UNIVERSITE DE LAUSANNE

FACULTE DES SCIENCES

Institut de Pharmacognosie et Phytochimie

Phytochemical investigation of plants used in African traditional medicine: *Dioscorea sylvatica* (Dioscoreaceae), *Urginea altissima* (Liliaceae), *Jamesbrittenia fodina* and *Jamesbrittenia elegantissima* (Scrophulariaceae).

Thèse de doctorat

présentée à la Faculté des Sciences de l'Université de Lausanne

par

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A mes parents

A Alain

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Scientific communications

The present work has been partially published and presented in international congresses as poster presentations.

Publications

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Posters

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Abbreviations and symbols

1,2,3	Symbols used for the compounds cited in the introduction but not isolated during this work
A, B, C	Symbols used for the isolated compounds
$[\alpha]_D$	Optical rotation
δ	Chemical shift (ppm) (NMR)
AChE	Acetylcholinesterase
AIDS	Acquired Immunodeficiency Syndrome
APCI	Atmospheric Pressure Chemical Ionization (MS)
BuOH	Butanol
CC	Open Column Chromatography
$CHCl_3$	Chloroform
CPC	Centrifugal Partition Chromatography
d	Doublet (NMR)
Da	Dalton (molecular weight unity)
D/CI	Desorbtion / Chemical Ionization (MS)
DCM	Dichloromethane
dd	Double doublet (NMR)
DEPT	Distorsionless Enhancement by Polarization Transfer (NMR)
DMSO	Dimethylsulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical
dqCOSY	Double Quantum Correlation Spectroscopy (NMR)
EI	Electron impact
EtOAc	Ethyl acetate
EtOH	Ethanol
Fuc	Fucose
gHMBC	^{1}H, ^{13}C, Gradient Heteronuclear Multiple Bond Correlation (NMR)
gHSQC	^{1}H, ^{13}C, Gradient Heteronuclear Single Quantum Coherence (NMR)
Glc	Glucose
HPLC	High Performance Liquid Chromatography
HR	High Resolution (MS)
Hz	Hertz

i.d.	Internal diameter
^{i}PrOH	Isopropanol
J	Coupling constant (NMR)
LC	Liquid Chromatography
LPLC	Low Pressure Liquid Chromatography
m	Multiplet (NMR)
MeCN	Acetonitrile
MeOH	Methanol
Mp	Melting point
MPLC	Medium Pressure Liquid Chromatography
MS	Mass Spectrometry
MW	Molecular Weight
m/z	Mass per electronic charge
n.d.	Not determined
NMR	Nuclear Magnetic Resonance
ppm	Parts per million (NMR unity)
q	Quadruplet (NMR)
Rha	Rhamnose
R_f	Retention Factor (TLC)
RP-18	Reversed Phase Silica with C-18 functional groups
s	Singlet (NMR)
sh	Shoulder (UV spectrum)
sp	Unspecified species (one)
spp	Species (several)
t	Triplet (NMR)
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane (NMR)
TOCSY	Total Correlation Spectroscopy (NMR)
TPA	Tetradecanoylphorbol Acetate
UV	Ultraviolet

Table of content

1 Aim of this work

African medicinal plants have a long history of providing important sources of healing drugs to local populations. In certain African countries, up to 90% of the population still relies exclusively on plants as a source of medicines (Hostettmann *et al*., 2000).

The available knowledge on the use of plant preparations in traditional medicine is important, but with the decreasing number of traditional healers, the dissemination of their valuable knowledge is progressively diminishing. An effort has been made to document this know-how. For this purpose, Gelfand *et al.* published in 1985 an ethnobotanical survey of the medicinal plants used by the traditional practitioners throughout Zimbabwe. Beside an enumeration of the plants, their preparations and indications, the book provides a detailed understanding of the medical practice of the traditional healer. This valuable information constitutes a basis for the investigation of the pharmacological and phytochemical aspects of these natural medications, and thus for the validation of their therapeutic benefits and their possible toxic effects.

With this aim, the present work will focus in the first part on the validation of use of plants frequently employed in traditional medicine for skin problems and rheumatism, but which are suspected of negative side effects.

Phytochemical aspects will be developed in a second part with the detection, isolation and characterisation of new natural compounds of potential therapeutic interest from plants of Zimbabwe.

With a very diverse climate and relief, the country possesses a very rich flora of more than 5000 species of higher plants and ferns; only a small percentage has been phytochemically investigated. Despite the constant development of synthetic pharmaceutical chemistry, including combinatorial chemistry and microbial fermentation, the pharmaceutical industry has realised that plants represent a huge reservoir of possible active substances. As stated by Farnsworth of the University of Illinois at Chicago, higher plants are unique chemical factories, "capable of synthesising unlimited numbers of highly complex and unusual chemical substances whose structures could otherwise escape the imagination forever[1]".

[1] **http://www.wri.org/biodiv/pharmacy.html**

Aim of this work

The strategy for the search of active compounds in this work is based on a chemical and biological screening performed on selected plant extracts. Different therapeutic targets are used. First is a search for antiinfectious molecules by means of fungicidal and antibacterial assays, combined with a screening for molluscicidal activity because snails are hosts to the vector of schistosomiasis, a widespread worldwide parasitic disease. Moreover, there is a search for new antioxidants, which have proved their role as protectors against cancer and atherosclerosis. Finally, an interest is taken in the discovery of new inhibitors of acetylcholinesterase, which are used as cholinergic replacement therapy in Alzheimer's disease.
The plants extracted and submitted to the screening are selected on the basis of their use in traditional medicine, the available literature data and chemotaxonomic criteria. The extracts presenting the most interesting activities are then selected for activity-guided phytochemical investigation, in order to identify the active compounds. Moreover, the isolation and characterisation of further molecules presenting original chemical structures and a potential therapeutic interest is performed.

A general description of the flora from Zimbabwe and a brief survey of traditional medicine in Zimbabwe precede this study.

2 Introduction

2.1 Plants in Zimbabwe

2.1.1 Flora of Zimbabwe

Zimbabwe, formerly called Rhodesia, is located in south-eastern Africa latitude 15°35' to 22°30'S and longitude 25°10' to 33°5'E. Its political borders follow natural borders: the Zambezi River and Lake Kariba to the north-west with Zambia, the Eastern Highlands to the east with Mozambique, the Limpopo River to the south with South Africa, and several small rivers to the south-west with Botswana (Seine, 1996).

The country falls within the pantropical belt of summer seasonal rainfall and has a pronounced dry season of 6 to 8 months. Most of the country's flora belongs to the Zambesian Regional Centre of Endemism (Müller, 1999), which consists of vegetation adapted to withstand prolonged periods without rain.

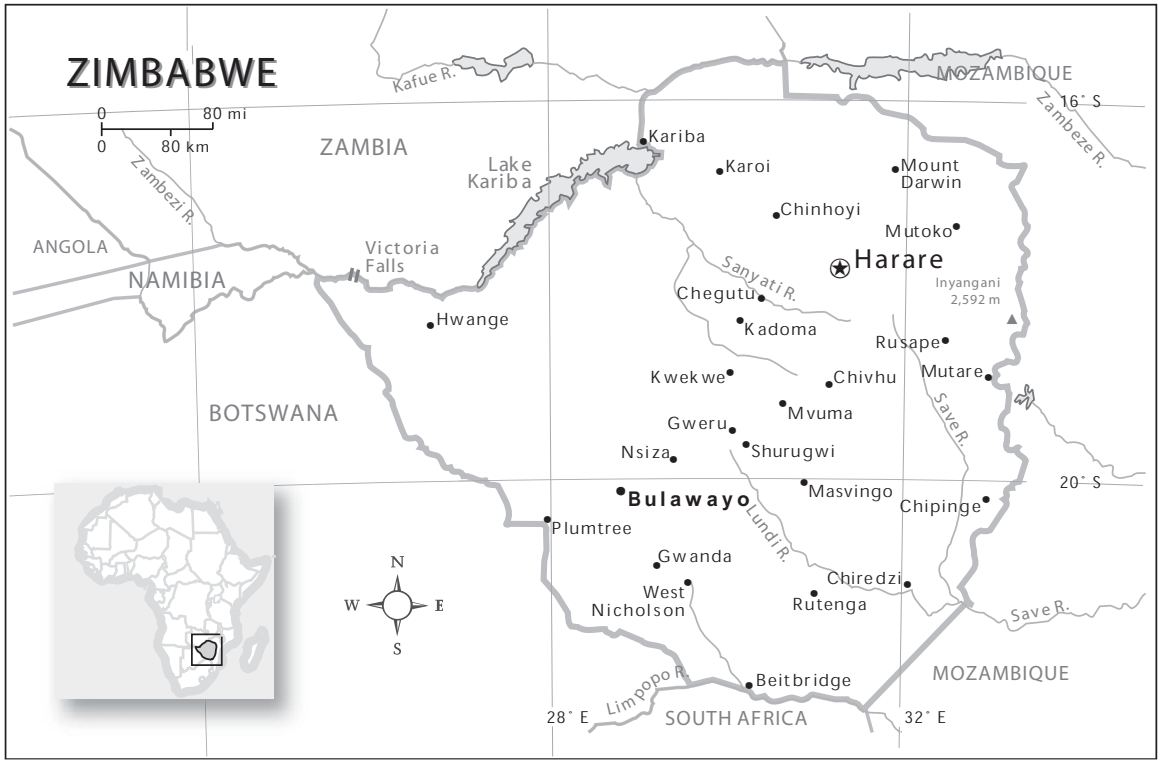


Figure 1: Map of Zimbabwe

The flora of Zimbabwe contains more than 5000 species (Gelfand *et al*., 1985).

Such diversity is allowed by the topography of the country that eases the tropical temperatures that might be expected at this latitude.
Six major physiognomic types are recognised in Zimbabwe: forest, thicket, woodland and savannah woodland, tree savannah, shrub savannah and grassland, as shown in Figure 2 (Wild and Grandvaux Barbosa, 1967):

-Forest

This covers most parts of the eastern districts of Zimbabwe, and generally occurs on steep slopes and sheltered valleys. *Erythrophleum suaveolens* Brenan (Leguminosae), *Zanha golungensis* Hiern (Burseraceae) and lianas like *Paullinia pinnata* L. (Sapindaceae) and *Saba comorensis* Pichon (Apocynaceae) are common in the moist evergreen forest of low altitude.
Above about 1700 m., the forest is dominated by *Cussonia umbellifera* Sond. (Araliaceae), *Albizia gummifera* C.A. Smith (Leguminosae), *Ilex mitis* Radlk. (Aquifoliaceae) and many other species.

-Thicket

Many species of *Commiphora* (Burseraceae) and *Combretum* (Combretaceae) grow on alluvial soils, grits or sandstones of the Lower Zambezi. *Dichrostachys cinerea* Wight & Am (Mimosaceae) and *Kirkia acuminata* Oliver (Simaroubaceae) often occur in this area.

-Savannah woodland

Known locally as miombo woodland, or *Brachystegia* woodland, because of the dominance of this genus, it represents most of central Zimbabwe. West of Harare, *Brachystegia boehmii* Taub. (Leguminosae) grows often associated with the mnondo (*Julbernardia globiflora* Troupin, Leguminosae*)*, at an altitude of 1000-1350 m.
In the Eastern Highlands, the msasa (*B. spicaeformis* Benth.) ascends to almost 2500 m.
In the lower area close to Lake Kariba, *J. globiflora* alternates with *Colophospermum mopane* J. Leonard (Leguminosae) zones, *Acacia* (Leguminosae) zones or *Combretum-Terminalia* (Combretaceae) zones.

-Tree savannah

The evergreen *Parinari cardiophylla* Ducke (Rosaceae) is characteristic of the northern plateau of Zimbabwe, where it grows on sandy humid soils. The grass cover is abundant in the intervening areas. It also grows around Chipinge in the east. *Terminalia sericea* Cambess (Combretaceae) is predominant south of Bulawayo. Different species of *Acacia* are very common around Bulawayo. *Colophospermum mopane* J. Leonard is abundant in the south, close to the borders with South Africa and Botswana, and alternates with *Comiphora-Combretum* zones.

-Shrub savannah

Colophospermum dominant shrub savannah appears on badly drained heavy clays in the south of the country.

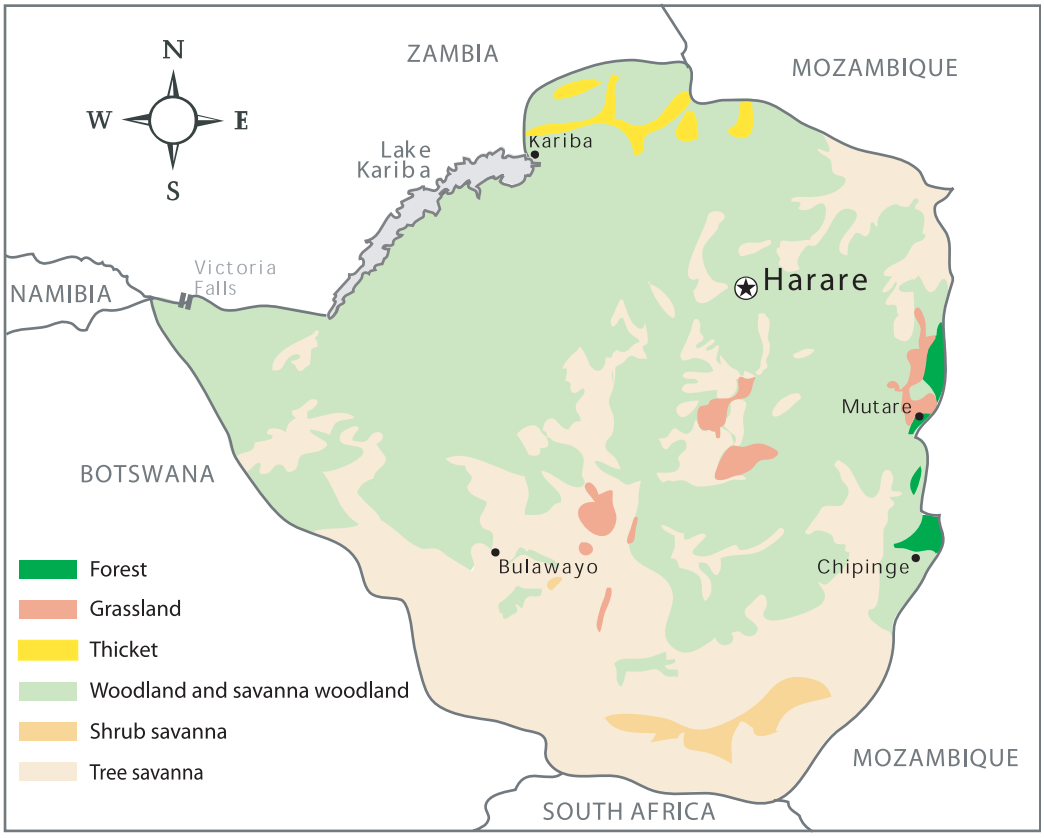


Figure 2: Vegetation map of Zimbabwe (Wild and Grandvaux Barbosa, 1967)

-Grassland

The serpentine grassland is confined to the serpentine areas of the Great 'Dyke', an extraordinary geological feature that runs more than 500 km north to south (see 2.3.2). The soils are stony, shallow and poor, and often on steep slopes. They carry almost no trees and are covered with short, stunted grass; the grass species principally concerned is *Andropogon gayanus* Kunth (Gramineae) commonly found with *Themeda triandra* Forsk. (Poaceae) co-dominant. Many plant species are endemic to this area.
Grassland also appears in the submontane areas of the Eastern Highlands. *Themeda* is dominant on the more fertile red soils, and *Loudetia* (Gramineae) on the poorer granite soils. Many types of grasses are associated.

The Zimbabwe flora is closely related to that of its neighbouring countries. Botswana, Zambia, Malawi, Zimbabwe and Mozambique united their efforts to create the Flora Zambesiaca in order to update the flora of all the territory drained by the Zambezi River. But further similarities can be established with South Africa and Angola vegetation (Wild, 1956).

2.1.2 Use of plants in traditional medicine

Out of the 5000 plant species growing in Zimbabwe, about 10 percent are used in traditional medicine. The knowledge of the traditional practitioners is thus of a great value. To document this precious oral tradition Gelfand *et al*. (1985) interviewed more than 200 traditional healers and noted all the plants they used, the symptoms of the patients and the ways of administration. This survey constitutes a valuable basis for the selection of plants and search for new active compounds.

As traditional healers do not generally make a clear distinction between 'medicine' in its medical sense and the broader sense which includes charms of various sorts, it seems important to give an overview of their methods.

2.1.2.1 The traditional healer's practice

Medicine is closely linked to religion in the faith of the Shona, the major ethnic community which occupies most of central and eastern Zimbabwe. The *n'anga*, or traditional folk practitioner, is as much a minister of religion as a diagnostician and healer.

N'anga's get their gift of healing and divining either from a *mudzimu* (ancestral spirit) or from a shave spirit (spirit of an unrelated *n'anga*) in the course of a dream.

A n'anga usually receives the patients in his house. Depending on the kind of illness, the *n'anga* will begin the consultation with divination. If the patient suspects the disease to be caused by a witch or an angered spirit, he will request divination. Different types of divination can be used; sometimes the *n'anga* will throw carved wooden bones (*hakata*) and interprets the combination of the pieces with their carved face uppermost.

Once the spiritual cause of the illness has been established, the patient may add information concerning his symptoms, and then the *n'anga* will administrate medicines to the patient. Some traditional healers will dispense treatments without divination, these are called herbalists.

Figure 2: A n'anga in his consultation room

2.1.2.2 Treatments

Medicines are commonly given in the form of powders, decoctions, infusions or ointments.

Fine powder to be taken orally is obtained by drying the drug in the sun, crushing it into small pieces and then grinding it into powder in a stamping block. The powder is usually taken in porridge or *sadza* (thickened porridge made out of pulverized grains, generally white maize), sometimes in water or beer.

Ointments are prepared by mixing the prepared potions with castor oil.

Inhalations are commonly used for respiratory disorders such as asthma and to drive away bad spirits. The aromatic ingredients are powdered and added to boiling water the vapour of which is inhaled by the patient.

A popular method of treatment is scarification. Incisions are made, and an irritant powder is rubbed into the incisions to increase blood supply to the affected part. This is often performed in case of rheumatism, headache, or abdomen and chest pains.

A *n'anga* carries with him a *gona,* a container made of a horn or a calabash containing special medicines. It generally consists of castor oil mixed with honey or sugar, small pieces of roots and twigs (often various species of *Loranthus*, Loranthaceae) and sometimes parts of animals. The content of the gona is claimed to have many beneficial effects; it can be added to medicines or used directly (Gelfand *et al*., 1985).

2.1.2.3 Importance of traditional medicine

When an individual has decided to seek professional help for a medical problem, he has to decide whether the illness should be referred to a modern or a traditional health practitioner. The decision depends largely upon the cost of each type of treatment, accessibility, knowledge of the probable effect of each type of treatment and the definition given to the illness by those involved. It was observed by Chavunduka (1994) that a patient often tries to find a social cause or a meaning to his illness. A western doctor does not produce an answer to this question and merely tries to cure the physical problem. If the patient fails to respond to the treatment, he will often turn to the traditional healer who, on the other hand, identifies himself with the patient's difficulties and social problems and helps find if the ancestral spirits have been offended; this gives the patient more confidence in his remedies. However, there are cases when the patient, dissatisfied with the treatment, turns back to the scientific practitioner (Gelfand *et al*., 1985).

Traditional healers have been represented since 1980 by an organization known as the Zimbabwe National Traditional Healers Association (ZINATHA) (Chavunduka, 1994). ZINATHA is involved in traditional health care with a cultural approach to HIV/AIDS education, prevention, counselling and palliative care. It represents most of the traditional healers (herbalists, spirit mediums, faith healers, and traditional midwives) practising in Zimbabwe. It should also promote a better recognition of the traditional practitioners.

