Analysis of Active Compounds in Functional Foods. Taylor & Francis.
Novobilsky A.,Mueller-Harvey I. & Thamsborg S. M. 2011. Condensed tannins act against cattle nematodes. Veterinary Parasitology 182:213-20.
Nunn C. L. & Altizer S. M. 2006. Infectious diseases in primates: behavior, ecology, and evolution. Oxford University Press., Oxford.
Nunn J. 2002. Ancient Egyptian Medicine. Red River Books, London.
Nyilasi I., Papp T., Takó M., Nagy E. & Vágvölgyi C. 2005. Iron gathering of opportunistic pathogenic fungi. Acta Microbiologica et Immunologica Hungarica 52:185.
O'Brien T. G., Kinnaird M. F., Dierenfeld E. S., Conklin-Brittain N. L., Wrangham R. W. & Silver S. C. 1998. What's so special about figs? Nature 392:668-68.
O'Donnell L.,Virjee J. & Heaton K. 1990. Detection of pseudodiarrhoea by simple clinical assessment of intestinal transit rate. British Medical Journal 300.
Oates J. F. 1977. The guereza and its food. Pages 276-321 *in* T. H. Clutton-Brock, Editor. Primate Ecology. Academic Press, New York.
Ochocka J. & Piotrowski A. 2002. Biologically active compounds from European mistletoe (*Viscum album* L.). Canadian review of Plant Pathology 24:21-28.
Olivares J. A. & Houk R. S. 1986. Suppression of analyte signal by various concomitant salts in inductively coupled plasma mass spectrometry. Analytical Chemistry 58:20-25.
Oliveira M. D. L., Andrade C. A. S., Santos-Magalha N. S., Coelho L. C. B. B., Teixeira J. A., Carneiro-da-Cunha M. G. & Correia M. T. S. 2008. Purification of a lectin from *Eugenia uniflora* L. seeds and its potential antibacterial activity. Letters in Applied Microbiology 46:371–76.
Olthof M. R.,Hollman P. C. H. & Katan M. B. 2001. Chlorogenic Acid and Caffeic Acid Are Absorbed in Humans. The Journal of Nutrition 131:66-71.
Omote J.,Kohno H. & Toda K. 1995. X-Ray fluorescence analysis utilizing the fundamental parameter method for the determination of the elemental composition in plant samples. Analytica Chimica Acta 307:117-26.
Onay-Ucar E.,Karagoz A. & Arda N. 2006. Antioxidant activity of *Viscum album* ssp album. Fitoterapia 77:556-60.
Ong K. W.,Hsu A. & Tan B. K. 2013. Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by ampk activation. Biochem Pharmacol 85:1341-51.
Onunogbo C., Ohaeri O. C., Eleazu C. O. & Eleazu K. C. 2012. Chemical composition of mistletoe extract (*Viscum album)* and its effect on protein, lipid metabolism and the antioxidant status of alloxan induced diabetic rats. Journal of medical Research 1:57-62.
Oomen A. G., Hack A., Minekus M., Zeijdner E., Cornelis C., Schoeters G., Verstraete W., Van de Wiele T., Wragg J., Rompelberg C. J. M., Sips A. & Van Wijnen J. H. 2002. Comparison of five *in vitro* digestion models to study the bioaccessibility of soil contaminants. Environmental Science & Technology 36:3326-34.
Oomen A. G., Rompelberg C. J. M., Bruil M. A., Dobbe C. J. G., Pereboom D. & Sips A. 2003. Development of an *in vitro* digestion model for estimating the bioaccessibility of soil contaminants. Archives of Environmental Contamination and Toxicology 44:281-87.
Oomen A. G., Rompelberg C. J. M., Van de Kamp E., Pereboom D., De Zwart L. L. & Sips A. 2004. Effect of bile type on the bioaccessibility of soil contaminants in an *in vitro* digestion model. Archives of Environmental Contamination and Toxicology 46:183-88.
Oppenheimer S. J. 2001. Iron and its relation to immunity and infectious disease. The Journal of Nutrition 131:616S-35S.
Orčić D., Francišković M., Bekvalac K., Svirčev E., Beara I., Lesjak M. & Mimica-Dukić N. 2014. Quantitative determination of plant phenolics in Urtica dioica extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. Food Chemistry 143:48-53.
Orhan D. D., Aslan M., Sendogdu N., Ergun F. & Yesilada E. 2005. Evaluation of the hypoglycemic effect and antioxidant activity of three *Viscum album* subspecies (European mistletoe) in streptozotocin-diabetic rats. J Ethnopharmacol 98:95-102.
Orhan D. D.,Calis I. & Ergun F. 2002. Two new flavonoid glycosides from *Viscum album* ssp album. Pharmaceutical biology 40:380-83.
Orhan D. D., Kupeli E., Yesilada E. & Ergun F. 2006. Anti-inflammatory and antinociceptive activity of flavonoids isolated from *Viscum album* ssp album. Journal of Biosciences 61:26-30.
Ortiz M., Azanon V., Melgar M. & Elias L. 1995. The corn tree (*Brosimum alicastrum*): a food source for the tropics. World Rev Nutr Diet 77:135-46.

Osadebe P. O.,Okide G. B. & Akabogu I. C. 2004. Study on anti-diabetic activities of crude methanolic extracts of *Loranthus micranthus* (Linn.) sourced from five different host trees. Journal of Ethnopharmacology 95:133-38.
Osadebe P. O., Omeje E. O., Uzor P. F., David E. K. & Obiorah D. C. 2010. Seasonal variation for the antidiabetic activity of *Loranthus micranthus* methanol extract. Asian Pacific Journal of Tropical Medicine 3:196-99.
Otto C. C., Cunningham T. M., Hansen M. R. & Haydel S. H. 2010. Effects of antibacterial mineral leachates on the cellular ultrastructure, morphology and membrane integrity of *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus*. Ann Clin Microbiol Antimicrob. 9.
Otto C. C. & Haydel S. E. 2013. Exchangeable Ions Are Responsible for the *In vitro* Antibacterial Properties of Natural Clay Mixtures. Plos One 8:e64068.
Ozçelik B.,Orhan I. & Toker G. 2006. Antiviral and antimicrobial assessment of some selected flavonoids. Z Naturforsch C. 61:632-8.
Pages G.,Lloyd E. & Suarez S. 2005. The impact of geophagy on ranging behaviour in Phayre's leaf monkeys (*Trachypithecus phayre*i) Folia Primatologica 76:342-6.
Pal A., Debreczeni J. E., Sevvana M., Gruene T., Zeeck A. & Sheldrick G. M. 2008. Structure of viscotoxin A1 and B2 from European mistletoe solved using native data alone. Acta Crystallo D 64:985-92.
Palacios G., Lowenstine L. J., Cranfield M. R., Gilardi K. V., Spelman L., Lukasik-Braum M., Kinani J. F., Mudakikwa A., Nyirakaragire E., Bussetti A. V., Savji N., Hutchison S., Egholm M. & Lipkin W. I. 2011. Human metapneumovirus infection in wild mountain gorillas, Rwanda. . Emerg Infect Disease.
Pan G. X.,Thomson C. I. & Leary G. J. 2002. UV–VIS. Spectroscopic characteristics of ferulic acid and related compounds. Journal of Wood Chemistry and Technology 22:137-46.
Panossian A., Kocharian A., Matinian K., Amroyan E., Gabrielian E., Mayr C. & Wagner H. 1998. Pharmacological activity of phenylpropanoids of the mistletoe, *Viscum album* L., host: *Pyrus caucasica* Fed. Phytomedicine 5:11-17.
Pansu M. & Gautheyrou J. 2007. Handbook of Soil Analysis: Mineralogical, Organic and Inorganic Methods. Springer.
Panvini A. D. & Eickmeier W. G. 1993. Nutrient and Water Relations of the Mistletoe *Phoradendron leucarpum* (Viscaceae): How Tightly are they Integrated? American Journal of Botany 80:872-78.
Park J. H.,Hyun C. K. & Shin H. K. 1999. Cytotoxic effects of the components in heat-treated mistletoe (*Viscum album*). Cancer Lett 139:207-13.
Parr N. A.,Melin A. D. & Fedigan L. M. 2011. Figs Are More Than Fallback Foods: The Relationship between Ficus and Cebus in a Tropical Dry Forest. International Journal of Zoology 2011.
Pebsworth P. A., Archer C. E., Appleton C. C. & Huffman M. A. 2012a. Parasite transmission risk from geophagic and foraging behavior in chacma baboons. Am J Primatol 74:940-7.
Pebsworth P. A.,Bardi M. & Huffman M. A. 2012b. Geophagy in *Chacma* baboons: patterns of soil consumption by age class, sex, and reproductive state. American Journal of Primatology 74:48-57.
Pebsworth P. A., Seim G. L., Huffman M. A., Glahn R. P., Tako E. & Young S. L. 2013. Soil Consumed by Chacma Baboons is Low in Bioavailable Iron and High in Clay. Journal of Chemical Ecology 39:447-49.
Pedersen A. & Davies T. J. 2009. Cross-Species Pathogen Transmission and Disease Emergence in Primates. EcoHealth 6:496-508.
Pelegrini P. B. & Franco O. L. 2005. Plant gamma-thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. International Journal of Biochemistry & Cell Biology 37:2239-53.
Pérez-Magariño S., Revilla I., González-SanJosé M. L. & Beltrán S. 1999. Various applications of liquid chromatography–mass spectrometry to the analysis of phenolic compounds. Journal of Chromatography A 847:75-81.
Perez-Vilar J. & Hill R. J. 1998. Identification of the half-cystine residues in porcine submaxillary mucin critical for multimerization through the D-domains. The Journal of Biological Chemistry 273:34527–34.
Perez E. 2011. Mistletoe poisoning. U.S. National Library of Medicine - National Institutes of Health.
Perkampus H. H.,Grinter H. C. & Threlfall T. L. 2012. UV-VIS Spectroscopy and Its Applications. Springer London, Limited.
Perry N. B.,Burgess E. J. & Glennie V. L. 2001. Echinacea standardization: Analytical methods for phenolic compounds and typical levels in medicinal species. Journal of Agricultural and Food Chemistry 49:1702-06.
Peumans W.J. & Van Damme E.J.M. 1995. Lectins as plant defence proteins. Pages 1-22 *in* Pusztai A. and S. B., Editors. Lectins – Biomedical Perspectives. Taylor & Francis.
Peumans W. J., Verhaert P., Pfuller U. & Van Damme E. J. M. 1996. Isolation and partial characterisation of a small chitin-binding lectin from mistletoe *(Viscum album)*. FEBS Lett. 396:261-65.
Pevzner I. B., Agapov I. I., Niwa H., Maluchenko N. V., Moisenovich M. M., Pfüller U. & Tonevitsky A. G. 2004. Differences in amino acid sequences of mistletoe lectin I and III B-subunits determining carbohydrate binding specificity. Biochimica et Biophysica Acta (BBA) - General Subjects 1675:155-64.
Pfüller U. 2000. Chemical Constituents of European Mistletoe (*Viscum Album* L.).*in* A. Bussing, Editor. Mistletoe: The Genus Viscum. Harwood Academic Publishers, Amsterdam.
Phillips T. 1999. Dietary clay in the chemoprevention of aflatoxin-induced disease. Toxicological Sciences 52:118-26.
Pieri L.,Bittelli M. & Pisa P. R. 2006. Laser diffraction, transmission electron microscopy and image analysis to evaluate a bimodal Gaussian model for particle size distribution in soils. Geoderma 135:118-32.
Pietrzak W.,Nowak R. & Olech M. 2013. Effect of extraction method on phenolic content and antioxidant activity of mistletoe extracts from *Viscum album* subsp. abietis. Chemical Papers:1-7.
Pietta P. G. 2000. Flavonoids as antioxidants. J Nat Prod 63:1035-42.
Pistole T. G. 1981. Interaction of bacteria and fungi with lectins and lectin like substances. Annual Review of Microbiology 35:85-112.
Plancon A. 2001. Order-disorder in clay mineral structures. Clay Minerals 36:1-14.

Plazonić A., Bucar F., Maleš Ž., Mornar A., Nigović B. & Kujundžić N. 2009. Identification and Quantification of Flavonoids and Phenolic Acids in Burr Parsley (*Caucalis platycarpos* L.), Using High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ionization Mass Spectrometry. Molecules 14:2466-90.

Pontes P. V., Moreira R. F. A., Trugo L. C. & Maria C. A. B. D. 2002. The content of chlorogenic acids in tropical fruits. Journal of the Science of Food and Agriculture 82:1177-81.

Popova M., Bankova V., Butovska D., Petkov V., Nikolova-Damyanova B., Sabatini A. G., Marcazzan G. L. & Bogdanov S. 2004. Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochemical Analysis 15:235-40.

Porter L. J. 1989. Tannins. Pages 389-419 *in* A. J. Harborne, Editor. Plant Phenolics. Academic Press, London.

Powell J. J.,Jugdaohsingh R. & Thompson R. P. H. 1999. The regulation of mineral absorption in the gastrointestinal tract. Proceedings of the Nutrition Society 58:147-53.

Prasad R. N., Viswanathan S., Devi J. R., Nayak V., Swetha V., Archana B., Parathasarathy N. & Rajkumar J. 2008. Preliminary phytochemical screening and antimicrobial activity of *Samanea saman*. Journal of Medicinal Plants Research 2:268-70.

Prior R. L.,Wu X. & Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53:4290-302.

Provenza F. D. & Villalba J. J. 2010. The role of natural plant products in modulating the immune system: An adaptable approach for combating disease in grazing animals. Small Ruminant Research 89:131-39.

Pryme I. F. & Tilrem P. 2007. Oral mistletoe lectins: A case for their use in cancer therapy. Cancer ther 5:287-300.

Puri M., Kaur I., Perugini M. A. & Gupta R. C. 2012. Ribosome-inactivating proteins: current status and biomedical applications. Drug discovery today 17:774-83.

Pusztai A. & Bardocz S. 2005. Lectins: Biomedical Perspectives. Taylor & Francis.

Pusztai A., Grant G., Ewen S. W. B., Peumans W. J., Van Damme E. M. J. & Bardocz S. 1995. Infection of the Gut by Pathogenic Bacteria is Inhibited by Dietary lectins : Chemical Probiosis. Pages 313-22 *in* A. Pusztai and Bardocz S., Editors. Lectins. Biomedical Perspectives. Taylor & Francis, London.

Pusztai A., Grant G., Gelencser E., Ewen S. W. B., Pfuller U., Eifler R. & Bardocz S. 1998. Effects of an orally administered mistletoe (type-2 RIP) lectin on growth, body composition, small intestinal structure, and insulin levels in young rats. Journal of Nutritional Biochemistry 9:31-36.

Pusztai A., Grant G., Brown D.S., Bardocz S., Ewen S.W.B., Baintner K., Peumans W.J. & Van Damme E.J.M. 1995. Lectins binding to the gut wall are growth factors for the pancreas: nutritional implications for transgenic plants,. Pages 141-54 *in* A. Pusztai and Bardocz S., Editors. Lectins – Biomedical Perspectives. Taylor & Francis.

Queralt I., Ovejero M., Carvalho M. L., Marques A. F. & Llabre´s J. M. 2005. Quantitative determination of essential and trace element content of medicinal plants and their infusions by XRF and ICP techniques. X-Ray Spectrometry 34:213-17.

Quinn G. P.,Axelrod J. & Brodie B. B. 1958. Species, strain and sex differences in metabolism of hexobarbitone, amidopyrine, antipyrine and aniline. Biochemical Pharmacology 1:152-59.

Rasmussen S. E., Frederiksen H., Struntze Krogholm K. & Poulsen L. 2005. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. Mol Nutr Food Res 49:159-74.

Reddy M. B. & Cook J. D. 1991. Assessment of dietary determinants of nonheme-iron absorption in humans and rats. The American Journal of Clinical Nutrition 54:723-8.

Reed J. D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. Journal of Animal Science 73:1516-28.

Reichardt F., Habold C., Chaumande B., Ackermann A., Ehret Sabatier L., Le Maho Y., Angel F., Liewig N. & Lignot J. H. 2009. Interactions between ingested kaolinite and the intestinal mucosa in rat: proteomic and cellular evidences. Fundamental & Clinical Pharmacology 23:69-79.

Reller L. B., Weinstein M., Jorgensen J. H. & Ferraro M. J. 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. Clinical Infectious Diseases 49:1749-55.

Ren J., Meng S., Lekka C. E. & Kaxiras E. 2008. Complexation of Flavonoids with Iron: Structure and Optical Signatures. J. Phys. Chem. B 112:1845-50.

Reniere M. L.,Pishchany G. & Skaar E. P. 2010. Iron uptake in *Staphylococci.in* P. Cornelis and Andrews S. C., Editors. Iron Uptake and Homeostasis in Microorganisms. Caister Academic Press.

Reppas C. & Augustijns P. 2010. Drug Solubility in the Gastrointestinal Tract. Pages 155-67 Oral Drug Absorption.

Reyes-López M., Serrano-Luna J. d. J., Negrete-Abascal E., León-Sicairos N., Guerrero-Barrera A. L. & de la Garza M. 2001. *Entamoeba histolytica*: Transferrin binding proteins. Experimental Parasitology 99:132-40.

Riba-Hernandez P.,Stoner K. E. & Lucas P. W. 2003. The sugar composition of fruits in the diet of spider monkeys (*Ateles geoffroyi*) in tropical humid forest in Costa Rica. Journal of Tropical Ecology 19:709-16.