2.2 Presentation of two plants used for skin diseases and rheumatism

The frequent use of two plants sold in Zimbabwe markets, *Dioscorea sylvatica* Ecklon (Dioscoreaceae) and *Urginea altissima* Baker (Liliaceae) was reported. Both plants were said to be used in case of rheumatism or skin disease: fresh underground parts are rubbed on the skin, causing a strong irritation. This could lead to problems associated with skin inflammation.
This work started with a short study of the possible causes of this irritant property.

2.2.1 Dioscorea sylvatica

2.2.1.1 Botanical description

The Dioscoreaceae, distributed throughout the tropics and some temperate regions, constitute a family consisting mainly of tropical climbers. The yams are its best known members.
Almost all species are perennial herbaceous or shrubby climbers with well developed tubers or rhizomes. They can climb to a great height by twining on a support (Heywood, 1993).

According to the Angiosperm Phylogeny Group classification (1998), the systematic position of the Dioscoreaceae is as shown below:

Superdivision	Spermatophytae
Division	Angiospermae
Class	Monocotyledons
	Non commelinoid
Order	Dioscoreales Hook f
Family	Dioscoreaceae

The history of nomenclature of *Dioscorea sylvatica* has presented many changes. The plant was named in 1830 by Ecklon, but was transferred to the genus *Testudinaria* by Kunth (1850). In 1924, Knuth reduced *Testudinaria* to a subgenus of *Dioscorea.* In 1952, Burkill divided his section *Testudinaria* into three species, one of which was *D. sylvatica*. In 1953, Rowley restored *Testudinaria* to generic rank and *T. sylvatica* included *D. sylvatica* and *D. hederifolia*. However, this treatment was not adopted by subsequent workers, and the taxonomy of the group is still in a fluid state (Blunden *et al*., 1971).

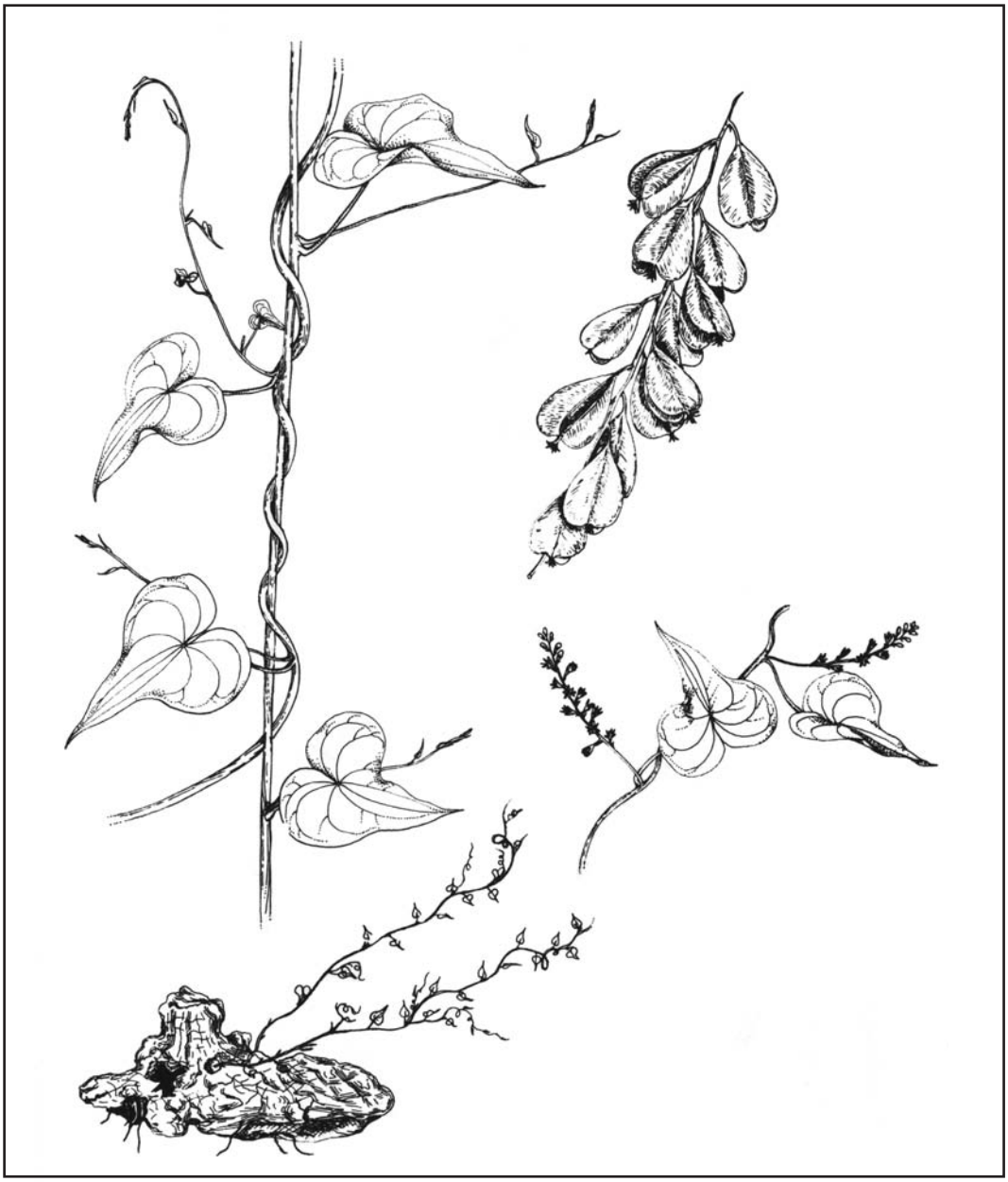


Figure 3: *Dioscorea sylvatica* Ecklon.

Dioscorea sylvatica Ecklon is a dioecious plant with the upper leafy parts dying back seasonally and being renewed either from the crown or, if the aerial stem persists, from aerial buds.

The tuber is perennial and hypogeal, or with up to one third exposed. The stem is solitary and climbing, leaves are generally alternate, deciduous and glabrous. Male and female flowers are solitary, the perianth is rotate and yellowish-cream or yellowish-green, respectively. There are 6 stamens or an inferior trilocular ovary, respectively. The fruit is a yellowish-brown oblong to obovate capsule (Archibald, 1967).

Figure 4: *Dioscorea sylvatica* Ecklon.

2.2.1.2 Traditional use

The tubers of numerous species of the genus *Dioscorea* (Dioscoreaceae), known as yam, are one of the most important sources of carbohydrates in some regions of the tropics (Wanasundera and Ravindran, 1994). The alkaloid dioscorin is characteristic of the genus. It is bitter and toxic. In the species cultivated for food in West Africa, the toxicity is generally very low. Certain wild species, like *D. dumetorum* Pax, *D. sylvatica* Ecklon, or *Dioscorea bulbifera* L. may be eaten, after soaking for some days in cold water and boiling, as famine food (Gelfand *et al*., 1985; Neuwinger, 1996).
The genus is also well known since the 1950s for the extraction of sapogenins, used as precursors in the semi-synthesis of sex hormones and corticosteroids (Norton, 1998).

Dioscorea sylvatica Ecklon has been reported to be sold on the markets in Zimbabwe for the treatment of skin diseases and *chitsinga* (physical disorder characterised by pain and swelling of the joints) (Gelfand *et al*., 1985). The fresh peeled rhizome is rubbed on the skin. A mild inflammation and itching soon appear.

It is interesting to underline that the use of other species for skin problems and rheumatic troubles is frequent throughout Africa:

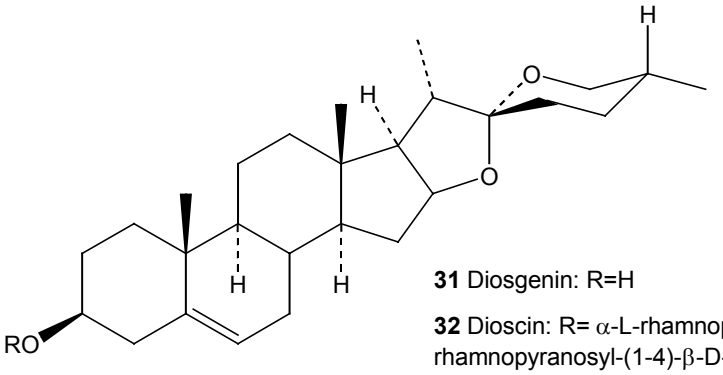
- In Zimbabwe, *Dioscorea bulbifera* L. tuber is also used as an infusion to apply on cuts and sores, both for humans and animals (Tredgold, 1990). In Cameroon and Madagascar, the pounded bulbs are applied to abscesses, boils and wound infections (Neuwinger, 1996).
- In Senegal, the tuber of *D. dumetorum* Pax. is sometimes used externally as a rubefacient (Kerharo, 1974). In Sudan, the root tuber is used against rheumatoid arthritis. In Southeast Tanzania, grated root is applied to suppurating abscesses (Neuwinger, 1996).
- Leaf juice of *D. alata* L. has been used as in applications to scorpion stings and the tuber to sores (Watt and Breyer-Brandwijk, 1962).
- Water heated in the scooped tuber of *D. dregeana* Dur. and Schinz is used as a lotion for cuts, sores and other minor superficial lesions in both human beings and animals (Watt and Breyer-Brandwijk, 1962).
- In Tanzania, the crushed tuber of *D. hirtiflora* Benth. is applied to scabies while the leaf juice is drunk in case of purulent abscess (Haerdi *et al*., 1964).

2.2.1.3 Previous phytochemical investigations on the genus

Steroid sapogenins, saponins and related steroids

The family is characterised by the rich occurrence of steroidal saponins, often consisting of glycosides of diosgenin (**31**). Dioscin (**32**) is toxic and produces convulsions (Hutchings, 1996).

Diosgenin, extracted from *D. mexicana* Scheidw. or *D. sylvatica* Ecklon., is used for the hemisynthesis of sex hormones and oral contraceptives; it can also serve as a precursor of the corticosteroid synthesis by the employment of a microbiological fermentation to introduce oxygen into the 11α-position (Trease and Evans, 1978).

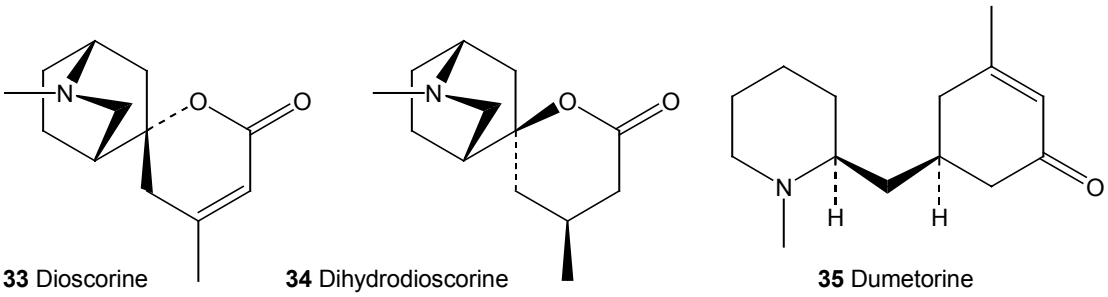


31 Diosgenin: R=H

32 Dioscin: R= α-L-rhamnopyranosyl-(1-2) -α-L-rhamnopyranosyl-(1-4)-β-D-glucopyranoside

Alkaloids

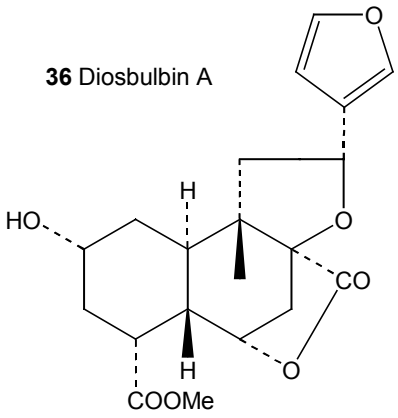
Alkaloids are responsible for the toxicity of many species of *Dioscorea*. The convulsant dioscorine (**33**) and dihydrodioscorine (**34**) are found in many toxic species, like *D. dumetorum* Pax. and *D. sansibarensis* Pax. (Neuwinger, 1996). Dumetorine (**35**) was isolated from the tubers of *D. dumetorum* Pax.(Corley *et al*., 1985).



33 Dioscorine **34** Dihydrodioscorine **35** Dumetorine

Bitter norditerpenoids

Diosbulbins A (**36**) to H, isolated from *Dioscorea bulbifera* L., all display an antitumor effect (Komori, 1997).



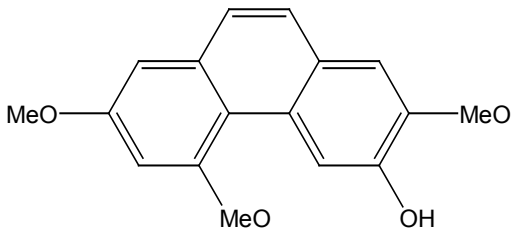
36 Diosbulbin A

Carbohydrates

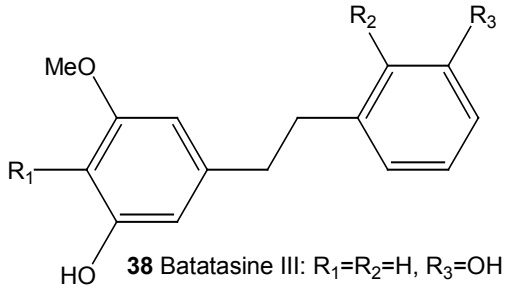
Starch represents about 80% of the tuber dry matter of *D. alata* (Wanasundera and Ravindran, 1994). Mucilage is also abundant (Hegnauer, 1986).

Dihydrostilbenes and phenanthrenes

Batatasines I to V (**37**-**40**) have been isolated from many species of *Dioscorea* (Hegnauer, 1986).



37 Batatasine I



38 Batatasine III: R_1=R_2=H, R_3=OH

39 Batatasine IV: R_1=R_3=H, R_2=OH

40 Batatasine V: R_1=OMe, R_2=OH, R_3=H

Other compounds

Oxalate salts are abundant: 300 to 400 mg were determined in 100 g of dry tuber of *Dioscorea alata*, most of it water soluble (Wanasundera and Ravindran, 1994).

2.2.2 *Urginea altissima*

2.2.2.1 Botanical description

The Liliaceae are one of the largest families of flowering plants; they are also very important in horticulture, as they include the ornamental lilies, tulips and hyacinths. The onion is the chief economic representative. The family is cosmopolitan, but smaller groups often have a limited distribution.

Most of the Liliaceae are herbs, most of them having swollen storage organs such as bulbs, corms, rhizomes or thick fleshy roots.

Many of the Liliaceae are pollinated by insects, attracted by honey secreted from the ovaries or nectarines exposed at the base of the perianth (Heywood, 1993).

According to the Angiosperm Phylogeny Group classification (1998), the systematic position of the Liliaceae is as shown opposite.

Superdivision	Spermatophytae
Division	Angiospermae
Class	Monocotyledons
Order	Liliales Perleb
Family	Liliaceae

Urginea is a heterogeneous, poorly understood genus in need of revision (Hutchings, 1997). It has at times been included in the genus *Drimia* (Jessop, 1977; Stedje, 1987). The genus is also placed in the Hyacinthaceae (Germplasm Resources Information Network)[1].

Urginea altissima Baker (or *Drimia altissima* Ker-Gawl or *Ornithogalum altissimum* L.f.) is a bulbous herb widespread in southern Africa.

The bulb is 9 to 20 cm long with whitish to pale green scales. Leaves are oblong, 20 to 40 cm long and 25 to 40 cm broad. Peduncles are 70 to 150 cm long, usually with 100 to 300 spirally arranged flowers. The white or cream-coloured perianth segments are all similar and united in the lower third or quarter. The stamens are inserted at the top of the perianth-tube.

[1] http://www.ars-grin.gov/var/apache/cgi-bin/npgs/html/taxon.pl?40903

The capsule is obovoid, 10-12 mm long and the seeds are flat and elliptic (Jessop, 1977).

Figure 4: *Urginea altissima* Baker

2.2.2.2 Traditional use

Urginea altissima Baker is sold on Harare markets for the same indications as *Dioscorea sylvatica,* that is *chitsinga* and skin troubles (Gelfand *et al*., 1985). The fresh bulb is rubbed on the skin, and again, an irritation appears quickly.

Indications of a similar use in other areas are less numerous in the literature.

Bulbs of *Urginea altissima* Baker are used in southern Africa as poultices for rheumatic swellings and gouty limbs (Hutchings, 1996).

Urginea physodes Baker is used by Zulu for the 'itch' of men and goats (Hutchings, 1996).

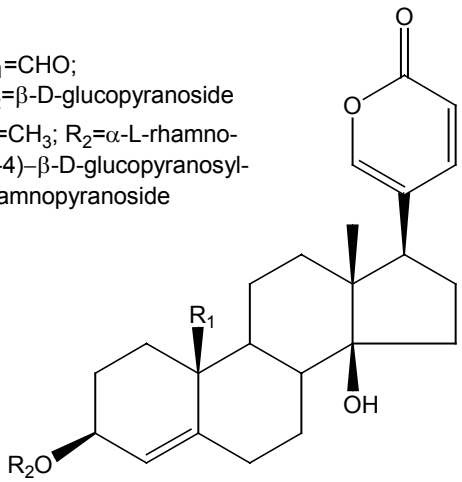
2.2.2.3 Previous phytochemical investigations

Cardiotonic glycosides

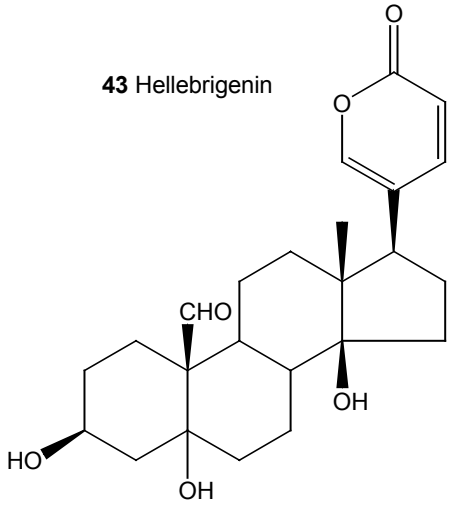
Urginea altissima contains a mixture of 6 bufadienolides that characterize the genus (Iwu, 1996). Amongst these are the glycoside altoside (**41**) the aglycone hellebrigenin (**43**) (Shimada *et al*., 1979), and the more recently isolated glycoside urginin (**42**) (Pohl *et al*., 2001).

41 Altoside: R_1=CHO; R_2=β-D-glucopyranoside

42 Urginin: R_1=CH_3; R_2=α-L-rhamno-pyranosyl(1-4)–β-D-glucopyranosyl-(1-3)-α-L-rhamnopyranoside

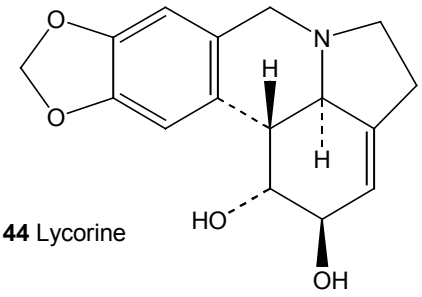


43 Hellebrigenin



Alkaloids

The alkaloid lycorine, isolated from the water extract of *U. altissima*, showed antimicrobial action against *Phytophtora capsici* (Miyakado *et al*., 1975).



44 Lycorine

Other compounds

Urginea species usually contain quercetin and kaempferol derivatives (Iwu, 1996). Their bulbs are rich in fructane (Hegnauer, 1963).

2.2.3 Plant contact dermatitis

Plants may induce skin reactions through mechanical/chemical irritancy or allergenicity. Sometimes, light is needed to ensure an adverse skin reaction, either phototoxic or photoallergic (Benezra and Ducombs, 1987).