Richter A., Popp M., Mensen R., Stewart G. R. & Vonwillert D. J. 1995. Heterotrophic carbon gain of the parasitic Angiosperm *Tapianthus oleifolius*. Australian Journal of Plant Physiology 22:537-44.

Rintoul L., Panayiotou H., Kokot S., George G., Cash G., Frost R., Bui T. & Fredericks P. 1998. Fourier transform infrared spectrometry: a versatile technique for real world samples. Analyst 123:571-77.

Ríos J. L. & Recio M. C. 2005. Medicinal plants and antimicrobial activity. Journal of Ethnopharmacology 100:80-84.

Rios L. Y., Bennett R. N., Lazarus S. A., Rémésy C., Scalbert A. & Williamson G. 2002. Cocoa procyanidins are stable during gastric transit in humans. The American Journal of Clinical Nutrition 76:1106-10.

Rivero-Cruz I., Acevedo L., Guerrero J. A., Martinez S., Bye R., Pereda-Miranda R., Franzblau S., Timmermann B. N. & Mata R. 2005. Antimycobacterial agents from selected Mexican medicinal plants. Journal of Pharmacy and Pharmacology 57:1117-26.

Robbins C. T. 1994. Wildlife Feeding and Nutrition. Academic Press, New York.
Robbins C. T., Hagerman A. E., Austin P. J., McArthur C. & Hanley T. A. 1991. Variation in mammalian physiological responses to a condensed tannin and its ecological implications. Journal of Mammalogy 72:480-86.
Robbins R. J. 2003. Phenolic Acids in Foods: An Overview of Analytical Methodology. J. Agric. Food Chem. 51:2866-87.
Robinson D. P. & Klein S. L. 2012. Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. Horm Behav 62:263-71.
Rockwood L. L. & Glander K. E. 1979. Howling monkeys and Leaf-cutting ants: Comparative foraging in a tropical deciduous forest. Biotropica 11:1-10.
Rode K. D., Chapman C. A., Chapman L. J. & McDowell L. R. 2003. Mineral resource availability and consumption by *Colobus* in Kibale National Park, Uganda. International Journal of Primatology 24:541-73.
Rodríguez-Burbano D.,Quijano-Celis C. E. & Pino J. A. 2010. Composition of the Essential Oil from Leaves of *Astronium graveolens J*acq Grown in Colombia. Journal of Essential Oil Research 22:488-89.
Rodríguez M., Hasegawa M., González-Mújica F., Motta N., Castillo A., Castillo J., Zea E., Mora K., Sousa L., González A. & Camejo D. 2008. Antidiabetic and antiradical activities of plants from Venezuelan Amazon. Revista Brasileira de Farmacognosia 18:331-38.
Romagnoli S., Fogolari F., Catalano M., Zetta L., Schaller G., Urech K., Giannattasio M., Ragona L. & Molinari H. 2003. NMR solution structure of viscotoxin C1 from *Viscum album species Coloratum ohwi*: toward a structure-function analysis of viscotoxins. Biochemistry 42:12503-10.
Romani A., Ieri F., Turchetti B., Mulinacci N., Vincieri F. F. & Buzzini P. 2006. Analysis of condensed and hydrolysable tannins from commercial plant extracts. J Pharm Biomed Anal 41:415-20.
Rosolova H.,Mayer Jr O. & Reaven G. 2000. Insulin-mediated glucose disposal is decreased in normal subjects with relatively low plasma magnesium concentrations. Metabolism 49:418-20.
Ross D. S. 1995. Recommended methods for determining soil cation exchange capacity. Recommended Soil Testing Procedures for the Northeastern United States. Northeastern Regional Publication:62-69.
Rowell D. L. 1994. Soil Science: Methods and Applications. Longman Scientific & Technical, Longman Group UK Limited, Harlow, Essex, UK.
Ruan Z., Liu S., Zhou Y., Mi S., Liu G., Wu X., Yao K., Assaad H., Deng Z. & Hou Y. 2014. Chlorogenic Acid Decreases Intestinal Permeability and Increases Expression of Intestinal Tight Junction Proteins in Weaned Rats Challenged with LPS. Plos One 9:e97815.
Ruby M. V., Davis A., Link T. E., Schoof R., Chaney R. L., Freeman G. B. & Bergstrom P. 1993. Development of an *in vitro* screening test to evaluate the *in vivo* bioaccessibility of ingested mine waste lead. Environmental Science & Technology 27:2870-77.
Ruby M. V., Davis A., Schoof R., Eberle S. & Sellstone C. M. 1996. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environmental Science & Technology 30:422-30.
Ruby M. V., Schoof R., Brattin W., Goldade M., Post G., Harnois M., Mosby D. E., Casteel S. W., Berti W., Carpenter M., Edwards D., Cragin D. & Chappell W. 1999. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. Environmental Science & Technology 33:3697-705.
Ruiz-Terán F.,Medrano-Martínez A. & Navarro-Ocaña A. 2008. Antioxidant and free radical scavenging activities of plant extracts used in traditional medicine in Mexico. African Journal of Biotechnology 7.
Russell J. D. 1987. Infra-red methods. Pages 133-73 *in* M. J. Wilson, Editor. Handbook of Determinative Methods in Clay Mineralogy. Blackie & Son, Glasgow.
Sahgal G., Ramanathan S., Sasidharan S., Mordi M. N., Ismail S. & Mansor S. M. 2009. Phytochemical and antimicrobial activity of *Swietenia mahagoni* crude methanolic seed extract. Trop Biomed 26:274-9.
Sajfrtova M., Sovova H., Karban J., Rochova K., Pavela R. & Barnet M. 2013. Effect of separation method on chemical composition and insecticidal activity of Lamiaceae isolates. Industrial Crops and Products 47:69-77.
Salatino A.,Kraus J. E. & Salatino M. L. F. 1993. Contents of tannins and their histological localization in young and adult parts of *Struthanthus vulgaris* Mart. (Loranthaceae). Annals of Botany 72:409-14.
Samuelsson G. 1961. Phytochemical and pharmacological studies on *Viscum album* L. Further improvements in the isolation methods for viscotoxin. Studies on viscotoxin from *Viscum album* growing on *Tilia cordata* Mill. Sven Farm Tidskr. 65:481-94.
Samuelsson G. 1973. Mistletoe Toxins. Systematic Zoology 22:566-69.
Samuelsson G. & Ekblad M. 1967. Isolation and properties of Phoratoxin, a toxic protein from *Phoradendron serotinum* (Loranthaceae). Acta Chem. Scand. 21:849-56.
Samuelsson G., Olson T., Mellstrand T. & Petterson B. 1970a. Amino acid sequences and disulfide bonds of small toxic proteins from plants of the family Loranthaceae. Toxicon 8:150-51.
Samuelsson G.,Pettersson B. & Ljunggren A. 1997. Toxic proteins from the mistletoe *Dendrophtora clavata*. I. Isolation and properties of denclatoxin A and denclatoxin B. Acta Pharmaceutica Suecica 14:237-44.
Samuelsson G. & Pettersson B. M. 1970b. Separation of viscotoxins from the European mistletoe *Viscum album* ViL.(Loranthaceae) by chromatography on sulfoethyl Sephadex. Acta Chem Scand 24:2751-56.
Sano A., Yamakoshi J., Tokutake S., Tobe K., Kubota Y. & Kikuchi M. 2003. Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. Biosci Biotechnol Biochem 67:1140-3.
Santisteban J., Mediavilla R., López-Pamo E., Dabrio J., C., Zapata M. B. R., García M. J. G., Castaño S. & Martínez-Alfaro P. E. 2004. Loss on ignition: a qualitative or quantitative method for organic matter and carbonate mineral content in sediments? Journal of Paleolimnology 32:287-99.
Santorelli C. J.,Schaffner C. M. & Aureli F. 2011a. Universal behaviors as candidate traditions in wild Spider monkeys. Plos One 6.