2.2.3.1 Irritant plants

Certain plants provoke microtraumatic injuries by mechanical means, with spines, thorns, or knife-like leaf or scale edge, which can develop into sores and granulomatous lesions (Ducombs and Schmidt, 1995).

A common irritant mechanism consists of a mechanical irritation promoting injection of irritant compounds into the skin. A good example is the case of *Diffenbachia picta* Schott (Araceae), an ornamental plant which is responsible for many accidents amongst children and domestic animals. Its toxicity is due to calcium oxalate raphides impregnated by lipid substance(s).This product injected by the raphides when in contact with the mouth mucosa produces an oedema (Carneiro *et al*., 1989).
Apart from causing injury because of its sharp spines, the sap from *Agave americana* L. (Agavaceae) also induces chemical irritant reactions (Ricks *et al*., 1999).

The term urticaria is derived from species of *Urtica*, the stinging nettle (Urticaceae): the tips of the leaf hairs containing histamine, 5-hydroxytryptamine, acetylcholine and leukotrienes break off along a specific line after blunt traumas, and the content of the hair is injected into the skin with the sharp end of the remaining hair (Czarnetzki *et al*., 1990).
This mechanism has also been described amongst Dioscoreaceae. The black bryony, or *Tamus communis* L., has been used in popular medicine as a rubefacient to remove blue marks resulting from bruises.

In 1937, Cortesi gave a description of very long and sharp calcium oxalate raphides he had observed in the rhizome of the plant, which could easily penetrate the epidermis when rubbed on the skin; he advised against its use in friction.

Schmidt and Moult (1983) demonstrated later that a second element, histamine, was responsible for the contact dermatitis. They suggested that the skin reaction in contact with the plant was caused by a mechanical irritation by the sharp oxalate needles, followed by an intracutaneous injection of histamine, starting the inflammatory process.

A case of allergy has been reported after contact with *Dioscorea batatas* Decaisne, but problems of irritation are more frequent. Calcium oxalate needles found in the plant certainly induce a mechanical irritation, but other non-identified substances may be responsible for the irritation or allergy (Kubo *et al*., 1988).

Some plants can induce a chemical irritation without concommitent irritation: *Euphorbia* species contain diterpene polyol esters which induce acute oedematous reactions, with pain rather than itching (Ducombs and Schmidt, 1995).

2.2.3.2 Allergic contact dermatitis

Some Liliaceae are well known amongst gardeners for producing contact dermatitis. *Tulipa gesnerana* L. causes “tulip fingers”, an allergic reaction consisting of a dry hyperkeratotic finger tip with eczema and pustules. Small particles of the bulb tecta, the dry and hard outer layer of the bulb, cause skin damage, which promotes penetration of the allergens tulipalin A and B. Calcium oxalate crystals are not considered to be responsible for a mechanical irritation because they are present only in very small amounts in the plant (Bruynzeel, 1997).
The bulb of *Hyacinthus orientalis* L., another Liliaceae, contains about 6 % of calcium oxalate crystals. Handling the bulbs causes eczematous eruptions, with itching and urticarial lesions. The itching disappears when the crystals are removed from the skin. Cases of possible allergic contact dermatitis have been sporadically described in the literature, but most publications are on irritant contact dermatitis (Bruynzeel *et al*., 1997).

2.3 Biological and chemical screening

2.3.1 Preliminary selection criteria

The search for new active compounds from higher plants being the aim of the second part of this work, the choice of the extracts to be studied was decided on the basis of a biological/chemical screening.

In the first instance, a selection of the plants to be extracted and tested had to be made according to different criteria.

- Most of the plants were chosen amongst recent collections from Zimbabwe kept at the Institute of Pharmacognosy and Phytochemistry. These were mainly random collections, from areas with a much diversified flora.

- A preliminary bibliographic search was performed to perceive little studied plants. The lack of previous phytochemical studies on a species, genus or family increases the chance of finding a new compound.

- Chemotaxonomic criteria can be of importance, as certain plant families possess therapeutically interesting compounds.

- The use of a plant in traditional medicine in Zimbabwe (or possibly another country) can be of value. It is necessary to emphasize, however, that for checking a specific traditional use, a battery of pharmacological and/or clinical tests is necessary. Nevertheless, the common use of a plant for various indications in different areas or countries might indicate the presence of interesting compounds.
  A short presentation of the Zimbabwean traditional medicine will be given in paragraph 2.1.2.

2.3.2 Biological and chemical screening

Final selection of the plant extracts to be studied was determined following the pharmacological/chemical screening of the plants chosen in a first instance.

2.3.2.1 Screening results

70 crude extracts were obtained from 28 selected plants. These consisted mainly of dichloromethane and methanol extracts. When sufficient plant material was available, an additional water extract was prepared. For the two fresh samples, *Dioscorea sylvatica* Ecklon (Dioscoreaceae) and *Urginea altissima* Baker (Liliaceae), water and methanol extracts were prepared with subsequent liquid-liquid extractions with solvents of different polarities (petrol ether, dichloromethane, ethyl acetate and butanol).

These extracts were tested on biological/chemical targets to determine the following activities:

- **Fungicidal activity** against *Cladosporium cucumerinum,* a phytopathogenic fungus
- **Fungicidal activity** against *Candida albicans,* a yeast notably responsible for oropharyngeal infections in immunodeficient HIV patients
- **Antibacterial activity** against *Bacillus subtilis*, a gram-positive aerobic bacteria
- **Inhibitory activity** on the enzyme *acetylcholinesterase*: inhibitors of this enzyme are already used as cholinergic replacement therapy in Alzheimer's disease
- **Molluscicidal activity** against *Biomphalaria glabrata,* the host of the vector of schistosomiasis (bilharzia), a parasitic disease widespread in Africa
- **Radical scavenging activity** with DPPH (1,1-diphenyl-2-picrylhydrazyl radical). Free radicals are known to participate in the pathogenesis of many disorders including cancer and atherosclerosis; natural antioxidants can thus serve as lead compounds for the development of new drugs.

Almost all these tests were performed on TLC, except for the molluscicidal test, allowing a visualization of the active compound(s). Procedures are detailed in paragraphs 5.5.3 and 5.7. Results of the screening are displayed in Table 1.

Table 1: Biological and chemical screening of plant extracts from Zimbabwean plants

Family and plant name (registration number)	Organ	Extract[2]	Biological/chemical target[1,3]					
			CC	CA	BS	AChE	BG	DPPH
Acanthaceae								
Dyschoriste alba S.Moore (98135)	Leaves & twigs	DCM	(+)	-	-	(+)	nd	(+)
		MeOH	-	-	-	-	nd	++
Amaryllidaceae								
Buphane disticha Herb. (95030)	Bulbs	DCM	(+)	-	(+)	(+)	nd	(+)
		MeOH	-	-	-	+	nd	-
Asclepiadaceae								
Asclepias densiflora N.E.Br (98051)	Aerial parts	DCM	-	-	+	-	nd	-
		MeOH	-	-	+	-	nd	+
Asteraceae								
Crassocephalum rubens S.Moore (98112)	Leaves	DCM	-	-	(+)	+	nd	-
		MeOH	-	-	-	(+)	nd	-
Geigeria schinzii. O.Hoffm (99033)	Aerial parts	DCM	-	-	(+)	+	nd	-
		MeOH	-	-	-	-	nd	+
Capparaceae								
Cadaba termitaria N.E.Br. (98038)	Whole plant	DCM	-	-	(+)	(+)	-	-
		MeOH	-	-	-	-	-	-
		H_2O	-	-	-	-	-	(+)
Celastraceae								
Hippocratea longipetiolata Oliver (98048)	Leaves & twigs	DCM	(+)	-	-	+	nd	-
		MeOH	-	-	-	(+)	nd	+
Combretaceae								
Combretum apiculatum Sond. (98146)	Leaves	DCM	++	++	++	-	nd	+
		MeOH	-	-	+	-	nd	++
Dioscoreaceae								
Dioscorea sylvatica Ecklon (99012)	Fresh bulbs	PE	(+)	-	+	(+)	nd	-
		DCM	(+)	-	+	(+)	nd	-
		EtOAc	-	-	+	(+)	nd	+
		BuOH						
		H_2O	-	-	-	-	nd	-
Euphorbiaceae								
Pseudolachnostylis maprouneifolia Pax (91131)	Roots	DCM	-	-	(+)	-	nd	(+)
		MeOH	-	-	(+)	(+)	nd	+
	Bark	DCM	-	-	(+)	-	nd	-
		MeOH	-	-	(+)	(+)	nd	+

Family and plant name	Organ	Extract[2]	Biological/chemical target[1,3]					
			CC	CA	BS	AChE	BG	DPPH
Acalypha petiolaris Sond. (2000039)	Root	DCM	-	-	(+)	(+)	nd	-
		MeOH	-	-	+	-	nd	(+)
		H_2O	-	-	nd	-	nd	++
	Leaves	DCM	-	-	-	-	nd	-
		MeOH	-	-	(+)	-	nd	(+)
Leguminosae								
Cordyla africana Lour. (98049)	Leaves	DCM	(+)	-	(+)	(+)	nd	-
		MeOH	-	-	-	(+)	nd	+
	Trunk bark	DCM	(+)	-	-	-	nd	-
		MeOH	-	-	(+)	-	nd	-
Macrotyloma densiflorum (Baker) Verdc. (98124)	Leaves	DCM	-	-	-	(+)	nd	-
		MeOH	-	-	-	-	nd	-
Tephrosia acaciaefolia. Welw ex Baker (98126)	Leaves & twigs	DCM	+	-	(+)	(+)	nd	-
		MeOH	-	-	-	-	nd	(+)
Liliaceae								
Urginea altissima Baker (2000043)	Fresh bulbs	DCM	-	-	(+)	(+)	nd	-
		BuOH	-	-	-	-	nd	-
		H_2O	-		-	-	nd	-
Malvaceae								
Sida cordifolia L. (98031)	Aerial parts	DCM	-	-	-	(+)	nd	-
		MeOH	-	-	-	-	nd	(+)
Meliaceae								
Ekebergia benguelensis Welw. Ex C.DC. (99027)	Bark	DCM	-	-	+	(+)	nd	-
		MeOH	-	-	+	(+)	nd	++
	Leaves	DCM	-	-	-	-	nd	-
		MeOH	-	-	-	-	nd	++
Moraceae								
Ficus capreaefolia Delile (98040)	Leaves & twigs	DCM	+	(+)	-	+	nd	-
		MeOH	-	-	-	-	nd	+
Myrtaceae								
Syzygium huillense Engl. (98042)	Trunk bark	DCM	-	-	-	(+)	nd	-
		MeOH	-	-	(+)	-	nd	+
Nyctaginaceae								
Boerhaavia erecta Burm f, (98068)	Aerial parts	DCM	-	-	(+)	+	nd	-
		MeOH	-	-	-	-	nd	+
Poaceae								
Brachiaria brizantha Stapf (98100)	Aerial parts	DCM	-	-	-	(+)	nd	(+)
		MeOH	-	-	-	-	nd	(+)

Family and plant name	Organ	Extract[2]	Biological/chemical target[1,3]					
			CC	CA	BS	AChE	BG	DPPH
Panicum maximum Jacq. (98092)	Aerial parts	DCM	-	-	(+)	(+)	nd	-
		MeOH	-	-	-	-	nd	(+)
Proteaceae								
Faurea speciosa Welw. (98111)	Leaves	DCM	-	-	-	(+)	nd	-
		MeOH	-	-	(+)	-	nd	+
Scrophulariaceae								
Jamesbrittenia elegantis-sima (Schinz) O.M.Hilliard (98184)	Whole plant	DCM	(+)	-	+	+	(+)	(+)
		MeOH	+	+	++	(+)	+	(+)
Jamesbrittenia fodina (Wild) O.M Hilliard (98182)	Whole plant	DCM	-	-	(+)	(+)	(+)	-
		MeOH	++	++	++	+	++	++
Sellaginellaceae								
Sellaginella dregei (C.Presl) Hieron (98167)	Whole plant	DCM	-	-	(+)	(+)	-	-
		MeOH	-	-	(+)	-	-	+
Verbenaceae								
Clerodendrum glabrum f. pubescens R.Fernandes (92001)	Leaves	DCM	+	(+)	+	(+)	nd	-
		MeOH	-	-	-	(+)	nd	++
Vitaceae								
Cissus rotundifolia Vahl (98044)	Aerial parts	DCM	-	-	(+)	(+)	nd	-
		MeOH	-	-	(+)	-	nd	+

[1]CC: *Cladosporium cucumerinum*
CA: *Candida albicans*
BS: *Bacillus subtilis*
AChE: Acetylcholinesterase
BG: *Biomphalaria glabrata*
DPPH: 1,1-diphenyl-2-picrylhydrazyl radical

[2]DCM: dichloromethane
MeOH: methanol
PE: petrol ether
EtOAc: ethyl acetate
BuOH: butanol

- no activity
(+) weak activity
+ moderate activity
++ strong activity
nd not determined

The activity scale was generally determined on a visual basis, by comparison between the extracts. For BG, the activity was determined using dilutions (see 5.7.4).

[3]The quantities of extract spotted on the plates were of 100 μg for CC, CA, BS and DPPH and 40 μg for AChE. The molluscicidal test (BG) was performed with 400 ppm aqueous solutions.

2.3.2.2 Discussion

Out of the 70 extracts tested, 7 (10%) displayed a significant activity against *Cladosporium cucumerinum* (moderate to strong activity), only 3 against *Candida albicans* (4%), 14 against *Bacillus subtilis* (20%).

8 extracts (11%) were inhibitors of acetylcholinesterase. This test was recently introduced at the Institute of Pharmacognosy and Phytochemistry as a routine test; it was not available when the present work was started. Activities were thus not taken into consideration for the choice of extracts to be studied. The results obtained were added to a database established with the results of a large scale screening initiated recently at the Institute of Pharmacognosy and Phytochemistry. The positive results can thus be part of further inverstigation.

Only 9 extracts were tested on *Biomphalaria glabrata* for practical reasons, 2 of which were molluscicidal.

Radical scavenging activity was not considered as a priority criterion: 21 out of the 22 antioxidant extracts (31%) were methanol extracts. Polar extracts are often rich in polyphenols like tannins and flavonoids, well documented for their antioxidant activity (Potterat, 1997). The only apolar extract to display a significant radical scavenging activity was the dichloromethane extract of *Combretum apiculatum.*

The amount of extract tested in the different assays was chosen according to previous work. It must be underlined, however, that a very active compound present in a very small amount in an extract may not be detected while major but not very active compounds will show a clear activity.

The extracts displaying the most promising activities were:

- *Combretum apiculatum* Sond.: strong antifungal, antibacterial and antioxidant activities of the polar and non-polar extracts.

Biological activities of *Combretum* are well documented. Acidic triterpenoids and their glycosides isolated from species of *Combretum* displayed molluscicidal, antifungal, antimicrobial and anti-inflammatory activities. Stilbenes isolated from *C. caffrum* were shown to be cytostatic, and phenanthrenes isolated from *C. apiculatum* demonstrated an antifungal activity (Rogers and Verotta, 1996).

- *Jamesbrittenia fodina* O.M. Hilliard and *J. elegantissima* O.M. Hilliard: strong antifungal, antibacterial, molluscicidal (and radical scavenging for the former) activities. The genus has hardly been studied.

- *Clerodendrum glabrum f. pubescens* R. Fernandes: antifungal and antibacterial activities of the non-polar extract. The hydroquinone uncinatone isolated from *C. uncinatum* was shown to be antifungal (Dorsaz *et al*., 1985).

- *Tephrosia acaciaefolia* Welw ex Baker: antifungal activity on CC and CA of the non-polar extract. Rotenoids isolated from species of *Tephrosia* have an insecticidal activity (Kole *et al*., 1992). Moreover, the genus has been the object of several studies.

- *Ficus capreaefolia* Delile: antifungal activity of the non-polar extract.

A TLC analysis and coelution with a standard showed that the active spot corresponded to psoralen, the antifungal activity of which had already been determined in *Ficus racemosa* (Deraniyagala *et al*., 1998).

Considering the activities mentioned above and the literature studies, *Jamesbrittenia fodina* O.M. Hilliard and *J. elegantissima* O.M. Hilliard were selected for a phytochemical study.

2.4 Presentation of Jamesbrittenia fodina *and* J. elegantissima

2.4.1 Botanical aspects

2.4.1.1 The family Scrophulariaceae

The Scrophulariaceae family is cosmopolite, and is mostly found in temperate areas of the northern hemisphere. It comprises 190-220 genera and 2600-4000 species (Spichiger *et al*., 2000).

This large family only has a limited economic use. The best known application is the extraction of digitalin and digoxin from certain species of *Digitalis*. Some species have medicinal properties, such as *Verbascum thapsus* Brot. or *Scrophularia nodosa* Linn. Many genera are well known as ornamental, such as species of *Antirrhinum* (snapdragons), *Veronica* (speedwells), *Mimulus* (monkey flowers) or *Digitalis* (foxglove).

Scrophulariaceae are usually herbs, shrubs or lianas, rarely trees, annual or perennial. Some representatives are hemi-parasitic on the roots of their hosts like the Rhinanthae. Their leaves are alternate or opposite, without stipules.

The type of inflorescence is variable. Flowers are cyclic, gamopetalous, hypogynous, bisexual, zygomorphic, with a persistent calyx and a corolla more or less bilabiate, sometimes spurred or actinomorphic.

Stamens are usually didynamous and inserted on the corolla, sometimes accompanied by staminodes. The ovary is superior, bicarpelled, bilocular, with one terminal stigma.

The fruit is usually a dry capsule, with various types of dehiscence or more rarely, indehiscent (Heywood, 1993; Philcox, 1990).

2.4.1.2 Classification

According to the Angiosperm Phylogeny Group classification (1998) based on plant phylogeny, the systematic position of the Scrophulariaceae is as shown opposite:

Superdivision	Spermatophytae
Division	Angiospermae
Class	Eudicotyledons
Subclass	Asteridae
Order	Lamiales
Family	Scrophulariaceae

This APG classification attributes 23 tribes to the Scrophulariaceae family. The genus *Jamesbrittenia* is one of the 17 genera belonging to the Manulae tribe.

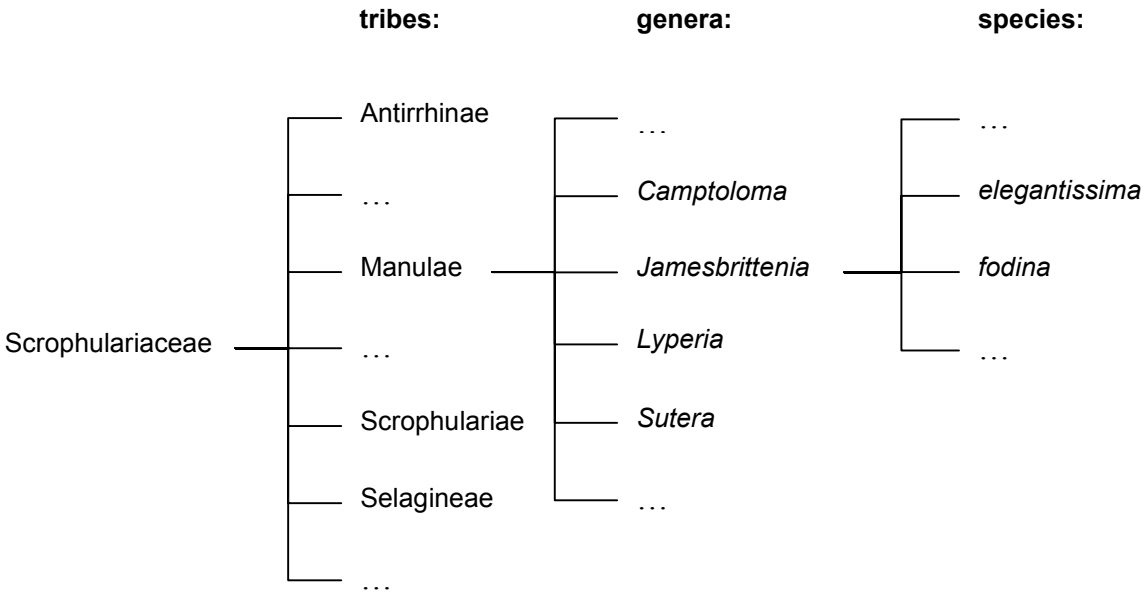


Figure 5: Classification within Scrophulariaceae according to Hilliard (1994)

The Manulae distinguish themselves from the allied tribe Selagineae (the erstwhile family Selaginaceae) by a multiovulate ovary, for Selagineae only have one ovule in each ovary loculus (Hilliard, 1994). Other distinguishing characteristics of Manulae are the arrangement of the petals in the bud, the synthecous anthers, and the four to many-seeded septicidal capsule.