Santorelli C. J., Schaffner C. M., Campbell C. J., Notman H., Pavelka M. S., Weghorst J. A. & Aureli F. 2011b. Traditions in Spider monkeys are biased towards the social domain. Plos One **6**(2):e16863.
Santos A. L. S., Sodre C. L., Valle R. S., Silva B. A., Abi-chacra E. A., Silva L. V., Souza-Goncalves A. L., Sangenito L. S., Goncalves D. S., Souza L. O. P., Palmeira V. F., d'Avila-Levy C. M., Kneipp L. F., Kellett A., McCann M. & Branquinha M. H. 2012. Antimicrobial Action of Chelating Agents: Repercussions on the Microorganism Development, Virulence and Pathogenesis. Current Medicinal Chemistry 19:2715-37.
Sarker S. D. & Nahar L. 2005. Hyphenated Techniques. Pages 233-67 *in* Z. L. Satyajit D. Sarker, Alexander I. Gray, Editor. Methods in Biotechnology Humana Press, New Jersey.
Sathiamoorthya, K. G., Chaturvedib A. K., Shuklab P. K. & Mauryaa R. 2007. New antifungal flavonoid glycoside from *Vitex negundo* Bioorganic & Medicinal Chemistry Letters 17: 239-42.
Sato M., Tsuchiya H., Akagiri M., Takagi N. & Iinuma M. 1997. Growth inhibition of oral bacteria related to denture stomatitis by anti-candidal chalcones. Aust Dent J 42:343-6.
Sato Y., Itagaki S., Kurokawa T., Ogura J., Kobayashi M., Hirano T., Sugawara M. & Iseki K. 2011. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. International Journal of Pharmaceutics 403:136-38.
Saxena K. 1989. Clinical features and management of poisoning due to potassium chloride. Med Toxicol Adverse Drug Exp 4:429-43.
Sayar S.,Saalatti R. & Naficy M. 1975. Studies on clinical haematological aspects and pathological changes in gastric mucosa in geophagia. Acta Medica Iran 18:137-47.
Schaefer H. M.,Schmidt V. & Winkler H. 2003. Testing the defence trade-off hypothesis: how contents of nutrients and secondary compounds affect fruit removal. Oikos 102:318-28.
Schaller G.,Urech K. & Giannattasio M. 1996. Cytotoxicity of different viscotoxins and extracts from the European subspecies of *Viscum album* L. Phytotherapy Research 10:473-77.
Schaller G., Urech K., Grazi G. & Giannattasio M. 1998. Viscotoxin composition of the three European sub specis of *Viscum ablum.* Planta Medica 64:677-78.
Scheer R.,A. S. & Errenst M. 1992. Two harvesting times, summer and winter: Are they essential for preparing pharmaceuticals from mistletoe (*Viscum album* L.). Page A594 *in* 40th Annual Congress of Medicinal Plant Research. Planta medica Trieste.
Schofield P.,Mbugua D. M. & Pell A. N. 2001. Analysis of condensed tannins: a review. Animal Feed Science and Technology 91:21-40.
Schrader-Fischer G. & Apel K. 1993. The Anticyclic timing of leaf senescence in the parasitic plant *Viscum album* is closely correlated with the selective degradation of Sulfur-rich Viscotoxins. Plant Physiology 101:745-49.
Schrader G. & Apel K. 1991. Isolation and characterization of cDNAs encoding viscotoxins of mistletoe (*Viscum album*). European Journal of Biochemistry 198:549-53.
Schubert M. L. 2007. Gastric secretion. Curr Opin Gastroenterol 23:595-601.
Schulkin J. 2000. Calcium Hunger: Behavioral and Biological Regulation. Cambridge University Press, Cambridge.
Schulze E. D., Lange O. L., Ziegler H. & Gebauer G. 1991. Carbon and nitrogen isotope ratios of mistletoes growing on nitrogen and non-nitrogen fixing hosts and CAM plants in the Namib desert confirm partial heterotrophy. Oecologia 88:457-62.
Schwertmann U. & Taylor R. M. 1989. Iron oxides. Pages 379–438. *in* J. B. Dixon and Weed S. B., Editors. Minerals in Soil Environments,. Soil Sci. Soc. America Book, Madison.
Serio-Silva J. C., Rico-Gray V., Hernandez-Salazar L. T. & Espinosa-Gomez R. 2002. The role of *Ficus* (Moraceae) in the diet and nutrition of a troop of Mexican Howler monkeys, *Alouatta palliata mexicana*, released on an island in southern Veracruz, Mexico. Journal of Tropical Ecology 18:913-28.
Sertie J. A., Basile A. C., Panizza S., Matida A. K. & Zelnik R. 1990. Anti-inflammatory activity and sub-acute toxicity of artemetin. Planta Med 56:36-40.
Setz E., Enzweiler J., Solferini V., Amendola M. & Berton R. 1999. Geophagy in the golden-faced saki monkey (*Pithecia pithecia chrysocephala)* in the Central Amazon. Journal of Zoology 247:91-103.
Severance H. W. J., Holt T., Patrone N. A. & Chapman L. 1988. Profound muscle weakness and hypokalemia due to clay ingestion. Southern Medical Journal 81:272-74.
Shah S.,Stapleton P. & Taylor P. 2008. The polyphenol (−)-epicatechin gallate disrupts the secretion of virulence-related proteins by *Staphylococcus aureus*. Letters in Applied Microbiology 46:181-85.
Sharon N. 2008. Lectins: past, present and future. Biochemical Society Transactions 36:1457-60.
Sharon N. & Lis H. 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 14:53R-62R.
Sharon N. & Lis H. 2007. Lectins. 2nd edition. Springer, Dordrecht, The Netherlands.
Shimooka Y. 2005. Sexual differences in ranging of *Ateles belzebuth belzebuth* at La Macarena, Colombia International Journal of Primatology 26:385-406.
Shinozuka K., Kikuchi Y., Nishino C., Mori A. & Tawata S. 1988. Inhibitory effect of flavonoids on DNA-dependent DNA and RNA polymerases. Experientia 44:882-85.
Siebner-Freibach H.,Hadar Y. & Chen Y. 2004. Interaction of Iron Chelating Agents with Clay Minerals. Soil Sci. Soc. Am. J. 68:470-80.
Silver S. 1996. Bacterial resistances to toxic metal ions - A review. Gene 179:9-19.
Simmen B., Tarnaud L., Bayart F., Hladik A., Thiberge A., Jaspart S., Jeanson M. & Marez A. 2005. Secondary metabolite contents in the forests of Mayotte and Madagascar, and their incidence on two leaf-eating lemur-species (*Eulemur* spp.). Revue d Ecologie - la terre et la vie 60:297-324.
Simonovska B., Vovk I., Andrensek S., Valentova K. & Ulrichova J. 2003. Investigation of phenolic acids in yacon

(*Smallanthus sonchifolius*) leaves and tubers. J Chromatogr A 1016:89-98.
Simpson S. J. & Raubenheimer D. 2012. The nature of nutrition: a unifying framework. Australian Journal of Zoology 59:350-68.
Singer M. S.,Mace K. C. & Bernays E. A. 2009. Self-Medication as Adaptive Plasticity: Increased Ingestion of Plant Toxins by Parasitized Caterpillars. Plos One 4:e4796.
Skoog D. A.,West D. M. & Holler F. J. 2006. Fundamentals of Analytical Chemistry. Sixth edition. Saunders College Publishing, Orlando, Florida, USA.
Smart G. 2002. LS 13 320 Particle size Analyser. Beckman Coulter Inc. Particle Characterization Group.
Smith B., Rawlins B. G., Cordeiro M., Hutchins M. G., Tiberindwa J. V., Sserunjogi L. & Tomkins A. M. 2000. The bioaccessibility of essential and potentially toxic trace elements in tropical soils from Mukono District, Uganda. Journal of the Geological Society 157:885-91.
Smith D. A. 1991. Species Differences in Metabolism and Pharmacokinetics: Are We Close to an Understanding? Drug Metabolism Reviews 23:355-73.
Smith T. 2000. Antibiotics from soil bacteria. Nat Struct Mol Biol 7:189-90.
Smyth J. D. & Halton D. W. 1987. Physiology of Trematode. Second, Revised edition. Cambridge University Press.
Snyder L. R., Barker J., Ando D. J., Kirkland J. J. & Glajch J. L. 2002. Practical HPLC Method Development. Wiley.
Söderlind E. & Dressman J. B. 2010. Physiological Factors Affecting Drug Release and Absorption in the Gastrointestinal Tract. Pages 1-20 Oral Drug Absorption.
Solecka D. 1997. Role of phenylpropanoid compounds in plant responses to different stress factors. Acta Physiologiae Plantarum 19:257-68.
Soler M. H., Stoeva S., Schwamborn C., Wilhelm S., Stiefel T. & Voelter W. 1996. Complete amino acid sequence of the A chain of mistletoe lectin I. FEBS Lett 399:153-7.
Soler M. H.,Stoeva S. & Voelter W. 1998. Complete amino acid sequence of the B chain of mistletoe lectin I. Biochem Biophys Res Commun 246:596-601.
Soloway S. & Wilen S. H. 1952. Improved Ferric Chloride Test for Phenols. Analytical Chemistry 24:979-83.
Sorensen T. C. & Fedigan L. M. 2000. Distribution of three monkey species along a gradient of regenerating tropical dry forest. Biological Conservation 92:227-40.
Sorgeloos P.,Remiche-Van Der Wielen C. & Persoone G. 1978. The use of *Artemia nauplii* for toxicity tests--a critical analysis. Ecotoxicol Environ Saf 2:249-55.
Souza S. M. M. M., C. S. S.; Couto, R. O.; Gil, E. S.; Fonseca, Y. M.; Conceição, E. C.S. 2012. *In vitro* antioxidant activity of *Apeiba tibourbou* Aubl. (Tiliaceae): A powerful antioxidant source of rosmarinic acid. Journal of Pharmacy Research 5:1414.
Sparks R. S. J.,Self S. & Walker G. P. L. 1973. Products of Ignimbrite Eruptions. Geology 1:115-18.
Sprynskyy M., Gadzała-Kopciuch R., Nowak K. & Buszewski B. 2012. Removal of zearalenone toxin from synthetic gastric and body fluids using talc and diatomite: A batch kinetic study. Colloids and Surfaces B: Biointerfaces 94:7-14.
Stalmach A., Edwards C. A., Wightman J. D. & Crozier A. 2012. Gastrointestinal stability and bioavailability of (poly)phenolic compounds following ingestion of Concord grape juice by humans. Mol Nutr Food Res 56:497-509.
Stan R. L., Hangan A. C., Dican L., Sevastre B., Hanganu D., Catoi C., Sarpataki O. & Ionescu C. M. 2013. Comparative study concerning mistletoe viscotoxins antitumor activity. Acta Biol Hung 64:279-88.
Stanford C. B. 2002. Avoiding predators: Expectations and evidence in primate antipredator behavior. International Journal of Primatology 23:741-57.
Stec B. 2006. Plant thionins – the structural perspective. Cellular and Molecular Life Sciences CMLS 63:1370-85.
Stec B., Markman O., Rao U., Heffron G., Henderson S., Vernon L. P., Brumfeld V. & Teeter M. M. 2004. Proposal for molecular mechanism of thionins deduced from physico-chemical studies of plant toxins. The Journal of Peptide Research 64:210-24.
Stein B., Stover L., Gillem A., Winters K., Lee J. H. & Chauret C. 2006. The effect of lectins on *Cryptosporidium parvum* oocyst *in vitro* attachment to host cells. Journal of Parasitology 92:1-9.
Stein G. M., Schaller G., Pfuller U., Schietzel M. & Bussing A. 1999. Thionins from *Viscum album* L: Influence of the viscotoxins on the activation of granulocytes. Anticancer Research 19:1037-42.
Stephens W. E. & Calder A. 2004. Analysis of non-organic elements in plant foliage using polarised X-ray fluorescence spectrometry. Analytica Chimica Acta 527:89-96.
Stewart G. R. & Press M. C. 1990. The physiology and biochemistry of parasitic angiosperms. Annu . Rev. Plant Physiol. Plant Mol. Biol. 41:127-51.
Stirpe F. 2005. Ribosome-Inactivating Proteins. Pages 9-29 *in* R. Wiley and Lappi D., Editors. Molecular Neurosurgery With Targeted Toxins. Humana Press.
Stolzenberg S. J.,Parkhurst R. M. & Reist E. J. 1976. Blastocidal and contraceptive actions by an extract and compounds from endod (*Phytolacca dodecandra*). Contraception 14:39-51.
Streckeisen A., Zanettin B., Le Bas M. J., Bonin B., Bateman P., Bellieni G., Dudek A., Efremova S., Keller J., Lamere J., Sabine P. A., Schmid R., Sorensen H. & Woolley A. R. 2002. Igneous Rocks: A Classification and Glossary of Terms, Recommendations of the International Union of Geological Sciences, Subcommission of the Systematics of Igneous Rocks. Cambridge University Press, 2002. ISBN 0-521-66215-X Cambridge University Press.
Stringham J., Bovier E., Wong J. & Hammond J. B. 2010. The Influence of Dietary Lutein and Zeaxanthin on Visual Performance. Journal of food science 75:R24-R29.
Sullivan J. H.,Teramura A. H. & Ziska L. H. 1992. Variation in UV-B sensitivity in plants from a 3000-m elevational gradient in Hawaii. American Journal of Botany 79:737-43.

Sumner P. & Mollon J. D. 2000. Catarrhine photopigments are optimized for detecting targets against a foliage background. J Exp Biol 203:1963-86.
Sun W. & Miller J. M. 2003. Tandem mass spectrometry of the B-type procyanidins in wine and B-type dehydrodicatechins in an autoxidation mixture of (+)-catechin and (-)-epicatechin. Journal of Mass spectrometry 38:438-46.
Sung W. S. & D.G. L. 2010. Antifungal action of chlorogenic acid against pathogenic fungi, mediated by membrane disruption. Pure Appl. Chem. 82:219-26.
Suntres Z. E.,Stone W. L. & Smith M. G. 2005. Ricin-induced toxicity: The role of oxidative stress. J. Med. CBR Def 3:1-21.
Svedstrom U., Vuorelaa H., Kostiainenb R., Huovinena K., Laaksoa I. & Hiltunen R. 2002. High-performance liquid chromatographic determination of oligomeric procyanidins from dimers up to the hexamer in hawthorn. Journal of Chromatography 968:53-60.
Swain S. S.,Ray D. K. & Chand P. K. 2012. ED-XRF spectrometry-based trace element composition of genetically engineered rhizoclones vis-a`-vis natural roots of a multi-medicinal plant, butterfly pea (*Clitoria ternatea* L.). J Radioanal Nucl Chem 293.
Symes C. T., Hughes J. C., Mack A. L. & Marsden S. J. 2006. Geophagy in birds of Crater Mountain Wildlife Management Area, Papua New Guinea. Journal of Zoology 268:87-96.
Symington M. M. 1988. Demography, ranging patterns and activity budgets of Black spider monkeys (*Ateles paniscus chamek*) in the Manu-National Park, Peru. American Journal of Primatology 15:45-67.
Szentmihályi K., May Z., Süle K. & Then M. 2013. Mineral element content of some herbs with anti inflammatory effect used in gastrointestinal diseases. Orvosi Hetilap 154:538-43.
Szentmihályi K., Taba G., Lado C., Fodor J., Then M. & Szőke É. 2005. Medicinal Plant Teas Recommended as nutritional source for element supplementation. Acta Alimentaria 34:161-67.
Tabiasco J., Pont F., Fournie J. J. & Vercellone A. 2002. Mistletoe viscotoxins increase natural killer cell-mediated cytotoxicity. European Journal of Biochemistry 269:2591-600.
Takemoto J. K.,Martinez S. E. & Davies N. M. 2012. Analysis of Flavonoids through Chromatography.*in* N. M. Davies and Yáñez J. A., Editors. Flavonoid Pharmacokinetics: Methods of Analysis, Preclinical and Clinical Pharmacokinetics, Safety, and Toxicology. John Wiley & Sons Inc, Hoboken, NJ, USA.
Takeuchi O. & Akira S. 2006. Innate Immunity. Encyclopedia of Molecular Cell Biology and Molecular Medicine. Wiley-VCH Verlag GmbH & Co. KGaA.
Tan K. H. 1998. Adsorption in Soils. Principles of Soil Chemistry, . Taylor & Francis.
Tasdemir D., Kaiser M., Brun R., Yardley V., Schmidt T. J., Tosun F. & Ruedi P. 2006a. Antitrypanosomal and antileishmanial activities of flavonoids and their analogues: *in vitro, in vivo*, structure-activity relationship, and quantitative structure-activity relationship studies. Antimicrob Agents Chemother 50:1352-64.
Tasdemir D., Lack G., Brun R., Rüedi P., Scapozza L. & Perozzo R. 2006b. Inhibition of *Plasmodium falciparum* fatty acid biosynthesis: Evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. Journal of Medicinal Chemistry 49:3345-53.
Tatara C. P., Newman M. C., McCloskey J. T. & Williams P. L. 1998. Use of ion characteristics to predict relative toxicity of mono-, di- and trivalent metal ions: *Caenorhabditis elegans* LC50. Aquatic Toxicology 42:255-69.
Taveira G. B., Mathias L. S., da Motta O. V., Machado O. L. T., Rodrigues R., Carvalho A. O., Teixeira-Ferreira A., Perales J., Vasconcelos I. M. & Gomes V. M. 2014. Thionin-like peptides from *Capsicum annuum* fruits with high activity against human pathogenic bacteria and yeasts. Peptide Science 102:30-39.
Taye B., Giday M., Animut A. & Seid J. 2011. Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia. Asian Pac J Trop Biomed 1:370-5.
Tegner C. 2013. Zoonotic respiratory infections and great ape conservation-an emerging challenge. Sveriges lantbruksuniversitet., Upsalla.
Tennakoon K. U.,Chak W. H. & Bolin J. F. 2011. Nutritional and isotopic relationships of selected Bornean tropical mistletoe–host associations in Brunei Darussalam. . Functional Plant Biology 38:505-13.
Tennakoon K. U., Chak W. H., Lim L. B. L. & Bolin J. F. 2014. Mineral nutrition of the hyperparasitic mistletoe *Viscum articulatum* Burm. f. (Viscaceae) in tropical Brunei Darussalam. Plant Species Biology 29:101-07.
Tennant S. M., Hartland E. L., Phumoonna T., Lyras D., Rood J. I., Robins-Browne R. M. & van Driel I. R. 2008. Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens. Infection and Immunity 76:639-45.
Testai L., Martelli A., Cristofaro M., Breschi M. C. & Calderone V. 2013. Cardioprotective effects of different flavonoids against myocardial ischaemia/reperfusion injury in Langendorff-perfused rat hearts. J Pharm Pharmacol 65:750-6.
Theng B. K. G. 2012. Chapter 1 - The Clay Minerals. Pages 3-45 *in* B. K. G. Theng, Editor. Developments in Clay Science. Elsevier.
Thirupathi K., Sathesh Kumar S., Raju V. S., Ravikumar B., Krishna D. R. & Krishna Mohan G. 2008. A Review of Medicinal Plants of the Genus *Cordia*: their Chemistry and Pharmacological Uses. Journal of Natural Remedies 8.
Thom E. 2007. The effect of chlorogenic acid enriched coffee on glucose absorption in healthy volunteers and its effect on body mass when used long-term in overweight and obese people. J Int Med Res 35:900-8.
Thomas A. W. & Frieden A. 1923. The Gelatin-Tannin Reaction. Industrial & Engineering Chemistry 15:839.
Thompson D. P. & Geary T. G. 1995. The structure and function of helminth surfaces. Page 204 *in* J. J. Marr and Muller M., Editors. Biochemistry and Molecular Biology of Parasites. Academic Press, new York.
Thunberg E. 1983. Phorotoxin-B, a toxic protein from the mistletoe *Phoradendron tomentosum* Subsp Macrophyllum. Acta Pharmaceutica Suecica 20:115-22.