Members of Manulae are almost confined to Africa south of the Sahara. One species of *Camptoloma* is endemic to Grand Canary, and one species of *Jamesbrittenia* extends from Egypt and Sudan to India (Hilliard, 1994).

2.4.1.3 *Jamesbrittenia fodina* and *J. elegantissima*

The name *Jamesbrittenia* was chosen by Kuntze in honour of James Britten, a keeper of Botany at the National History British Museum. In 1891, Kuntze judged two plants named *Sutera foetida* Roth and *Sutera glandulosa* Roth to be generically different, and provided a new name for the latter. At that time, the change concerned only *J. dissecta* O.Kuntze [=*Sutera glandulosa* Roth]. However it proves to be a large genus (83 species recognised), many species of which were originally described by Bentham under his generic name *Lyperia*.

In 1992, Hilliard published a simple key to distinguish the genera *Sutera*, *Lyperia*, *Jamesbrittenia* and *Camptoloma* (see Table 2). He renamed amongst many others *Sutera fodina* and *Lyperia elegantissima* into *Jamesbrittenia fodina* and *Jamesbrittenia elegantissima*.

Table 2: Simple key for the distinction of *Sutera*, *Lyperia*, *Jamesbrittenia* and *Camptoloma* (Hilliard, 1992)

1	Calyx with distinct tube, 1 or 2 pairs of stamen exerted, posticous filaments not decurrent down corolla tube, seeds amber colored, pallid or blue	*Sutera*
	Calyx lobed nearly or quite to base, stamens usually included, posticous filaments decurrent, seeds black or shades of brown	⇨**2**
2	Leaf base decurrent forming narrow wings or ridges, seeds black, patterned with longitudinal rows of transversely elongated pits	*Lyperia*
	Leaf bases not decurrent, seeds shades of brown, testa generally reticulate	⇨**3**
3	Corolla tube cylindric, abruptly expended near apex, stamens included, seeds with many longitudinal rows of cells forming a reticulum	*Jamesbrittenia*
	Corolla tube cylindric, scarcely expanded near apex, anticous stamens very shortly exserted, seeds with 6-8 longitudinal ribs, reticulate between the ribs	*Camptoloma*

***Jamesbrittenia fodina* Wild**

Jamesbrittenia fodina (Wild) O.M. Hilliard was formely called *Sutera fodina* Wild (1965).

This shrublet is 0.3 to 1 m tall and up to 2 m across. Its branches are glandular-pubescent, and its twigs very leafy.

The leaves are opposite, oblong to narrowly elliptic, with an obtuse to subacute apex and crenate margins often revolute; their texture is thick, and their surface glandular-pubescent, with hairs up to 0.3-2 mm long.

The sweetly scented flowers are solitary in upper leaf-axils forming short terminal racemes. The corolla tube is cylindric and is 14 to 20 mm long; its color is creamy-white with yellow or orange throat.

Both corolla and calyx are glandular-pubescent outside. Filaments of the stamens are bearded with clavate hairs. Pedicels are up to 1-3 mm long. A remarkable feature is the pair of bracts immediately below the calyx, the lobes of which they closely resemble.
The fruit is a 4 to 6 mm long capsule and is either slightly glandular-pubescent or clad in glistening glands. Seeds are 0.8-1 mm long and 0.4-0.5 mm wide (Hilliard, 1994).

Figure 6: Serpentine endemic *Jamesbrittenia fodina* O.M. Hilliard

***Jamesbrittenia elegantissima* Schinz**

Jamesbrittenia elegantissima (Schinz) O.M. Hilliard was formely called *Lyperia elegantissima* Schinz (1896).
It is a perennial herb with a woody taproot, 0.15 to 1 m in length, erect or prostrate. Its numerous branches are spindly, glandular-pubescent, and its twigs very leafy.
The leaves are opposite, soon alternate upwards, oblong, the apex is obtuse to subacute. The margins are very different to those of *J. fodina* as they are pinnatipartite to bipinnatisect; leaves' surface is glandular-pubescent, with hairs up to 0.5-1 mm long.
The flowers are solitary in upper leaf axils. The corolla tube is cylindric and abruptly dilatated near apex; it is much smaller than *J. fodina*, with 5.5 to 7.5 mm length. Its color varies from dull to bright yellow. Both corolla and calyx are glandular-pubescent outside. Filaments of the stamens are minutely puberulous. Pedicels are up to 8-18 mm long and as hairy as the stems. Unlike *J. fodina*, there are no bracts.

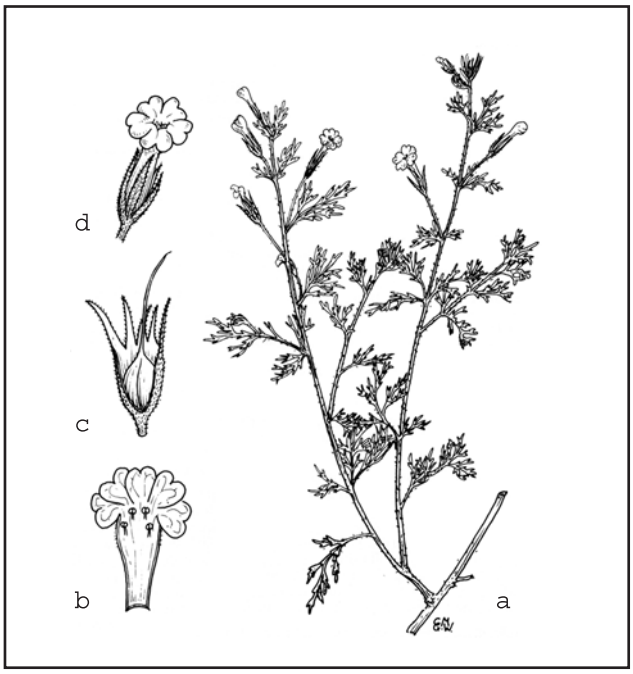


Figure 7: *Jamesbrittenia elegantissima*, (a) flowering branch, (b) corona opened showing stamen, (c) fruit, (d) flower

The fruit is a 4.5 to 6 mm long capsule, glabrous except for a few glistening glands on sutures. Seeds are 0.25-0.3 mm long (Hilliard, 1994).

2.4.2 Distribution

2.4.2.1 Jamesbrittenia fodina

Jamesbrittenia fodina is one of the 14 taxa endemic to the ultramafic and mafic rocks of the Great 'Dyke' of Zimbabwe.

This remarkable geological phenomenon, which bisects Zimbabwe along a north-south line of 530 km for an average of 8 km in width, carries some of the world's greatest deposits of chrome and asbestos, and significant ore deposits (see Figure 8).

The Great 'Dyke' is overall not a true dyke, but rather a line of 4 elongated mafic-ultramafic layered complexes arranged end to end. The successive horizontal layers were formed by intrusion of magma through a fissure and solidification, after a differentiation process at depth (Wilson, 1982). The layers, probably originally flat, subsided along the middle to form series of shallow elongated basins overlaying one another (Brooks, 1987).

The serpentine comes from the hydrothermic alteration of ultramafic rocks. Soils rich in serpentine are soft, but are hardened by circulating waters rich in silica and iron, especially on the hills. This hard stone is used by many sculptors throughout the country (Camerapix, 1990).

These soils are very rich in nickel and chromium. Wild reported extremely high levels of chromium in *Jamesbrittenia fodina*, with a content of 4800 ppm in ashed leaves (corresponding to about 2400 µg/g in dried leaves) (Wild, 1974; Brooks, 1987).

Jamesbrittenia fodina grows among rock outcrops in grassland or woodland; flowering has been recorded in most months.

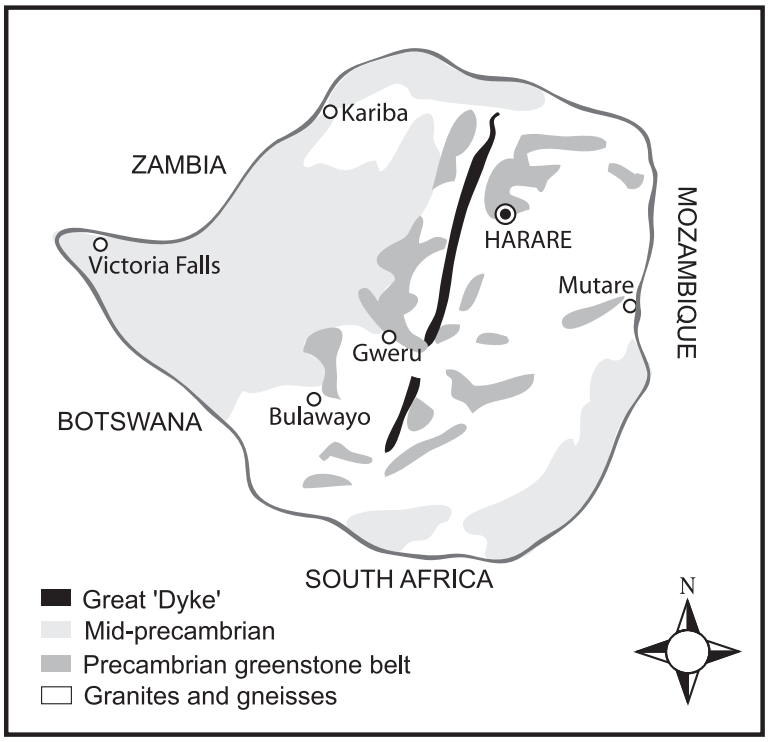


Figure 8: Geology map of Zimbabwe

Three other species that were part of the screening (2.2.2) also grow in the serpentine soils of the Great 'Dyke' area, but are not endemic to this area: *Faurea speciosa* Welw. (Proteaceae), *Combretum apiculatum* Sond. (Combretaceae), *Buphane disticha* Herb. (Amaryllidaceae) (Brooks, 1987).

2.4.2.2 Jamesbrittenia elegantissima

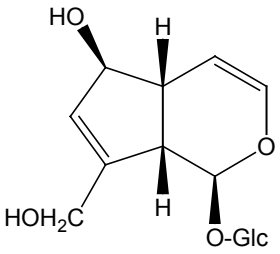
The species is confined to the Cunene –Cubango –Cuanavale –Cuando –Okavango – Zambezi drainage systems, which cover part of Zambia, Botswana, Angola, Namibia and Zimbabwe, east of Binga (on Lake Kariba). Many records are from riverbanks, the margin of islands in floodplains, islands in the Okavango swamps, around pans. Drier sites have also been noted, such as *Terminalia* woodland and dry grassland. Flowers can be seen in any month (Hilliard, 1994).

2.4.3 Previous phytochemical studies

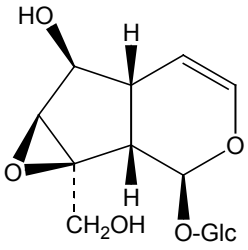
2.4.3.1 Main metabolites in Scrophulariaceae

Iridoid glycosides

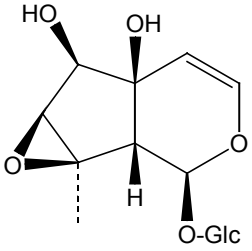
Iridoid glycosides are common amongst Scrophulariaceae, especially C_9-iridoids, like aucubin (**1**), found in many genera, and often accompanied by catalpol (**2**) or numerous derivatives and esters.



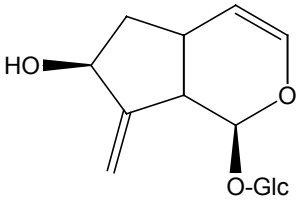
1 Aucubin



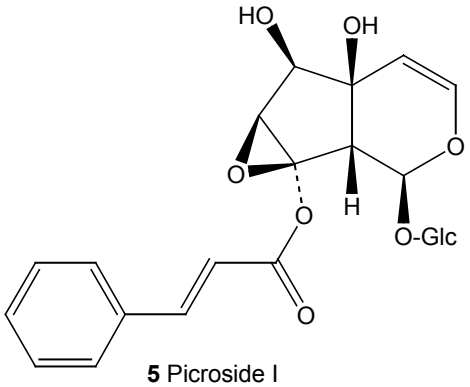
2 Catalpol



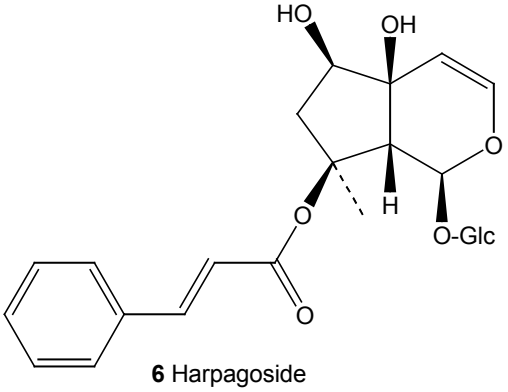
3 Antirrhinosid



4 Antirrhid

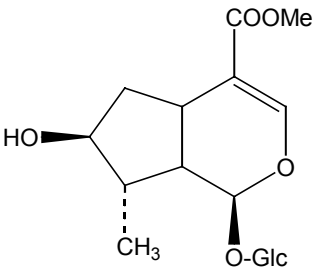


5 Picroside I

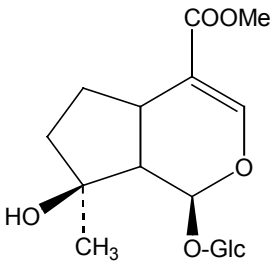


6 Harpagoside

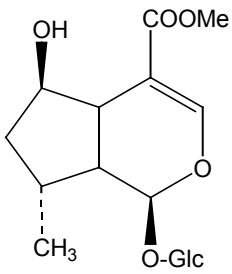
Moreover, certain taxa contain C_{10}-iridoids. 8-Epiloganin (**7**) and its isomer mussaenosid (**8**) are relatively frequent (Hegnauer, 1973; 1990).



7 8-Epiloganin



8 Mussaenosid



9 Penstemonoside

Many biological activities have been attributed to these iridoids:

Picroliv is a standardised extract of the underground parts of *Picrorhiza kurroa* Royle ex Benth., containing at least 60% of kuktoside and picroside I (**5**), 10-ester derivatives of catalpol, used in Ayurvedic medicine. This mixture has been the object of different studies, which demonstrated antihepatotoxic and choleretic effects, hypolipidemic action and antiviral activity (Ghisalberti, 1998).

Peracetates of aucubin (**1**), catalpol (**2**) and penstemonoside (**9**) isolated from *Parentucillia latifolia* L. inhibited the uterine muscular contractions induced by acetylcholine in a way similar to papaverine (De Urbina *et al*., 1994).

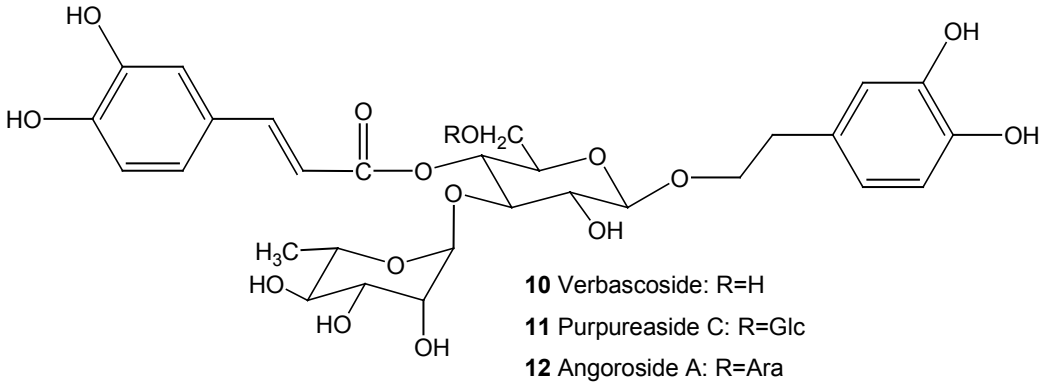
Aucubin (**1**) showed anti-inflammatory activity both *in vitro* and *in vivo* (Bermejo Benito *et al*., 1999; Recio *et al*., 1993). The anti-inflammatory effect of *Harpagophytum procumbens* DC. (Pedaliaceae) seems to be linked to its high content in harpagoside (**6**), but other compounds have to be involved (Loew *et al*., 2001; Recio *et al*., 1993).

Phenolic compounds

Aromatic acids are widely represented in the Scrophulariaceae family. They are often esterified with sugars, flavonoid moieties or iridoid glycosides (Hegnauer, 1990).

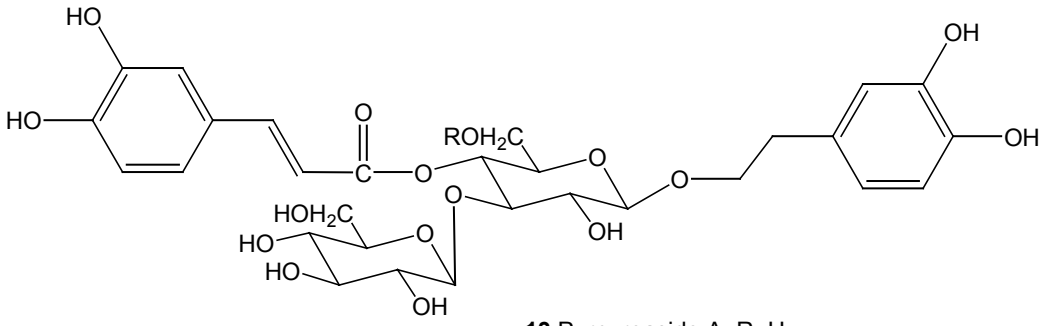
Verbascoside (**10**) was first isolated from *Verbascum sinuatum* L. (Hegnauer, 1973), but was further found in species of *Scrophularia*, *Verbascum* and *Digitalis*. Verbascoside demonstrated an inhibitory effect on protein kinase C (PKC) that could explain in part the antitumor activity measured *in vitro* (Herbert *et al.*, 1991). An antiproliferative effect on aortic smooth muscle could result in a preventive effect on arteriosclerosis (He *et al.*, 2001).

More generally, verbascoside-type phenolic ester glycosides showed various biological activities, from antibiotic and antifeedant to immunodepressive (Hegnauer, 1990; Shoyama *et al*., 1986).



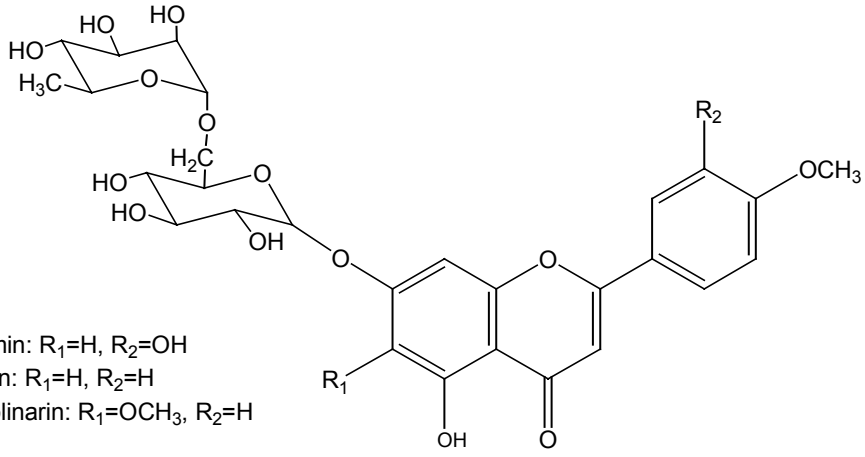
10 Verbascoside: R=H
11 Purpureaside C: R=Glc
12 Angoroside A: R=Ara

Purpureasides A, B and C (**13**, **14**, **11**) have been isolated from *Digitalis purpurea* L. (Matsumoto *et al*., 1987), and angoroside A (**12**) from the roots of *Scrophularia scopolii* Hoppe ex Pers. and *Verbascum spinosum* Linn. (Calis *et al*., 1987).



13 Purpureaside A: R=H
14 Purpureaside B: R=Rha

Flavones such as luteolin and apigenin glycosides are frequently found in Scrophulariaceae leaves. Their 6-OH, 6-OMe and 8-OH derivatives are frequently encountered, often as 7-glycosides or 7-glucuronides (Hegnauer, 1990).
Diosmin (**15**) was isolated from the leaves of *Verbascum floccosum* Kuntze, *V. lychnitis* Linn. and *V. thapsiforme* Guss. Linarin (**16**), pectolinarin (**17**), and acetylated derivatives were isolated from *Linaria japonica* Miq. (Otsuka, 1992).
8-Hydroxyflavones are common in some *Veronica* species and in *Gratiola officinalis* L. Flavonols are rather rarely encountered.

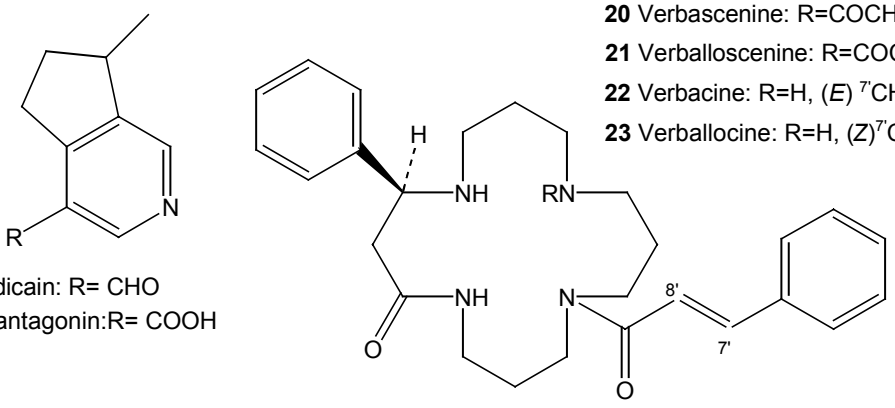


15 Diosmin: R_1=H, R_2=OH
16 Linarin: R_1=H, R_2=H
17 Pectolinarin: R_1=OCH_3, R_2=H

Alkaloids

Originally, the Scrophulariaceae were considered as free of alkaloids. It was later demonstrated that several genera contained alkaloids. Indicain (**18**) and plantagonin (**19**) were isolated from *Pedicularis olgae* Regel (Hegnauer, 1973).

Macrocyclic lactam alkaloids were isolated in Scrophulariaceae, like verbascenine (**20**) from the aerial parts of *Verbascum phoeniceum* L. and *V.nigrum* L. (Seifert *et al*., 1982), verballoscenine (**21**) from the leaves of *Verbascum phoeniceum* L., verbacine (**22**) and verballocine (**23**) from the leaves of *Verbascum pseudonobile* Stoj. et Stef (Dandarov, 1997).



18 Indicain: R= CHO
19 Plantagonin:R= COOH

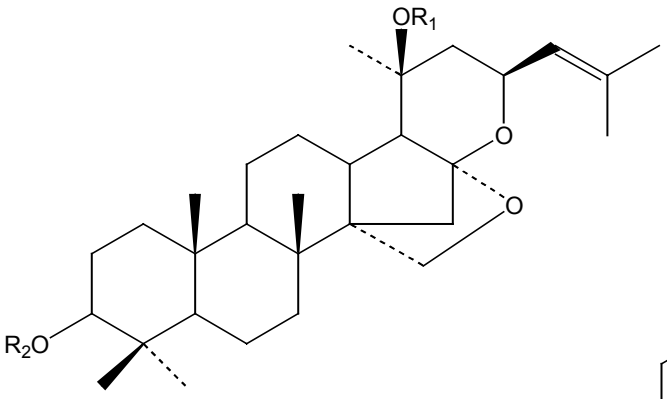
20 Verbascenine: R=$COCH_3$, (*E*) $^{7'}CH=^{8'}CH$
21 Verballoscenine: R=$COCH_3$, (Z) $^{7'}CH=^{8'}CH$
22 Verbacine: R=H, (*E*) $^{7'}CH=^{8'}CH$
23 Verballocine: R=H, (*Z*)$^{7'}CH=^{8'}CH$

A transfer of alkaloids was observed in the case of root parasitism, between the host plant and the parasite. Pyrrolizidine, quinolizidine and norditerpene alkaloids, encountered in species of *Castilleja* (Scrophulariaceae), were proved to come from their host plants (*Senecio*, *Lupinus* respectively *Delphinum* species) (Marko and Stermitz, 1997).

Saponins

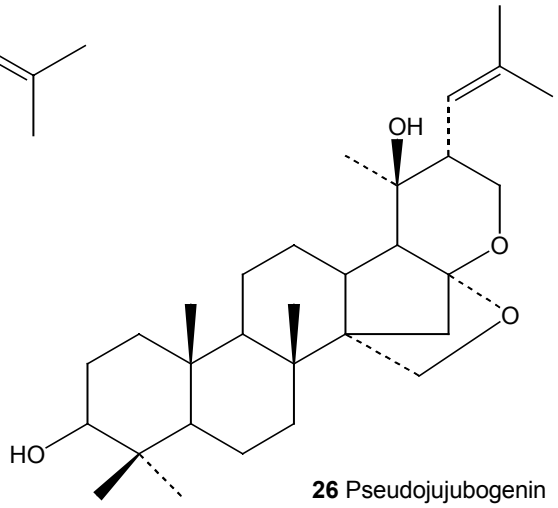
As early as 1936, Hohman analysed 307 genera belonging to the Scrophulariaceae. He determined that the presence of saponins was often limited to one or two organs of a species, while the rest of the plant was saponin-free (Hegnauer, 1973).

A series of dammarane-type glycosides was isolated from *Bacopa monniera* Wettst., named bacopasaponins A-F, all derivatives of the aglycones jujubogenin (**24**) or pseudojujubogenine (**26**) (Garai *et al*., 1996a; 1996b).



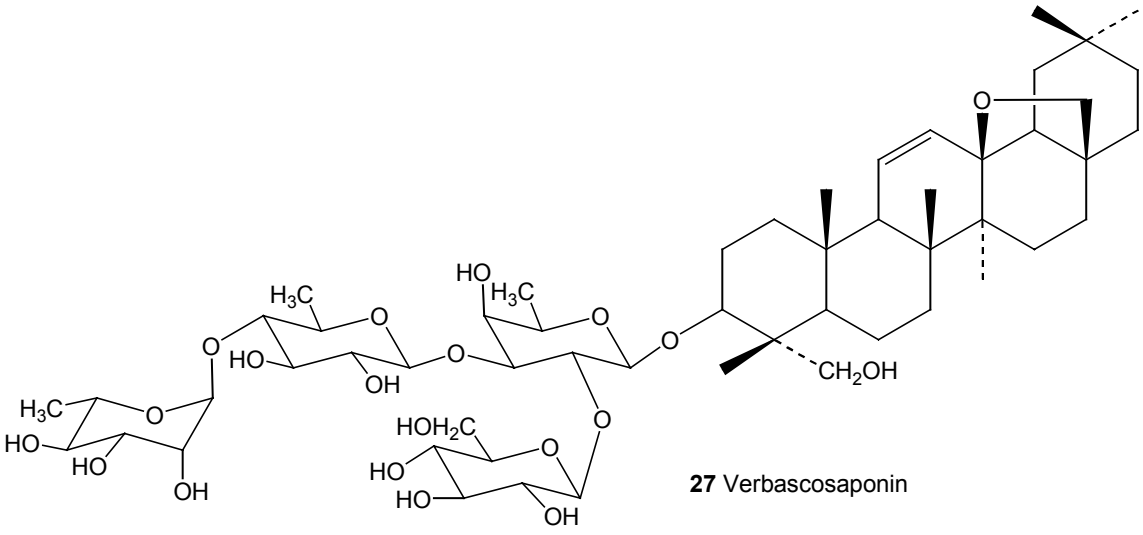
24 Jujubogenin: $R_1=R_2=H$

25 Bacopasaponine A: $R_1=R_2=\alpha$–L-arabinose



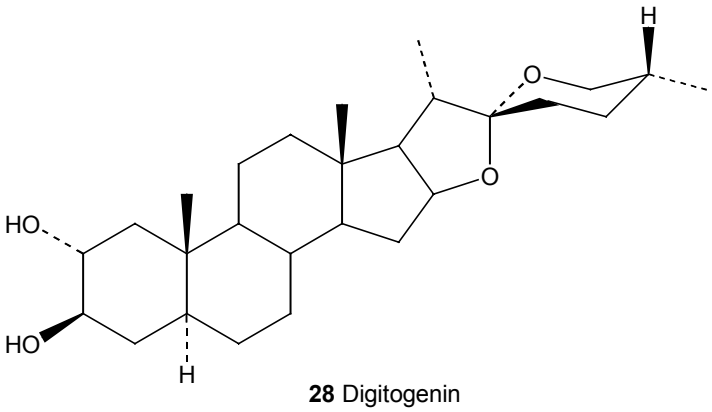
26 Pseudojujubogenin

Tschetsche *et al*. isolated in 1980 from *Verbascum phlomoides* L. the oleanane saponin verbascosaponin (**27**), the structure of which was revised by Schröder and Haslinger (1992). This saponin demonstrated anti-inflammatory activities *in vivo* (Giner *et al*., 2000). Scrokoelziside A, isolated from *Scrophularia koelzii* Pennell, is a quite similar saponin, with a different sugar sequence (Bhandari *et al*., 1996).



27 Verbascosaponin

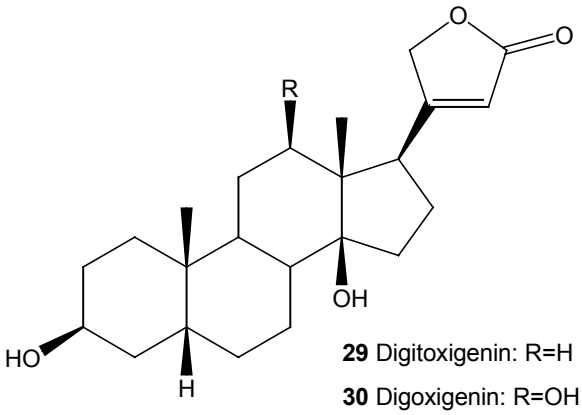
Glycosides of the steroidic spirostanol aglycone digitogenin (**28**) were isolated from species of the genus *Digitalis* (Muhr *et al*., 1996).



28 Digitogenin

Cardiotonic glycosides

Heterosides of the steroid genins digitoxigenin (**29**), digoxigenin (**30**) and other derivatives are isolated from many *Digitalis* species. *D. purpurea* L. and *D. lanata* Ehrh. are well known for the industrial extraction of digitoxin and digoxin. 2, 6-didesoxyhexoses and 6-desoxyhexoses are specific to this class of compounds (Bruneton, 1999).



29 Digitoxigenin: R=H

30 Digoxigenin: R=OH

Other compounds

An antibiotic and antifungic diterpene quinone has been isolated from *Capraria biflora* L. (Hegnauer, 1973).

Cyanogenetic compounds were discovered in the leaves of *Linaria bipartita* (Vent.) Willd., *Mecardonia dianthera* Pennell and *Veronica persica* Hort. ex Poir. (Hegnauer, 1990).

2.4.3.2 Previous phytochemical studies on the genus *Jamesbrittenia*

Phytochemical studies concerning the genus *Jamesbrittenia* are almost inexistent. There is only one publication reporting the isolation of aucubin (**1**) from *Jamesbrittenia dissecta* Kuntze (formerly *Sutera dissecta* Walp.) (Forgacs *et al*., 1986).

2.4.4 Use in traditional medicine

- *Jamesbrittenia albobadia* Hilliard (formerly *Sutera burkeana* Hiern) is used in Zimbabwe for various troubles. The powdered root is prescribed for abdominal pains and an infusion of the leaves is administered as an enema in cases of constipation. An infusion of the leaves is dropped into the eyes for cataract and sore eyes or is used to wash the baby in cases of inflammation of the navel cord. Inhalation of the burnt plant has been performed in cases of madness (Gelfand *et al*., 1985).

- The stigma of *Jamesbrittenia atropurpurea* (Benth.) Hilliard (formerly *Sutera atropurpurea* (Benth.) Hiern) is one of the commercial sources of saffron, though it is more commonly derived from *Crocus sativus* L. (Iridaceae). In South Africa, the whole flower appears to be used; it contains the carotenoid crocetin, its ester crocin and picrocrocin, a bitter derivative of cyclocitral. It was formerly used in the treatment of convulsions in children, as an antispasmodic, as a stimulant and in case of exanthema. Today it is used as flavouring and colouring agent (Watt and Breyer-Brandwijk, 1962).

- *Jamesbrittenia filicaulis* Hilliard (formerly *Sutera filicaulis* (Benth.) Hiern and *Sutera pinnatifida* Hiern) is an African remedy for chest trouble; the juice of the leaves is used for treating sore eyes in the sheep (Watt and Breyer-Brandwijk, 1962).

- Pounded leaf infusions of *Jamesbrittenia kraussiana* (Bernh.) Hilliard (formerly *Sutera Kraussiana* (Bernh.) Hiern) are used by Zulu to relieve menstrual pains (Hutchings, 1996).

- A hot infusion of the pounded leaves of *Sutera floribunda* (Benth.) O.Kuntze is a Zulu remedy for menstrual pains (Hutchings, 1996). A root decoction of the same plant is used in Lesotho for children with chest colds. *Sutera hispida* (Thunb.) Bruce is recommended more generally for chest troubles (Watt and Breyer-Brandwijk, 1962).

- The Zulu administer an infusion of the leaf and stem of a *Sutera* sp. per os or as an enema for feverishness (Watt and Breyer-Brandwijk, 1962).

3 Results

3.1 *Study of* Dioscorea sylvatica *and* Urginea altissima

The frequent use of two plants sold in Zimbabwe markets, *Dioscorea sylvatica* Ecklon (Dioscoreaceae) and *Urginea altissima* Baker (Liliaceae) was reported for the treatment of rheumatism or skin disease. However, application of these plants on the skin leads to irritation and inflammation.

As described in paragraph 2.2.3, contact dermatitis is a complex phenomenon that can involve many factors.

It is known, for exemple, that a member of the Dioscoreaceae, *Tamus communis*, causes contact irritation; the rhizome was shown to contain calcium oxalate raphides and histamine, responsible, at least in part, for the inflammatory response to a contact of the rhizome with the skin (Schmidt and Moult, 1983).

This prompted an investigation of *Dioscorea sylvatica* and *Urginea altissima* in order to determine the possible presence of oxalate crystals and histamine in the underground organs.

3.1.1 Microscopic examination

The first step of our investigation was a microscopic investigation of *Dioscorea sylvatica* and *Urginea altissima* in order to search for the presence of oxalate crystals.

Dry powdered rhizome and bulbs of both plants were observed under a light microscope (magnification 100x).

Microscopic observation revealed in both plants long needles of calcium oxalate, grouped in bundles called raphides (Trease and Evans, 1978).

The needles were about 300-400 μm long in *Dioscorea sylvatica* (Figure 9) and about 200-300 μm long in *Albuca melleri* (Figure 10).

The preparations were also observed after addition of methylene blue, which reveals mucilage. In *D. sylvatica*, the raphides appeared to be gathered in mucilaginous cells, like in *Tamus communis*. In *Urginea altissima*, on the contrary, the cells containing the raphides were not coloured in blue after the addition of methylene blue.

Figure 9: Calcium oxalate raphide in a mucilaginous cell of *Dioscorea sylvatica*

The chemical composition of the raphides could be confirmed by the observation of a group of crystals after addition of 20 % sulphuric acid. The crystals partially dissolved, and after a few minutes, bunches of smaller needles began to form, attached to the remainder of the longer crystals. The small crystals are the result of the crystallisation of poorly soluble calcium sulphate (Schmidt and Moult, 1983).

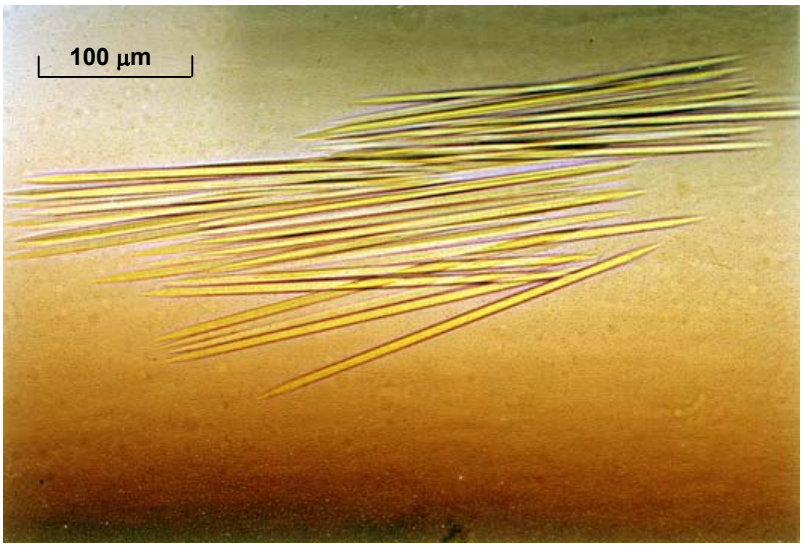


Figure 10: Bundle of sharp needles of calcium oxalate of *Urginea altissima*

The preparations were also observed after addition of Sudan Red. This dye has been used by Carneiro *et al.* (1989) to detect the toxic lipid substance impregnating the raphides in *Dieffenbachia picta* Schott. The calcium oxalate needles did not turn red, indicating the absence of lipid substances associated with the crystals.

The presence of the sharp needles in both plants gives a first explanation of the dermatitis observed after contact with the skin.

3.1.2 Search for histamine

3.1.2.1 TLC analysis

As histamine has been associated with with the inflammatory response of the skin to a few agents causing contact dermatitis, efforts were made to detect this mediator.
Histamine could be identified in the methanol extract of the rhizome of *Tamus communis* by thin layer chromatography (Schmidt and Moult, 1983). This simple method was chosen as a first approach for the search for histamine in the methanol and water extracts of both plants.

The extracts were eluted with histamine standard on TLC plates (acetone/H_2O/NH_3 (90 :7 :3) as mobile phase). 600 µg of the extracts and 2 µg of the standard were spotted on the plates. The plates were sprayed with a solution of 30 mg of ninhydrin in 10 ml of butanol and 0.3 ml of acetic acid 98% and heated at 100°C (Wagner *et al*., 1996). This reagent allows the specific detection of amino acids.

The TLC of the extract of *Dioscorea sylvatica* is presented in Figure 11. The same operation was performed with *Urginea altissima* and with the water extracts.

Histamine was not detected in either of the extracts by TLC. The histamine reference standard appeared as a purple spot (R_f 0.37) with ninhydrin reagent.