Thunberg E. & Samuelsson G. 1977. Isolation and properties of ligatoxin A, a toxic protein from the mistletoe *Phoradendron liga*. Acta Pharmaceutica Suecica 14:64.
Thunberg E. & Samuelsson G. 1982. Isolation and properties of Ligatoxin-A, a toxic protein from the mistletoe *Phoradendron liga*. Acta Pharmaceutica Suecica 19:285-92.
Tiburski J. H., Rosenthal A., Deliza R., de Oliveira Godoy R. L. & Pacheco S. 2011. Nutritional properties of yellow mombin, *Spondias mombin* L. pulp. Food Research International 44:2326-31.
Tistaert C.,Dejaegher B. & Heyden Y. V. 2011. Chromatographic separation techniques and data handling methods for herbal fingerprints: A review. Analytica Chimica Acta 690:148-61.
Tonevitsky A., Agapov I., Solopova O., Yurkova M., Demina I., Pfueller K. & Pfueller U. 2001. Epitope specificity of mouse immune response on short polypeptides isolated from *Viscum album.* Arzneimittelforschung 51:964-9.
Tongaonkar P., Tran P., Roberts K., Schaal J., Ösapay G., Tran D., Ouellette A. J. & Selsted M. E. 2011. *Rhesus macaque* θ-defensin isoforms: expression, antimicrobial activities, and demonstration of a prominent role in neutrophil granule microbicidal activities. Journal of leukocyte biology 89:283-90.
Tortora G. J. & Grabowski S. R. 2003. Principles of Anatomy and Physiology. 10th edition. John Wiley & Sons., Hoboken, N.J.
Tosserams M.,Magendans E. & Rozema J. 1997. Differential effects of elevated ultraviolet-B radiation on plant species of a dune grassland ecosystem. Plant Ecology 128:267-81.
Toutain P. L.,Ferran A. & Bousquet-Melou A. 2010. Species differences in pharmacokinetics and pharmacodynamics. Handb Exp Pharmacol:19-48.
Tran D., Tran P., Roberts K., Ösapay G., Schaal J., Ouellette A. & Selsted M. E. 2008. Microbicidal Properties and Cytocidal Selectivity of *Rhesus Macaque* Theta Defensins. Antimicrobial agents and chemotherapy 52:944-53.
Trejo-Macias G. & Estrada A. 2012. Risk factors connected to gastrointestinal parasites in mantled *Alouatta palliata mexicana* and black howler monkeys *Alouatta pigra* living in continuous and in fragmented rainforests in Mexico.
Trentin D. S., Silva D. B., Amaral M. W., Zimmer K. R., Silva M. V., Lopes N. P., Giordani R. B. & Macedo A. J. 2013. Tannins possessing bacteriostatic effect impair *Pseudomonas aeruginosa* adhesion and biofilm formation. Plos One 8:e66257.
Tullet J. M., Araiz C., Sanders M. J., Au C., Benedetto A., Papatheodorou I., Clark E., Schmeisser K., Jones D. & Schuster E. F. 2014. DAF-16/FoxO Directly Regulates an Atypical AMP-Activated Protein Kinase Gamma Isoform to Mediate the Effects of Insulin/IGF-1 Signaling on Aging in *Caenorhabditis elegans*. PLoS genetics 10:e1004109.
Tunnicliffe J. M. & Shearer J. 2008. Coffee, glucose homeostasis, and insulin resistance: physiological mechanisms and mediators. Applied Physiology, Nutrition, and Metabolism 33:1290-300.
Türe C.,Böcük H. & Aşan Z. 2010. Nutritional relationships between hemi-parasitic mistletoe and some of its deciduous hosts in different habitats. Biologia 65:859-67.
Urech K.,Jäggy C. & Schaller G. 2009. Viscotoxin and mistletoe lectin contents in *Viscum album* L. - pharmaceutical implications. Pages 67-78 *in* R. Scheer, Alban S., Becker H., Holzgrabe U., Kemper F. H., Kreis W., Matthes H., and Schilcher H., Editors. Die Mistel in der Tumortherapie 2: Aktueller Stand der Forschung und klinische Anwendung.
Urech K.,Schaller G. & Jaggy C. 2006. Viscotoxins, mistletoe lectins and their isoforms in mistletoe (*Viscum album* L.) extracts Iscador. Arzneimittelforschung 56:428-34.
Urech K., Schaller G., Ramm H. & Baumgartner S. 2011. Organ specific and seasonal accumulation of viscotoxin isoforms in *Viscum album* spp. *album.in* 5th Mistletoe Symposium: Mistletoe in Cancer Therapy – Basic Research and Clinical Practice, November 10-12, 2011. Phytomedicine (Elsevier-Verlag), European Academy of Otzenhausen, Nonnweiler, Saarland.
Urech K., Schaller G., Ziska P. & Giannattasio M. 1995. Comparative study on the cytotoxic effect of viscotoxin and mistletoe lectin on tumor cells in culture. Phytother Res. 9:49-55.
US Food and Drug Administration. 2004. Guidance for Industry Botanical Drug Product., Rockville.
Valgas C., Souza S. M. d., Smânia E. F. A. & Smânia Jr. A. 2007. Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology 38:369-80.
van Acker S. A. B. E., van Balen G. P., van den Berg D. J., Bast A. & van der Vijgh W. J. F. 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochemical Pharmacology 56:935-43.
Van Dam R. M. & Feskens E. J. 2002. Coffee consumption and risk of type 2 diabetes mellitus. The Lancet 360:1477-78.
Van Damme E. J. M., Peumans W. J., Pusztai A. & Bardocz S. 1998. Handbook of Plant Lectins: Properties and Biomedical Applications. John Wiley, Chichester.
van den Berg R., Haenen G. R. M. M., van den Berg H. & Bast A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chemistry 66:511-17.
van Dooremolen W. A., Wielemaker W. G., van Breemen N., Meijer E. M. & van Reewijk L. P. 1990. Chemistry and Mineralogy of Andosols of various age in a soil chronosequence on Andesitic lahars in Costa Rica. Pages 139-41 *in* Geochemistry of the Earth's Surface and of Mineral Formation 2nd International Symposium Aix en Provence, France.
van Vuuren S. F. 2008. Antimicrobial activity of South African medicinal plants. Journal of Ethnopharmacology 119:462-72.
Vandecasteele C.,Vanhoe H. & Dams R. 1993. Inductively coupled plasma mass spectrometry of biological samples. Journal of Analytical Atomic Spectrometry 8:781-86.
Vandeputte O. M., Kiendrebeogo M., Rajaonson S., Diallo B., Mol A., El Jaziri M. & Baucher M. 2010. Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. Applied and Environmental