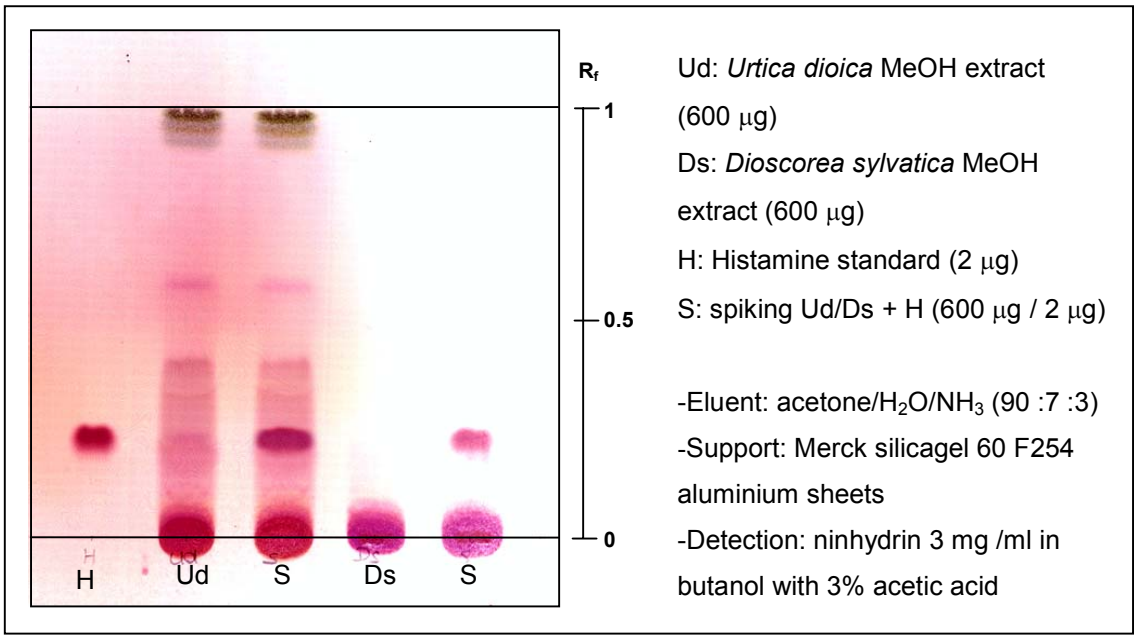


Figure 11: Comparative TLC of the methanol extract of *Dioscorea sylvatica* with histamine standard

In order to verify the method, the same conditions were used to detect histamine in the nettle plant *Urtica dioica* L. (Urticaceae), known to contain histamine (Oliver *et al*., 1991). A fresh sample of the aerial parts of *Urtica dioica* was extracted with methanol and water, as described for *Dioscorea sylvatica* and *Urginea altissima.* Traces of histamine were detected in both extracts (Figure 11).

Schmidt and Moult could detect and isolate histamine from the methanol extract of the rhizome of *Tamus communis*, or black bryony (Dioscoreaceae), by analytical and preparative TLC (1983). There was no information as to the quantities of extract and standard applied on the plate.

The limit of detection of histamine was measured by applying geometrical dilutions on TLC. 0.025 µg of histamine were detected with ninhydrin reagent.

However, smaller quantities of the amine could remain undetected by this method. Therefore, a HPLC analysis was performed.

3.1.2.2 LC/UV analysis

The elaboration of a rapid LC/UV method for the detection of histamine was attempted. The chromatographic method described by Beljaars *et al*. (1998) for the quantification of histamine in food samples was followed.

The samples were eluted on a Spherisorb® ODS-2 reversed-phase C-18 column (250x4.6 mm, 5 μm particle size, Macherey-Nagel) with an isocratic mixture of 12.5 % acetonitrile and 87.5 % phosphate buffer pH 3.0. The phosphate buffer was prepared with 2.9 g of sodium-1-heptane sulfonate and 1.9 g of potassium dihydrogen phosphate in 900 ml water. The pH was adjusted to 3.0 with phosphoric acid, using a pH-meter. The solution was then diluted to 1000 ml with water.

The histamine reference standard was prepared with a solution of 5 mg of histamine base in 0.25 ml perchloric acid made up to 10.0 ml with water. Geometrical dilutions were then injected onto the column (Beljaars *et al.*, 1998).

The plant extracts (30 mg) were prepared with 100 μl of perchloric acid and made up to 1 ml with water/MeOH. The filtered solutions were injected onto the column (20 μl). Histamine was detected at 210 nm, which corresponds to its maximum UV absorption.

No signal corresponding to histamine was observed by LC/UV analysis. The detection level of histamine, measured at 0.05 μg, was not lower than with the TLC method.

In order to validate the method, the same conditions were used to detect histamine in the nettle plant *Urtica dioica* L.. No peak corresponding to histamine was detected; small peak with the same retention time as histamine displayed different UV spectra.

3.1.3 Discussion

In order to cause irritation, pieces of freshly cut tubers of *Dioscorea sylvatica* and bulbs of *Urginea altissima* have to be rubbed on the skin. Simple application of the methanol extracts is not sufficient to cause a cutaneous reaction.

The presence of sharp needles, observed in the underground organs of both plants by light microscopy, might be the only factor responsible for a mechanical irritation sufficient to cause mild inflammation and itching. Glass wool fibres with diameters greater than 5.3 μm cause a reaction of this type (Snyder *et al*., 1978).

The mechanical irritation may also aid the penetration of irritant substances and/or allergens through the cutaneous barrier. However, histamine could not be detected in tubers of *Dioscorea sylvatica* and bulbs of *Urginea altissima* by TLC or LC/UV analyses. The latter method could not be validated as histamine was not detected from the aerial parts of *Urtica dioica*. Oliver *et al*. determined in 1991 the mean histamine content of a nettle hair to be 6.1 ng.
The other available data concerned the content of histamine in the juice obtained from *Urtica urens* measured by fluorometric methods and determined to be 1.6 μg/ml of juice. An extrapolation to the content in the methanol extract of the aerial parts of the plant is difficult.
Whatever the content in the methanol extract may be, the UV detection at 210 nm appeared to be not specific enough, as it reveals almost all compounds extracted. In contrast, the ninhydrin detection on TLC was shown to be more specific as it revealed selectively the amino acid derivatives. HPLC separation followed by derivatisation and detection with fluorometric methods, as described by Beljaars *et al*. (1998), allows a higher specificity of the detection. The elaboration of a more rapid detection method by LC/UV/MS could be considered for a continuation of this work.

In addition to histamine, the different types of irritant compounds found in plants and involved in skin reactions are numerous: alkaloids, tulipaline, sesquiterpene lactones, quinones, isothiocyanates, diterpenes, long chain phenols, aldehydes, ketones, etc. (Benerza and Ducombs, 1987).

A first step for a solution to this problem would be to perform *in-vivo* and *in-vitro* experiences in order to determine the type of reaction involved with *Dioscorea sylvatica* and *Urginea altissima*: a pure mechanic irritation, a non allergic contact urticaria or an allergic reaction. The only data available on the skin reaction are oral reports from Zimbabwe. These reports are too approximate to provide the necessary information.

Oliver *et al*. (1991) described two methods for the evaluation of the reaction to *Urtica dioica*:

- a clinical study on volunteers with symptoms reports and biopsies at the site of exposure to determine the histological features
- *in-vitro* experiments on dispersed-rat-skin mast cells to determine the release of endogenous histamine.

It has to be taken into consideration that different clinical forms may arise from an irritant contact dermatitis (ICD), with changes like skin barrier disruption, epidermal cellular change and cytokine release. Recent hypotheses suggest that ICD might be linked to allergic contact dermatitis (ACD) by lowering the threshold of sensibility to an allergen (Smith *et al*., 2002). Contact dermatitis can thus be a very complex phenomenon, and many factors have to be estimated.

Independently of the presence or absence of irritant or allergenic compounds in the two plants under investigation, a conclusion can be drawn from our work concerning the validation of the traditional use in terms of possible toxic effects:
Considering the presence of sharp calcium oxalate needles, the frequent application on the skin of the two plants causes repeated microtraumatisms, and granulomatous lesions might develop (Ducombs and Schmidt, 1995). Therefore a long-term use in traditional medicine is not judicious.

3.2 Phytochemical investigation of the methanol extract of *Jamesbrittenia fodina*

In a second part of this work, *Jamesbrittenia fodina* Wild (Scrophulariaceae) was selected for phytochemical investigation. The important activity of the methanol extract detected in the biological and chemical screening performed previously (2.3.2) and the lack of studies concerning the genus prompted this choice.

3.2.1 Activity-guided isolation of compounds A and B

3.2.1.1 Activity of the extract

The aerial parts methanol extract of *Jamesbrittenia fodina* (Scrophulariaceae) displayed several activities: amongst these, the antifungal activities against *Candida albicans* and *Cladosporium cucumerinum* and the antibacterial activity against *Bacillus subtilis* all appeared as white spots ain TLC bioautography. The R_f of these spots were close, around 0.3 – 0.4, as shown in Figure 12. Considering that two types of supports were used (aluminium and glass), and that the migration of a compound is very dependant on the conditions in the chromatographic tank, it was assumed that these similar activities might be due to the same compound(s).

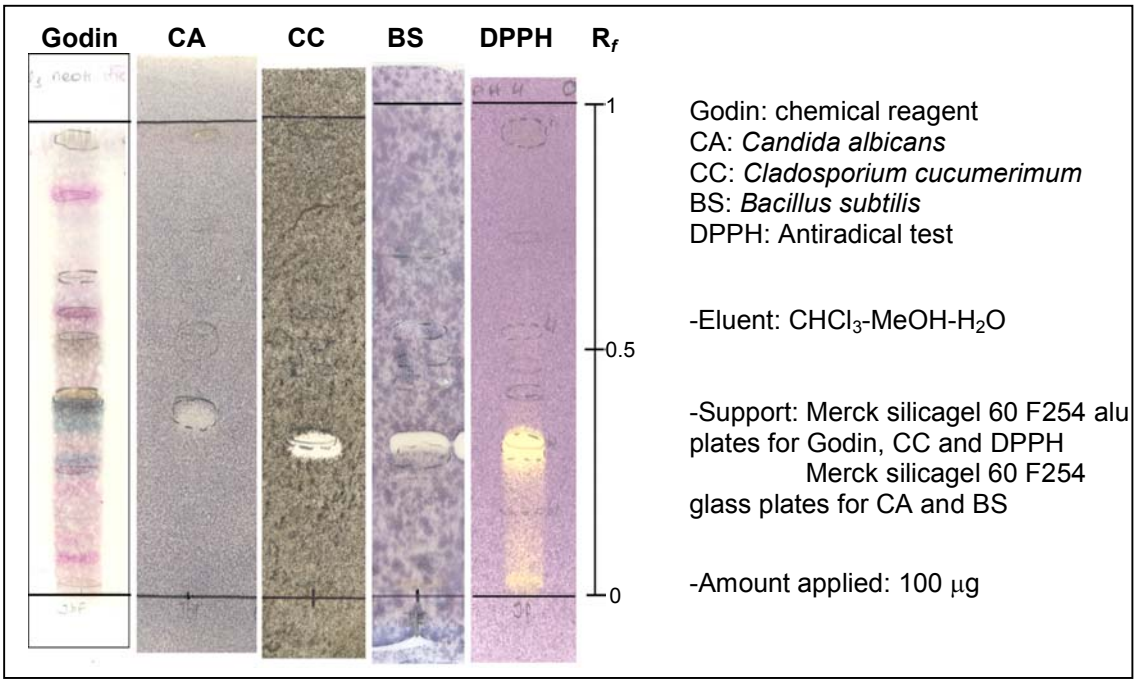


Figure 12: Detection of the main activities of *Jamesbrittenia fodina* methanol extract

The spot indicating the antiradicalar activity appeared to have a similar R_f , but that might be a coincidence, as this activity involves different mechanisms.

An activity-guided isolation of the active compound was undertaken, using the *Cladosporium cucumerinum* test as a guide, as this test is the most rapid and easy to perform.

3.2.1.2 Fractionation and isolation of compounds A and B

A first fractionation was carried out by means of open column silicagel chromatography, with an isocratic eluent composed of ethyl acetate-methanol-water starting from 50:5:1 and reaching 50:20:5 in 3 steps.

Out of the 18 fractions obtained, fractions 12 to 15 displayed antifungal activity. Fraction 15 was further fractionated to yield fractions 15-4 and 15-6. Interestingly, despite the very good MPLC conditions, the separation did not give completely pure compounds **A** and **B**. In fraction 15-4, a majority of **B** was accompanied by about 15% of **A** and vice versa in fraction 15-6, suggesting a transformation of **A** into **B** and **B** into **A**.

In order to prevent this transformation, other separation conditions were tested. The purification of the compounds was achieved with a Lobar LPLC using acetonitrile as the organic solvent instead of methanol (Figure 13).

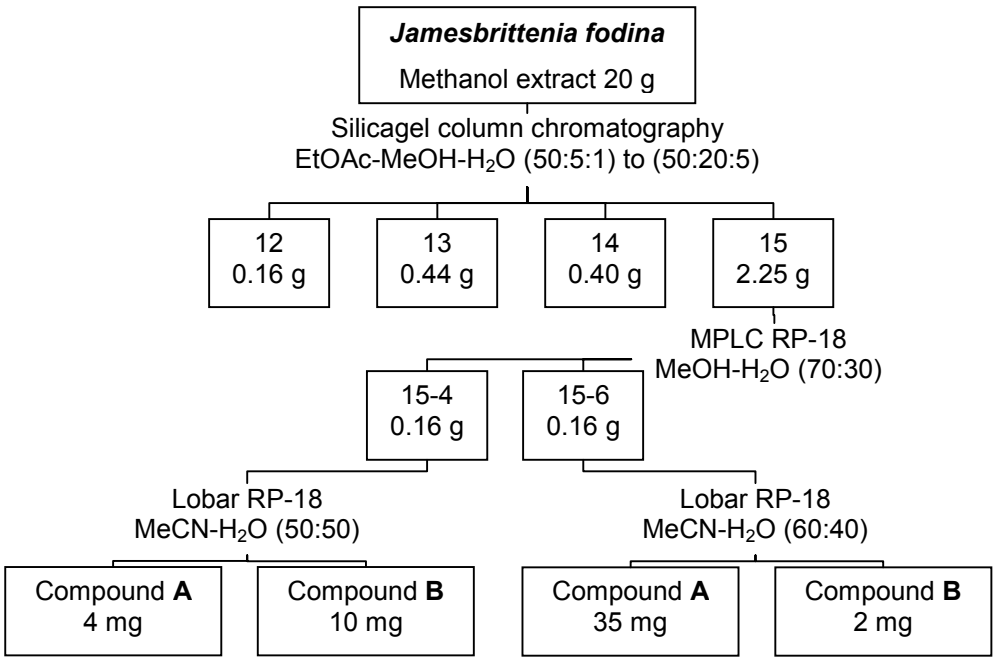


Figure 13: Isolation scheme of compounds **A** and **B**

Once the pure compounds **A** and **B** were obtained, stability experiments were performed, by keeping the products in different solvents and at different temperatures. Methanol was shown to be responsible at least in part, for the instability of **A**: a clear transformation of **A** into **B** was observed when it was incubated at 37°C in methanol containing 0.05 % TFA (Figure 14). However, the transformation of **B** into **A** could not be induced intentionally.

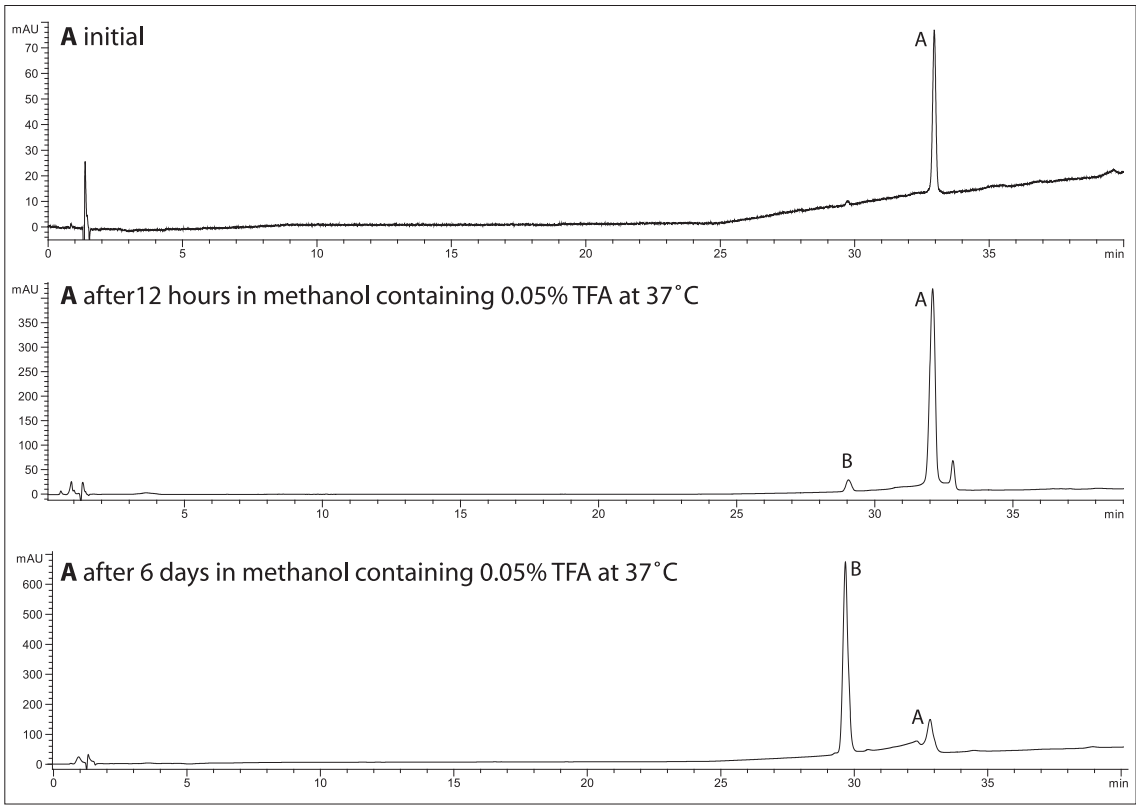


Figure 14: LC/UV analysis showing the progressive transformation of compound **A** into **B** when heated at 37°C in methanol containing TFA.

No conclusion concerning the origin of the transformation could be drawn at this stage. The structure elucidation of both compounds **A** and **B** was undertaken.

3.2.1.3 Structure determination of compounds A and B

Compounds **A** and **B** could finally be obtained as pure compounds. They appeared as white amorphous powders; TLC coloration with Godin was green.

Their structure elucidation was performed by means of mass spectrometry and nuclear magnetic resonance spectroscopy.

3.2.1.3.1 MS analyses

ESI-MS analysis in the positive ion mode indicated a high molecular weight, with a pseudomolecular ion at *m/z* 1073.1 $[M+H]^+$ for **A** and 1105.0 $[M+H]^+$ for **B**.

In the case of compound **A**, fragments indicated the departure of four sugar units corresponding to two hexoses and two deoxyhexoses (ions at *m/z* 927.1 $[M+H-146]^+$, *m/z* 911.1 $[M+H-162]^+$, *m/z* 765.3 $[M+H-308]^+$, *m/z* 603.3 $[M+H-470]^+$ and *m/z* 457.0 $[M+H-616]^+$) (Figure 15).