Microbiology 76:243-53.
Varela B. G., Fernandez T., Ricco R. A., Zolezzi P. C., Hajos S. E., Gurni A. A., Alvarez E. & Wagner M. L. 2004. *Phoradendron liga* (Gill. ex H. et A.) Eichl. (Viscaceae) used in folk medicine: anatomical, phytochemical, and immunochemical studies. Journal of Ethnopharmacology 94:109-16.
Vasconcellos C. L. C., Vitório K. C. D., Andrade P. A., Cambuí É. V. F., Lira A. F., Cavalcante S. C. H., Estevam C. S., Antoniolli Â. R. & Thomazzi S. M. 2009. Antinociceptive, anti-inflammatory, and antioxidant properties of *Phoradendron piperoides* leaves. Pharmaceutical biology 47:645-52.
Vasconcelos I. M., Cavada B. S., Moreira R. D. A. & Oliveira J. T. A. D. 1991. Purification and partial characterisation of a lectin from seeds of *Dioclea guianensis*. Journal of Food Biochemistry 15:137-54.
Vasconcelos I. M. & Oliveira J. T. A. 2004. Antinutritional properties of plant lectins. Toxicon 44:385-403.
Veiga L. M. & Ferrari S. F. 2007. Geophagy at termitaria by Bearded Sakis (*Chiropotes satanas*) in Southeastern Brazilian Amazonia. American Journal of Primatology 69:816-20.
Versantvoort C. H. M., Oomen A. G., Van de Kamp E., Rompelberg C. J. M. & Sips A. 2005. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology 43:31-40.
Versantvoort C. H. M.,van de Kamp E. & Rompelberg C. J. M. 2004. Development and applicability of an *in vitro* digestion model in assessing the bioaccessibility of contaminants from food (RIVM 320102002). National Institute for Environmental Public Health and the Environment, Bilthoven.
Vicas S.,Rugina D. & Socaciu C. 2011a. Comparative Study about Antioxidant Activities of *Viscum album* from Different Host Trees, Harvested in Different Seasons. Journal of Medicinal Plant Research 5:2237-44.
Vicas S. I.,Rugina D. & Socaciu C. 2012. Antioxidant Activity of European Mistletoe (*Viscum album*).*in* D. V. Rao., Editor. Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health. InTech.
Vicas S. I., Rugina O. D., Leopold L., Pintea A. & Socaciu C. 2011b. HPLC fingerprint of bioactive compounds and antioxidant activities of *Viscum album* from different host trees. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 39:48-57.
Villalba J. J. & Provenza F. D. 2007. Self-medication and homeostatic behaviour in herbivores: learning about the benefits of nature's pharmacy. Animal 1:1360-70.
Villalba J. J. & Provenza F. D. 2009. Learning and Dietary Choice in Herbivores. Rangeland Ecology & Management 62:399-406.
Villalba J. J. & Provenza F. D. 2010. Challenges in Extrapolating *In vitro* Findings to *In vivo* Evaluation of Plant Resources. Pages 233-42 *in* P. E. Vercoe, Makkar H. P. S., and Schlink A. C., Editors. *In vitro* screening of plant resources for extra-nutritional attributes in ruminants: nuclear and related methodologies. Springer Netherlands.
Villalba J. J.,Provenza F. D. & Shaw R. 2006. Sheep self-medicate when challenged with illness-inducing foods. Animal Behaviour 71:1131-39.
Villanua D., Perez-Rodriguez L., Gortazar C., Hofle U. & Vinuela J. 2006. Avoiding bias in parasite excretion estimates: the effect of sampling time and type of faeces. Parasitology 133:251-59.
Vogel H. G. 2006. Drug Discovery and Evaluation : Pharmacological Assays. 3 edition. Springer-Verlag, Berlin.
Vogel T. A., Patino L. C., Alvarado G. E. & Gans P. B. 2004. Silicic ignimbrites within the Costa Rican volcanic front: evidence for the formation of continental crust. Earth and Planetary Science Letters 226:149-59.
Voigt C. C., Capps K. A., Dechmann D. K. N., Michener R. H. & Kunz T. H. 2008. Nutrition or Detoxification: Why Bats Visit Mineral Licks of the Amazonian Rainforest. Plos One 3.
Voigt C. C., Dechmann D. K., Bender J., Rinehart B. J., Michener R. H. & Kunz T. H. 2007. Mineral licks attract neotropical seed-dispersing bats. International Journal of Ecology 2007.
Voinot F., Fischera C., Boeuf B., Schmidt C., Delval-Dubois V., Reichardt F., Liewiga N., Chaumand B., Ehret-Sabatier L., Lignot J. & Angel F. 2012. Effects of controlled ingestion of kaolinite (5%) on food intake, gut morphology and *in vitro* motility in rats. Fundamental & Clinical Pharmacology 26:565–76.
Vondruskova H., Slamova R., Trckova M., Zraly Z. & Pavlik I. 2010. Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. Veterinarni Medicina 5:199-224.
Voros J., Mahaney W. C., Milner M. W., Krishnamani R., Aufreiter S. & Hancock R. G. V. 2001. Geophagy by the Bonnet macaques (*Macaca radiata*) of southern India: A preliminary analysis. Primates 42:327-44.
Vos G. H. & Vos D. 1979. Heterophile Antibodies. Part I. The Specificity of Agglutinating and Hemolytic Sheep Red Blood Cell Antibodies in Human Sera. Transfusion 19:562-66.
Wacker R., Stoeva S., Pfüller K., Pfüller U. & Voelter W. 2005. Complete structure determination of *N*-acetyl-D-galactosamine-binding mistletoe lectin-3 from *Viscum album* L. *album*. Journal of Peptide Science 11:289-302.
Wada K. 1987. Minerals formed and mineral formation from volcanic ash by weathering. Chemical Geology 60:17-28.
Wada K. & Kakuto Y. 1985. Embryonic halloysites in Ecuadorian soils derived from volcanic ash. Soil Sci.Soc. Amer. J. 49:1309-18.
Waghorn G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—Progress and challenges. Animal Feed Science and Technology 147:116-39.
Wagner H. 1999. Phytomedicine Research in Germany. Environmental Health Perspectives 107.
Wagner M. L., Fernández T., Varela B. G., Alvarez E., Ricco R. A., Hajos S. & Gurni A. A. 1998. Anatomical, Phytochemical and Immunochemical studies on *Ligaria cuneifolia* (R. et P.) Tiegh (Loranthaceae) Pharmaceutical Biology (Formerly International Journal of Pharmacognosy), 46:131-39.
Wakibara J. V., Huffman M. A., Wink M., Reich S., Aufreiter S., Hancock R. G. V., Sodhi R., Mahaney W. C. & Russel S. 2001. The adaptive significance of geophagy for Japanese macaques (*Macaca fuscata*) at Arashiyama, Japan. International Journal of Primatology 22:495-520.