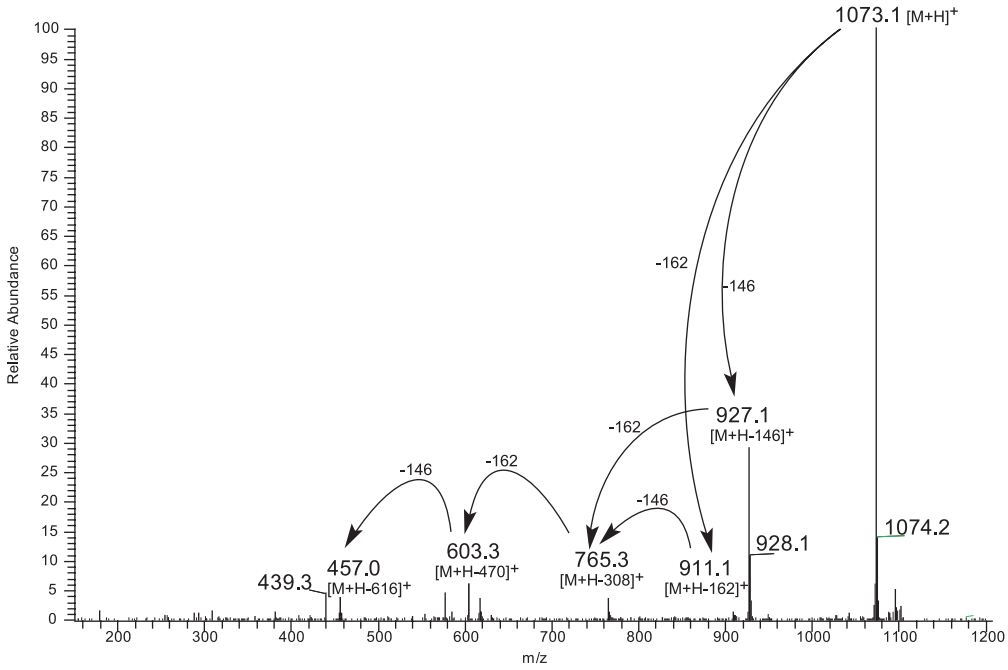


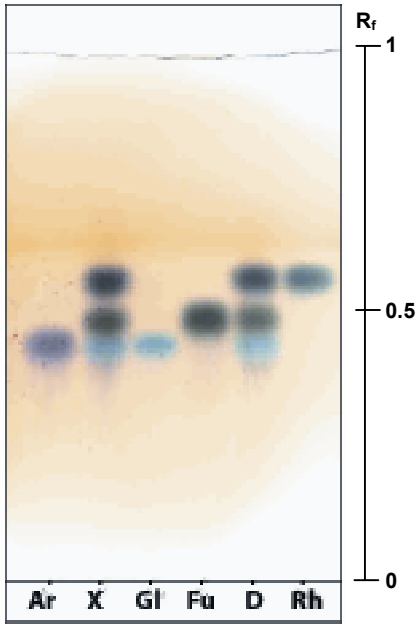
Figure 15: LC/ESI-MS spectrum of compound **A** in the positive ion mode

In the case of compound **B**, fragmentation was not as clear, and the departure of only two sugar units could be observed (ions at *m/z* 957.0 $[M+H-146]^+$, *m/z* 941.1 $[M+H-162]^+$ and *m/z* 795.1 $[M+H-308]^+$).

A high molecular weight and the presence of sugar moieties, foam formation in solution and a lack of absorption in the UV spectrum suggested the presence of saponins.

3.2.1.3.2 Identification of the sugar units

The identity of the sugar moieties was determined by acidic hydrolysis of **A** and **B** with HCl 2N, followed by a liquid-liquid extraction to separate the sugars from the aglycone. In both cases, the aqueous phase yielded glucose, fucose and rhamnose, determined by comparison with standards on TLC, and detected with naphthoresorcin reagent (illustrated for **A** in Figure 16).



X: sugar mixture from hydrolysis of **A**
Ar: arabinose
Gl: D-glucose
Fu: D-fucose
Rh: L-rhamnose
D: X + Ar + Gl + Fu + Rh

-Eluent: ^{i}PrOH-H_2O (85:15)

-Support: Merck silicagel 60 F254 aluminium sheets

-Amount applied: 10 μg of standards/ 40 μg of X

-Detection: naphthoresorcin

Figure 16: Comparison of the sugars resulting from the hydrolysis of compound **A** with standards.

3.2.1.3.3 Structure elucidation of compound A

The composition of the sugar chain was confirmed with the help of 1D and 2D NMR analysis.

Four anomeric carbons, detected at δ 104.94, 104.03, 103.95 and 102.78, corresponding to four anomeric protons at δ 5.28, 4.90, 5.59 and 5.85, were found to belong to α-rhamnose, β-fucose and two β-glucose (Figure 17, Figure 18). Anomeric configurations were determined by observing the coupling constants of the anomeric protons and the chemical shifts of the sugar carbons, which differ for α and β configurations (Agrawal *et al*., 1985; Hostettmann and Marston, 1995).

The ^{13}C-NMR spectrum of **A**, recorded in pyridine-*d5*, exhibited 30 remaining carbons belonging to the aglycone part.

With the help of the gHSQC spectrum, these carbon signals were shown to be constituted of a double bond (δ 131.96 and 131.67), one oxygenated quaternary carbon (δ 84.82), six quaternary carbons (δ 31.69, 36.26, 41.65, 41.96, 43.79 and 44.07), one oxygenated tertiary carbon (δ 82.50), three tertiary carbons (δ 36.26, 47.80 and 51.40), two hydroxyl methyl groups (δ 64.59 and 76.98), nine methylene moieties (δ 17.50, 25.50, 25.81, 25.99, 30.90, 31.47, 33.59, 37.28, 38.40), and six methyl groups (δ 12.68, 18.63, 19.53, 19.81, 23.54 and 35.00) (Figure 17).

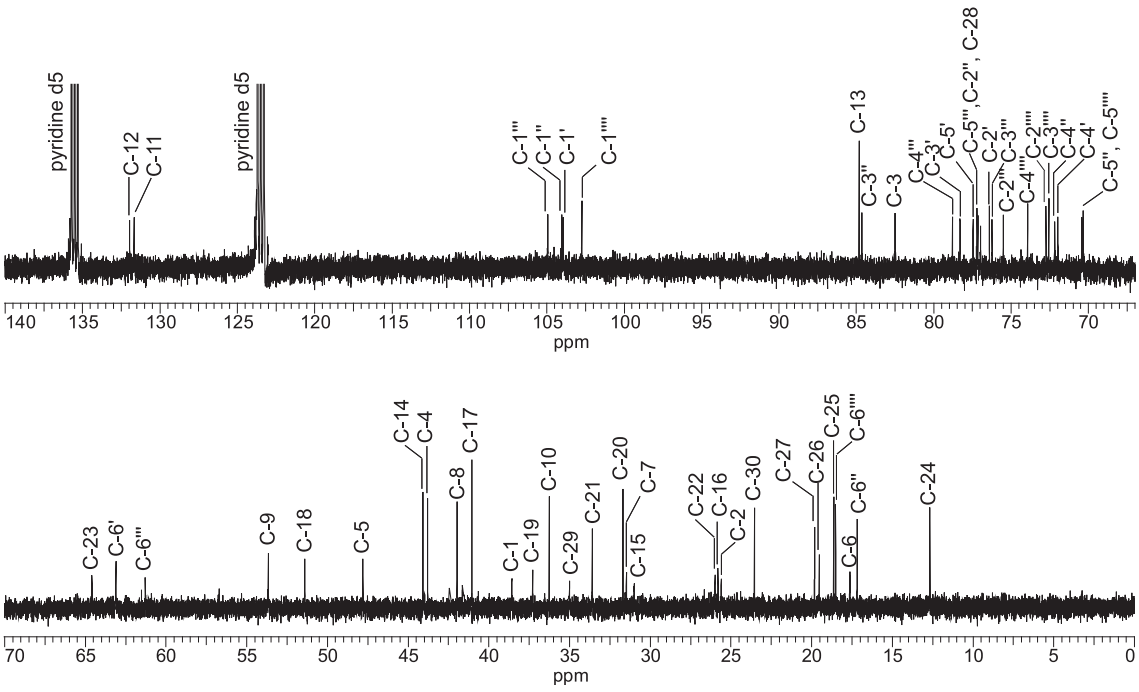


Figure 17: ^{13}C-NMR of **A** (125 MHz, in pyridine-*d5*). C-1' to C-6': terminal Glc signals, C-1'' to C-6'': Fuc signals, C-1''' to C-6''': internal Glc signals, C-1'''' to C-6'''': Rha signals.

This corresponds to a β-amyrin type triterpene, with a double bond between C-11 and C-12 and an ether bridge between C-13 and C-28 (Hostettmann and Marston, 1995; Schröder and Haslinger, 1993).

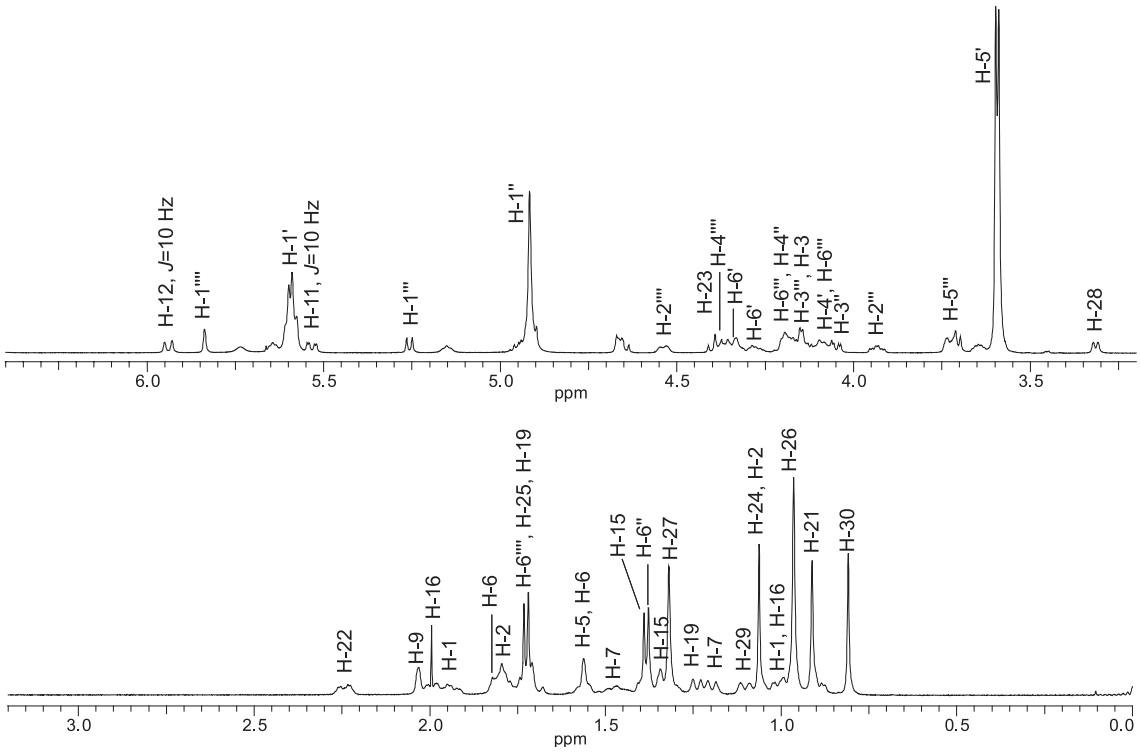


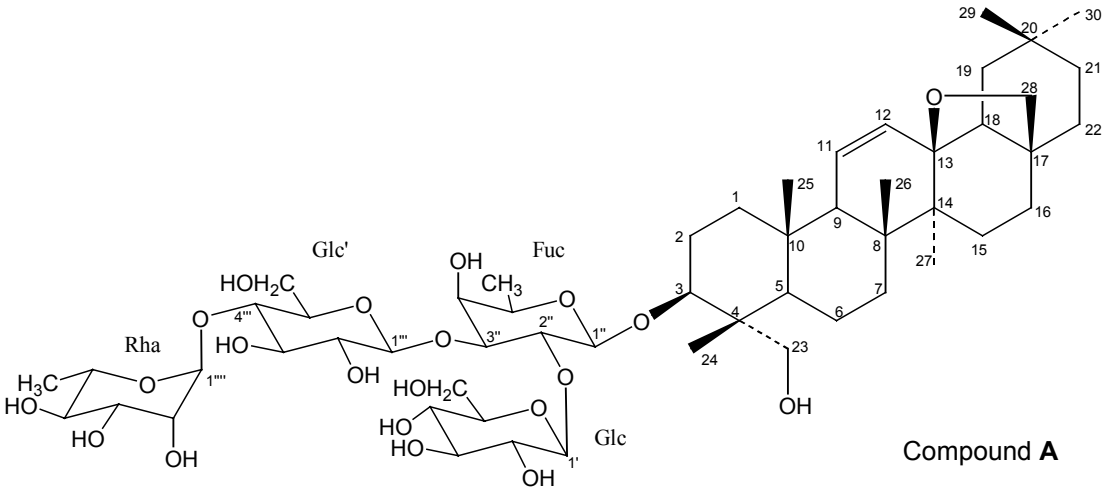
Figure 18: ^{1}H-NMR of **A** (500 MHz, in pyridine-*d5*, TMS as internal standard). H-1' to H-6': terminal Glc signals, H-1'' to H-6'': Fuc signals, H-1''' to H-6''': internal Glc signals, H-1'''' to H-6'''': Rha signals.

Interglycosidic linkages were determined from gHMBC connectivities.

Compound **A** was finally identified as 13, 28-epoxy-3β-*O*-[α-rhamnopyranosyl-(1→4)-β-glucopyranosyl-(1→3)-[β-glucopyranosyl-(1→2)]-β-fucopyranosyl]-11-oleanen-23-ol, also called mimengoside A, ilwensisaponin A or verbascosaponin.

This saponin has been isolated from *Buddleja officinalis* Maxim., *B. madagascariensis* Lamk. (Buddlejaceae), *Scrophularia ilwensis* C.Koch, *S. kakudensis* Franch. and *Verbascum phlomoides* L. (Scrophulariaceae) (Ding *et al.*, 1992; Calis *et al.*, 1993; Yamamoto *et al.*, 1993 and Emam *et al.*, 1996).

The relative configuration of the molecule was determined to be identical to that of the β-amyrine triterpene, as chemical shifts were exactly similar to those displayed in the literature. An α configuration of the C-3 carbon instead of the usual β configuration would cause significative upfield shifts of signals corresponding to C-3 and C-5 (Mahato and Kundu, 1994). Moreover, the optical rotation $[\alpha]_D$=+31.7 (MeOH, c.0.1) measured was in accordance with the literature (Ding *et al.*, 1992).



3.2.1.3.4 Structure elucidation of compound B

The ^{1}H- and ^{13}C-NMR spectra of **B** displayed many similarities with those of **A**. 31 carbons constituted the aglycone part instead of 30 for **A** (Figure 19). The exceptions concerned mainly signals at δ 122.55 and 149.38, attributed to a double bond in C-12-13, signal at δ 68.71, corresponding to a hydroxymethyl group associated with two protons at δ 3.60 and 3.80, and signal at δ 53.91, characteristic for a methoxyl group, corresponding to the proton at δ 3.80.

As expected from the hydrolysis results, the composition of the sugar chain was determined to be identical to that of **A**. gHMBC correlations confirmed the attachement position of the different sugar units (Figure 20).

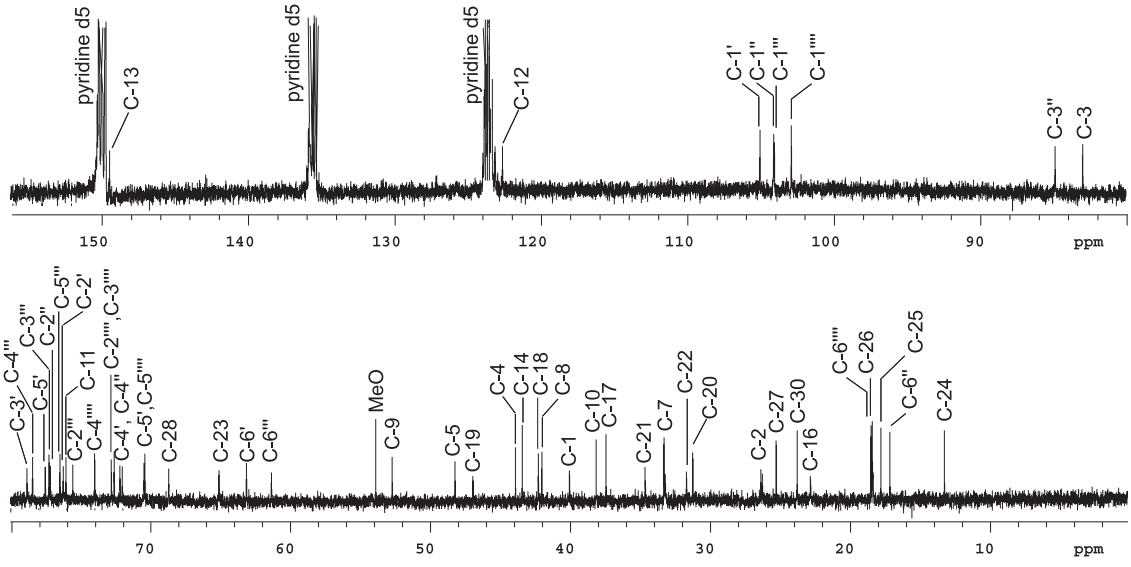


Figure 19: ^{13}C-NMR of B (125 MHz, in pyridine-*d5*). C-1' to C-6': terminal Glc signals, C-1'' to C-6'': Fuc signals, C-1''' to C-6''': internal Glc signals, C-1'''' to C-6'''': Rha signals.

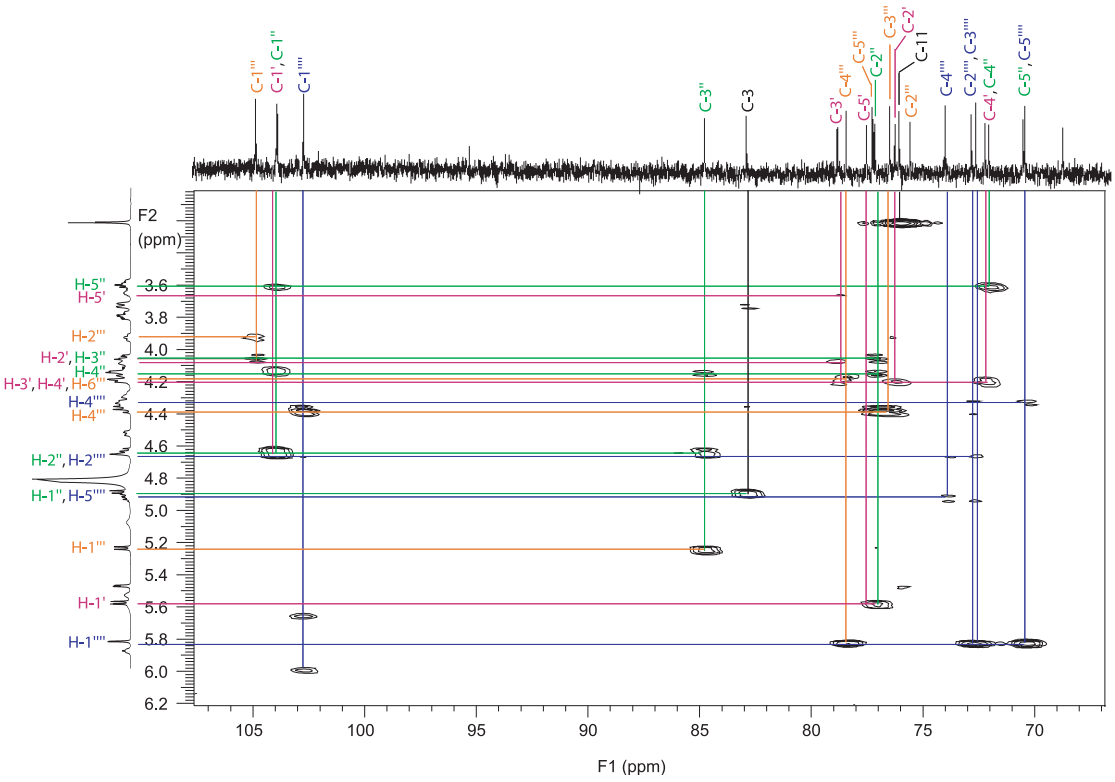
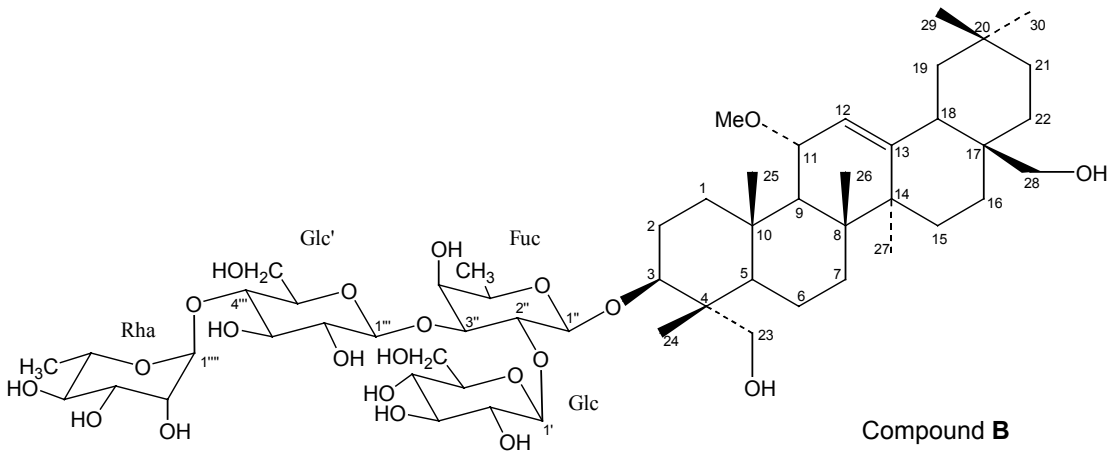


Figure 20: gHMBC-NMR of A (500 MHz, in pyridine-*d5*, TMS as internal standard). H-1' to H-6': terminal Glc signals, H-1'' to H-6'': Fuc signals, H-1''' to H-6''': internal Glc signals, H-1'''' to H-6'''': Rha signals.