Waksmundzka-Hajnos M. & Sherma J. 2010. High Performance Liquid Chromatography in Phytochemical Analysis. Taylor & Francis.
Wall M. E., Taylor H., Ambrosio L. & Davis K. 1969. Plant antitumor agents III: A convenient separation of tannins from other plant constituents. Journal of Pharmaceutical Sciences 58:839-41.
Wall M. E., Wani M. C., Brown D. M., Fullas F., Olwald J. B., Josephson F. F., Thornton N. M., Pezzuto J. M., Beecher C. W. W., Farnsworth N. R., Cordell G. A. & Kinghorn A. D. 1996. Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. Phytomedicine 3:281-85.
Wallace R. B. 2005. Seasonal variations in diet and foraging behavior of *Ateles chamek* in a southern amazonian tropical forest. International Journal of Primatology 26:1053-75.
Wallace R. B. 2008. habitat use and ranging patterns.*in* C. J. Campbell, Editor. Spider Monkeys Behaviour, Ecology and Evolution of the Genus *Ateles*. Cambridge University Press, Cambridge.
Wandersman C. 2010. Heme uptake and iron extraction by bacteria.*in* P. Cornelis and Andrews S. C., Editors. Iron Uptake and Homeostasis in Microorganisms Caister Academic Press.
Wang, Teng H., Lee, Min H., Su & Nan W. 2009. Screening of lectins by an enzyme-linked adsorbent assay. Elsevier Ltd.
Wang Q. & Xie M. 2010. Antibacterial activity and mechanism of luteolin on *Staphylococcus aureus*. Wei Sheng Wu Xue Bao = Acta microbiologica Sinica. 50:1180-4.
Watanabe T., Arai Y., Mitsui Y., Kusaura T., Okawa W., Kajihara Y. & Saito I. 2006. The blood pressure-lowering effect and safety of chlorogenic acid from green coffee bean extract in essential hypertension. Clin Exp Hypertens 28:439-49.
Watson D. M. 2001. Mistletoe - A keystone resource in forests and woodlands worldwide. Annual Review of Ecology and Systematics 32:219-49.
Watson D. M. & Herring M. 2012. Mistletoe as a keystone resource: an experimental test. Proc Biol Sci 279:3853-60.
Watson R. R.,Preedy V. R. & Zibadi S. 2014. Polyphenols in Human Health and Disease. Elsevier.
Weinberg E. D. 1975. Nutritional Immunity - Hosts attempt to withhold iron from microbial invaders. Journal of the American Medical Association 231:39-41.
Weinberg E. D. 1996. Iron withholding: a defense against viral infections. Biometals. 9:393-9.
Weinberg E. D. 1999. The role of iron in protozoan and fungal infectious diseases. Journal of Eukaryotic Microbiology 46:231-38.
Weinberg E. D. 2009. Iron availability and infection. Biochimica Et Biophysica Acta 1790:600-05.
Weinberg E. D. & Miklossy J. 2008. Iron withholding: A defense against disease. Journal of Alzheimers Disease 13:451-63.
Weinberg E. D. & Moon J. 2009. Malaria and iron: history and review. Drug Metabolism Reviews 41:644-62.
Weissenstein U., Kunz M., Urech K. & Baumgartner S. 2014. Interaction of standardized mistletoe (*Viscum album)* extracts with chemotherapeutic drugs regarding cytostatic and cytotoxic effects *in vitro*. Bmc Complementary and Alternative Medicine 14:6.
Welker B. J., Konig W., Pietsch M. & Adams R. P. 2007. Feeding selectivity by mantled howler monkeys (*Alouatta palliata*) in relation to leaf secondary chemistry in *Hymenaea courbaril*. Journal of Chemical Ecology 33:1186-96.
Welkos S., Cote C., Hahn U., Shastak O., Jedermann J., Bozue J., Jung G., Ruchala P., Pratikhya P. & Tang T. 2011. Humanized θ-defensins (retrocyclins) enhance macrophage performance and protect mice from experimental anthrax infections. Antimicrobial agents and chemotherapy 55:4238-50.
Wen-Feng C., Guo-Fen Q., Yan-Jie L., Zhen-Wei P., Xian-Mei P., Yun-Long B., Hong-Li S. & Bao-Feng Y. 2006. Flavonoids from Chinese *Viscum coloratum*: antiarrhythmic efficacy and ionic mechanisms. Phytotherapy Research 20:1100-02.
Wendeln M. C. & Runkie J. R. 2000. Nutritional values of 14 fig species and bat feeding preferences in Panama. Biotropica 32:489-501.
Wesp E. F. & Brode W. R. 1934. The Absorption Spectra of Ferric Compounds. I. The Ferric Chloride—Phenol Reaction. Journal of the American Chemical Society 56:1037-42.
West M. E. & Feng P. C. 1967. Rubrine C, a pharmacologically active alkaloid from *Phoradendron rubrum*. J Pharm Pharmacol 19 (3):197-8.
Wiart C. 2007. Ethnopharmacology of Medicinal Plants: Asia and the Pacific. Humana Press.
Wijnands L.,Dufrenne J. & van Leusden F. 2003. *Bacillus cereus*: characteristics, behaviour in the gastro-intestinal tract, and interaction with Caco-2 cells. RIVM 250912003/2005, National Institutes for Environmental Public Health and the Environment, Bilthoven.
Wiley A. S. & Katz S. H. 1998. Geophagy in pregnancy: A test of a hypothesis. Current Anthropology 39:532-45.
Wilkinson J. M. 2006. Methods for Testing the Antimicrobial Activity of Extracts. Pages 157-71 in Modern Phytomedicine. Wiley-VCH Verlag GmbH & Co.
Williams L. B. & Haydel S. E. 2010. Evaluation of the medicinal use of clay minerals as antibacterial agents. Int Geol Rev 52:745-70.
Williams L. B., Metge D. W., Eberl D. D., Harvey R. W., Turner A. G., Prapaipong P. & Poret-Peterson A. T. 2011. What makes a natural clay antibacterial? Environmental Science and Technology 45:3768-73.
Wilson M. E. & Britigan B. E. 1998. Iron acquisition by parasitic protozoa. Parasitology Today 14:348-53.
Wilson M. J. 1987. X-ray powder diffraction methods. Pages 26-98 *in* M. J. Wilson, Editor. A handbook of determinative methods in clay mineralogy. Chapman and Hall, New York.
Wilson M. J. 2003a. Clay mineralogical and related characteristics of geophagic materials. Journal of Chemical Ecology 29:1525-47.
Wilson M. J. 2003b. A review of clay mineralogical and other characteristics of geophagy materials ingested by animals

and man. Pages 321-26 *in* J. L. P. Rodríguez, Editor. Applied Study of Cultural Heritage and Clays. Consejo Superior de Investigaciones Científicas.
Wilson M. J. 2004. Weathering of the primary rock-forming minerals: processes, products and rates. Clay Minerals 39:233-66.
Wink M. 2010. Annual Plant Reviews, Functions and Biotechnology of Plant Secondary Metabolites. Wiley.
Winter M. & Herrmann K. 1984. Analysis of hydroxycinnamic acid esters and their glucosides by reversed-phase high-performance liquid chromatography after polyamide separation. Journal of Chromatography A 315:243-51.
Wong S. K., Lim Y. Y., Abdullah N. R. & Nordin F. J. 2011a. Antiproliferative and phytochemical analyses of leaf extracts of ten Apocynaceae species. Pharmacognosy Res 3:100-6.
Wong S. K., Lim Y. Y., Abdullah N. R. & Nordin F. J. 2011b. Assessment of antiproliferative and antiplasmodial activities of five selected Apocynaceae species. BMC Complement Altern Med 11:3.
Wong S. K., Lim Y. Y., Ling S. K. & Chan E. W. 2014. Caffeoylquinic acids in leaves of selected Apocynaceae species: Their isolation and content. Pharmacognosy Res 6:67-72.
Wragg J. 2012. Development, Validation and Application of a Harmonised BARGE Method. Taipei International Conference on Remediation and Management of Soil and Groundwater Contaminated Sites & Soil and Groundwater Exhibition. Environmental Protection Agency, Taiwan, NTUH International Convention Center, Taipei, Taiwan (R.O.C.).
Wrangham R. W. 1995. Relationship of chimpanzee leaf-swallowing to a tapeworm infection American Journal of Primatology 37:297-303.
Xiao Z. P., Peng Z. Y., Peng M. J., Yan W. B., Ouyang Y. Z. & Zhu H. L. 2011. Flavonoids health benefits and their molecular mechanism. Mini Rev Med Chem 11:169-77.
Yasin B., Wang W., Pang M., Cheshenko N., Hong T., Waring A. J., Herold B. C., Wagar E. A. & Lehrer R. I. 2004. θ defensins protect cells from infection by *herpes simplex* virus by inhibiting viral adhesion and entry. Journal of virology 78:5147-56.
Yearsley J. M., Villalba J. J., Gordon I. J., Kyriazakis I., Speakman J. R., Tolkamp B. J., Illius A. W. & Duncan A. J. 2006. A theory of associating food types with their postingestive consequences. American Naturalist 167:705-16.
Young S., Wilson M., Miller D. & Hillier S. 2008. Toward a Comprehensive Approach to the Collection and Analysis of Pica Substances, with Emphasis on Geophagic Materials. Plos One.
Young S. L., Goodman D., Farag T. H., Ali S. M., Khatib M. R., Khalfan S. S., Tielsch J. M. & Stoltzfus R. J. 2007. Geophagia is not associated with *Trichuris* or hookworm transmission in Zanzibar, Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 101:766-72.
Young S. L., Khalfan S. S., Farag T. H., Kavle J. A., Ali S. M., Hajji H., Rasmussen K. M., Pelto G. H., Tielsch J. M. & Stoltzfus R. J. 2010. Association of Pica with Anemia and Gastrointestinal Distress among Pregnant Women in Zanzibar, Tanzania. The American Journal of Tropical Medicine and Hygiene 83:144-51.
Young S. L., Sherman P. W., Lucks J. B. & Pelto G. H. 2011. Why on earth?: Evaluating hypotheses about the physiological functions of human geophagy. The Quarterly Review of Biology. 86:97-120.
Yusuf L.,Oladunmoye M. & Ogundare A. 2013. In-vivo Antibacterial Activities of Mistletoe *(Viscum album*) Leaves Extract Growing on Cocoa Tree in Akure North Nigeria. European Journal of Biotechnology and Bioscience 1:37-42.
Zamora N., Méndez J., Barahona M. & Sjöbohm L. 2004. Volcano - Estratigrafía associada al campo de domos de Cañas Dulces, Guanacaste, Costa Rica. Revista Geológica de América Central 30:41-58.
Zarkovic N., Vukovic T., Loncaric I., Miletic M., Zarkovic K., Borovic S., Cipak A., Sabolovic S., Konitzer M. & Mang S. 2001. An overview on anticancer activities of the *Viscum album* extract Isorel (R). Cancer Biotherapy and Radiopharmaceuticals 16:55-62.
Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389-95.
Zhao Y.-L., Fan R.-H., Yuan H.-X., Yu M., Bi K.-S. & Yu Z.-G. 2011. Development of the fingerprints for the quality evaluation of *Viscum coloratum* by high Performance liquid chromatography. Journal of Pharmaceutical Analysis 1:113-18.
Zhao Z.-h.,Sakagami Y. & Osaka T. 1998. Relationship between Residual Metal Ions in a Solution and the Inhibitory Capability of the Metal Ions for Pathogenic Bacterial Growth. Bulletin of the Chemical Society of Japan 71:939-45.
Zuk M. & McKean K. A. 1996. Sex differences in parasite infections: Patterns and processes. International Journal for Parasitology 26:1009-24.