Compound **B** was thus identified as 3β-*O*-[α-rhamnopyranosyl-(1→4)-β-glucopyranosyl-(1→3)-[β-glucopyranosyl-(1→2)]-β-fucopyranosyl]-11α-methoxy-olean-12-en-3, 23, 28-triol, also called mimengoside B or ilwensisaponin C.

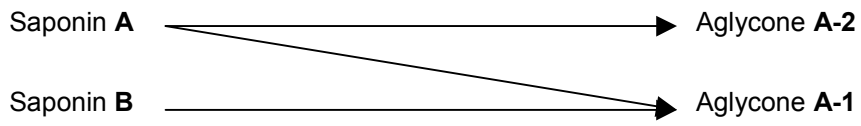
It has been isolated from *Buddleja officinalis* Maxim., *B. madagascariensis* Lamk. (Buddlejaceae), *Scrophularia ilwensis* C. Koch and *Verbascum nigrum* L. (Scrophulariaceae) (Ding *et al.*, 1992; Calis *et al.*, 1993; Klimek *et al.*, 1992 and Emam *et al.*, 1997).



Compound **B**

3.2.1.3.5 Structure elucidation of the aglycones

In order to confirm the structure of compounds **A** and **B**, structure determination of the aglycones obtained after the acidic hydrolysis mentioned above (3.2.1.3.2) was also performed. The acidic hydrolysis led in the case of **A** to two distinct aglycones, named **A-1** and **A-2**, and to **A-1** alone in the case of **B**. The isolation of **A-2** was performed by means of semi-preparative HPLC with a Symmetry C-18 preparative column under isocratic conditions with 50% acetonitrile.



Aglycone **A-1** displayed pseudomolecular ions at *m/z* 457.3 $[M+H]^+$ and *m/z* 474.3 $[M+NH_4]^+$ in DCI-MS, with ammonia used as a reagent gas.

^{13}C-NMR analysis performed on **A-1** showed the presence of 30 carbons. A comparison with the aglycone parts of compounds **A** and **B** indicated the presence of a second double bond between C-13 (δ 136.3) and C-18 (δ 135.9) (Figure 21).

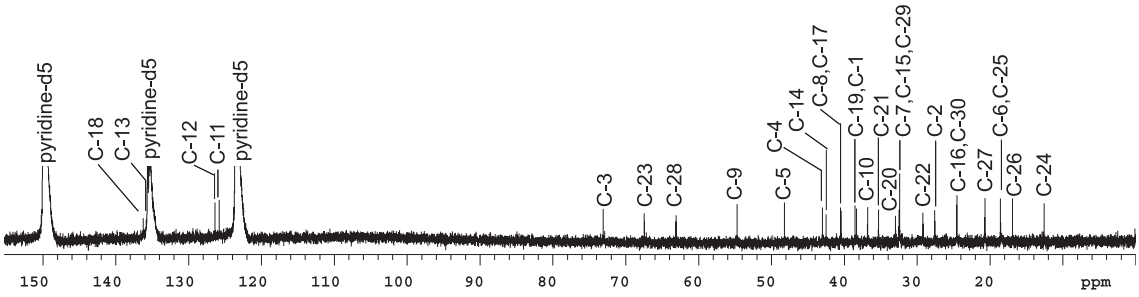
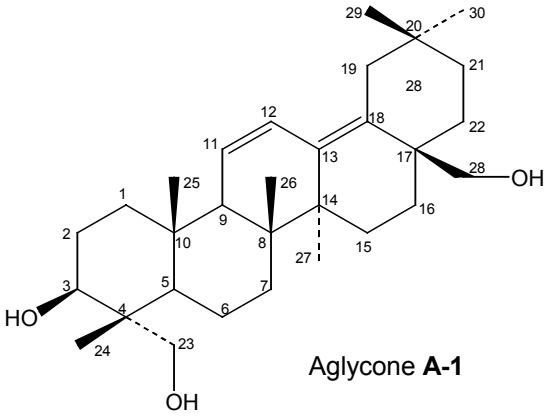


Figure 21: ^{13}C-NMR of aglycone **A-1** (125 MHz, in pyridine-*d5*).

The structure of the aglycone **A-1** was determined to be 3, 23, 28-trihydroxyoleanan-11, 13(18)-diene, also called Triterpen A (Tschesche *et al*., 1980). This is in accordance with the structure of one of the aglycones obtained by acidic hydrolysis of Mimengoside A, described by Ding *et al*. (1992).



Aglycone **A-1**

The ^{13}C-NMR spectrum of aglycone **A-2** displayed 29 signals (Figure 22). In comparison with **A-1**, signal 28 was missing, and the two double bonds were placed between C-12 (δ 117.3) and C-13 (δ 129.0), and C-17 (δ 140.2) and C-18 (δ 125.7).

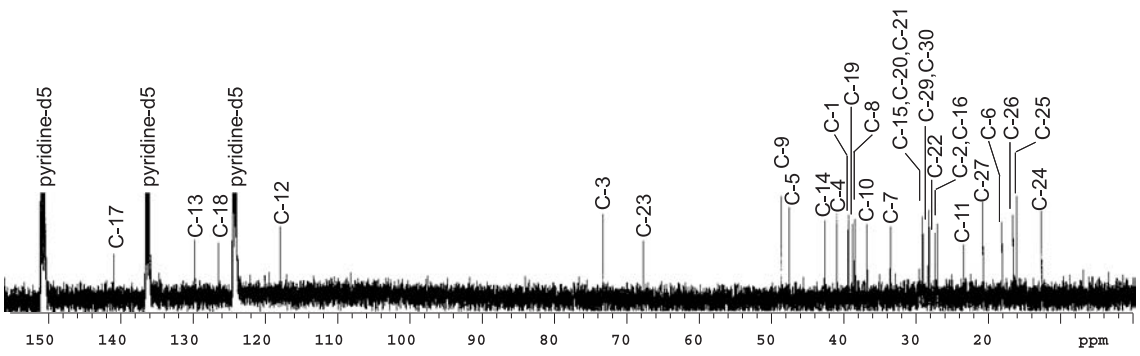
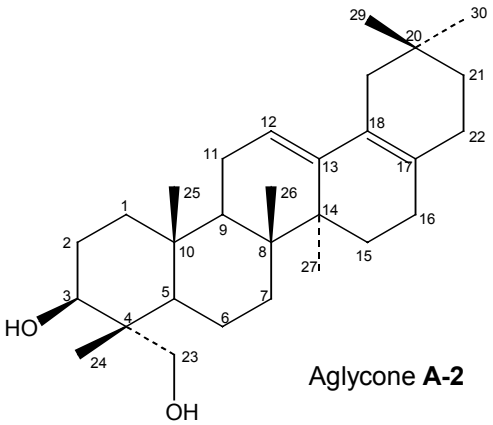


Figure 22: ^{13}C-NMR of aglycone **A-2** (125 MHz, in pyridine-*d5*).

The structure of **A-2** was thus established as 3, 23-dihydroxy-28-nor-oleanan-12, 17-diene, or Triterpen B (Tschesche *et al*., 1980), corresponding to the second aglycone described by Ding *et al*., obtained by acidic hydrolysis of Mimengoside A (1992).



Aglycone **A-2**

3.2.1.4 Activities of compounds A and B

Pure compounds **A** and **B** were tested against *Cladosporium cucumerinum*, *Candida albicans* and as inhibitors of the acetylcholinesterase. In order to avoid transformations between **A** and **B**, ethanol was used instead of methanol in TLC eluents.

The activities were quantified by observing the activity of geometrical dilutions of the pure compounds (Table 3).

Compound **A** was much more active against *C. cucumerinum* and *C. albicans* than **B**. While 10 μg of **A** could inhibit the growth of *C. albicans* and 5 μg the growth of *C. cucumerinum*, 50 μg of **B** were necessary to inhibit *C. cucumerinum* and **B** was inactive against *C. albicans*. This activity, however, was not as important as that observed for the positive control miconazole, active at 1 μg against both *C. cucumerinum* and *C. albicans*.

The activity of **A** against *Candida albicans* has already been described (Emam *et al*., 1995).

Table 3: Quantification of the biological activities of **A** and **B** (amounts spotted in μg, i:inactive)

	C. cucumerinum	*C. albicans*	Acetylcholinesterase
Compound **A**	5	10	1
Compound **B**	50	i	1
Miconazole	1	1	-
Galanthamine	-	-	0.01

Both saponins displayed an activity as inhibitors of the acetylcholinesterase (Figure 23).

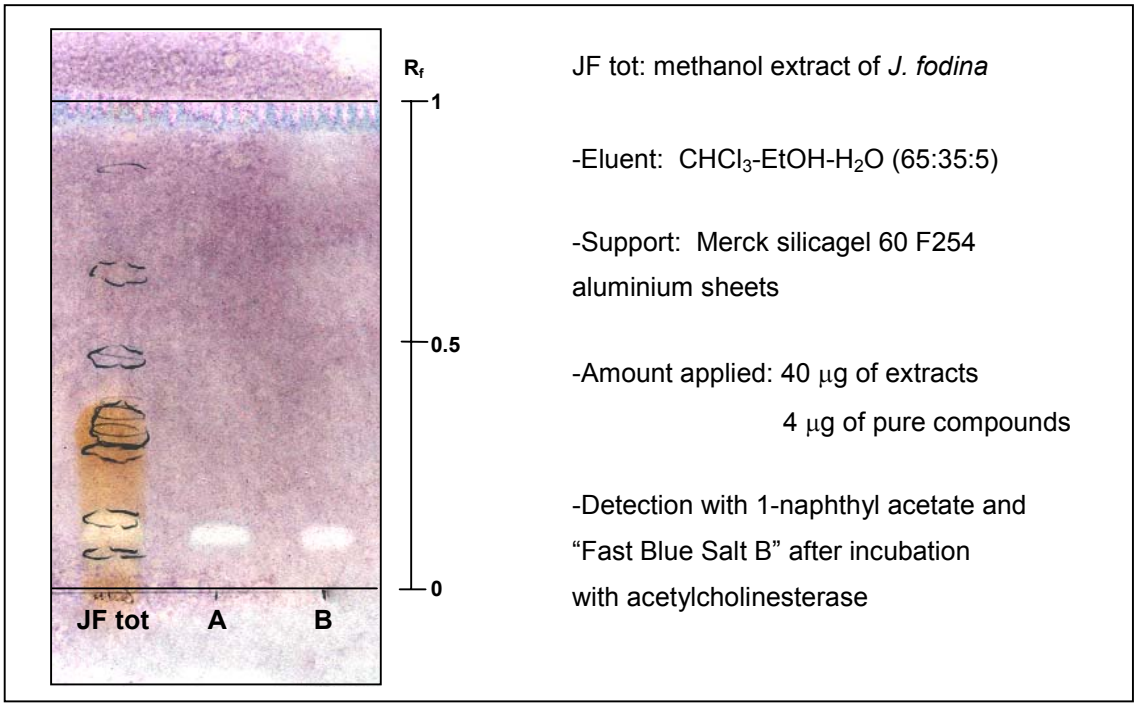


Figure 23: Inhibitory activity of compounds **A** and **B** against acetylcholinesterase

Saponins **A** and **B** were equally active against acetylcholinesterase down to 1 µg. This activity was clearly noticeable, but was much weaker than the positive standards galanthamine (active down to 0.01 µg) and physostigmine (active down to 0.001 µg) (Marston *et al*., 2002). To our knowledge, this inhibition property has not been reported previously for this class of compounds.

3.2.1.5 Discussion

The instability of saponin **A** and its transformation into saponin **B** observed during isolation process (Figure 14) have been described by Ding *et al.* (1992): compound **A** treated with p-toluene sulfonic acid in methanol led to compound **B**. Moreover, a reverse reaction was observed when **B** was heated at 37°C in acetate buffer (pH 4) for one day (Figure 24).

Compounds **A** and **B** could be isolated as pure compounds by avoiding the use of methanol and acid.

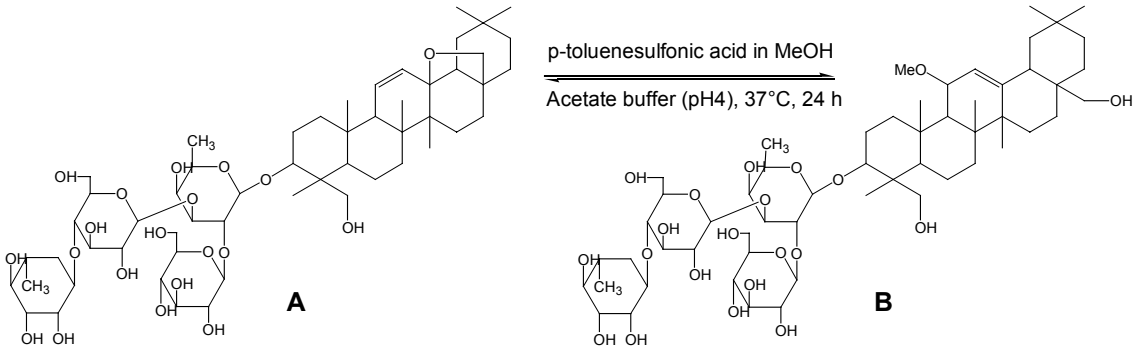


Figure 24: Transformation between saponins **A** and **B** according to Ding *et al.* (1992)

The mechanism proposed for this reaction consists of the protonation of the epoxy group, forming a cyclic oxonium ion, leading to its opening and the subsequent formation of a carbocation, followed by nucleophile addition of methanol on the carbocation as described in Figure 25.

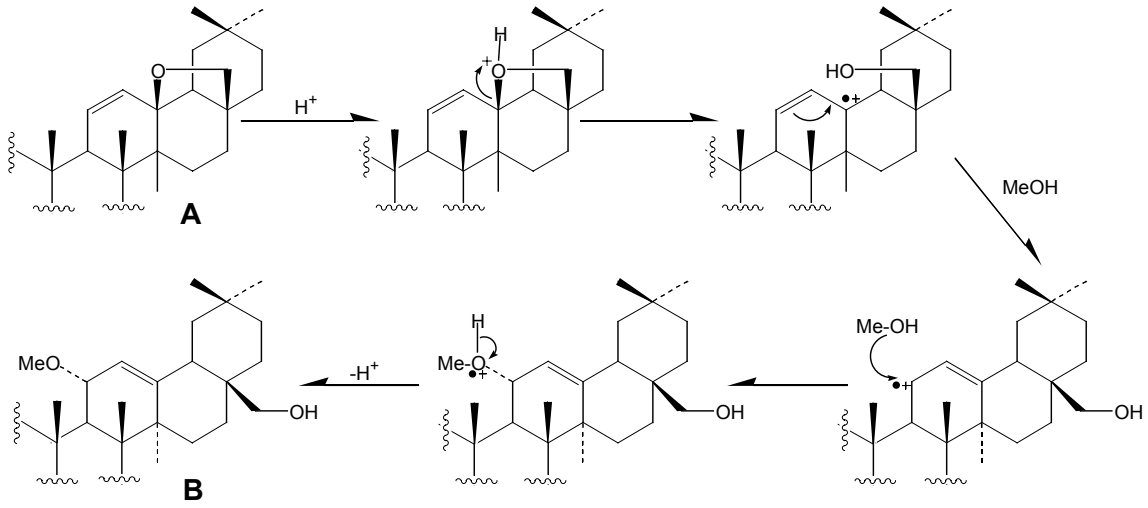


Figure 25: Proposed mechanism for the transformation of **A** into **B**

In addition to the antifungal, antibacterial, molluscicidal and enzymatic inhibitory activities described previously, mimengoside A was recently shown to possess a significant anti-inflammatory activity in various acute and chronic experimental models. It suppressed the edematous response 1 h after the carrageenan injection in the carrageenan-induced mouse paw edema. It was also effective in both acute and chronic TPA models, the potency of mimengoside A being similar to that of indomethacin. It was effective against tissue damage and cellular infiltration (Giner *et al*., 2000).

3.2.2 LC/UV/MS analysis of *Jamesbrittenia fodina* methanol extract

After the active components had been isolated, a further study of the remaining compounds present in the extract was undertaken.

Before continuing fractionation, chemical screening was initiated with LC/UV-DAD and LC/ESI-MS analyses in order to obtain preliminary information on the number and type of the extract components.

Good resolution of the constituents was obtained using a Symmetry C-18 column with a methanol-water gradient. The chromatogram at 210 nm showed major compounds at retention times (R_t) of 17.6, 20.2, 26.2, 33.6, 41.5, 58.2 and 64.2 minutes (Figure 26).

Peaks at retention times 58.2 and 64.2 minutes represented the isolated compounds **B** and **A**, respectively.

Examination of the UV spectra revealed that most of the remaining peaks presented a characteristic absorption for cinnamic acid derivatives (Waldron *et al*., 1996). Compounds with an absorption maximum at about λ 325-335 nm with a minimum at λ 260-265 nm like compounds at R_t 20.2, 33.6, 36.0, 36.8, 38.1 minutes could correspond to ferulic or caffeic acid-like derivatives. Compounds with an absorption maximum at about λ 305-310 nm with a minimum at λ 250-255 nm like compounds at R_t 39.0, 40.6, 41.5 and 42.9 minutes could correspond to *p*-coumaric acid-like derivatives (Waldron *et al*., 1996).

LC/MS analysis performed just after UV-DAD detection provided additional useful information. The analysis presented in Figure 26 was performed with an APCI interface in the positive ion mode. Other analyses with an APCI interface in the negative ion mode and an electrospray interface in the positive ion mode confirmed the mass of most of the compounds.

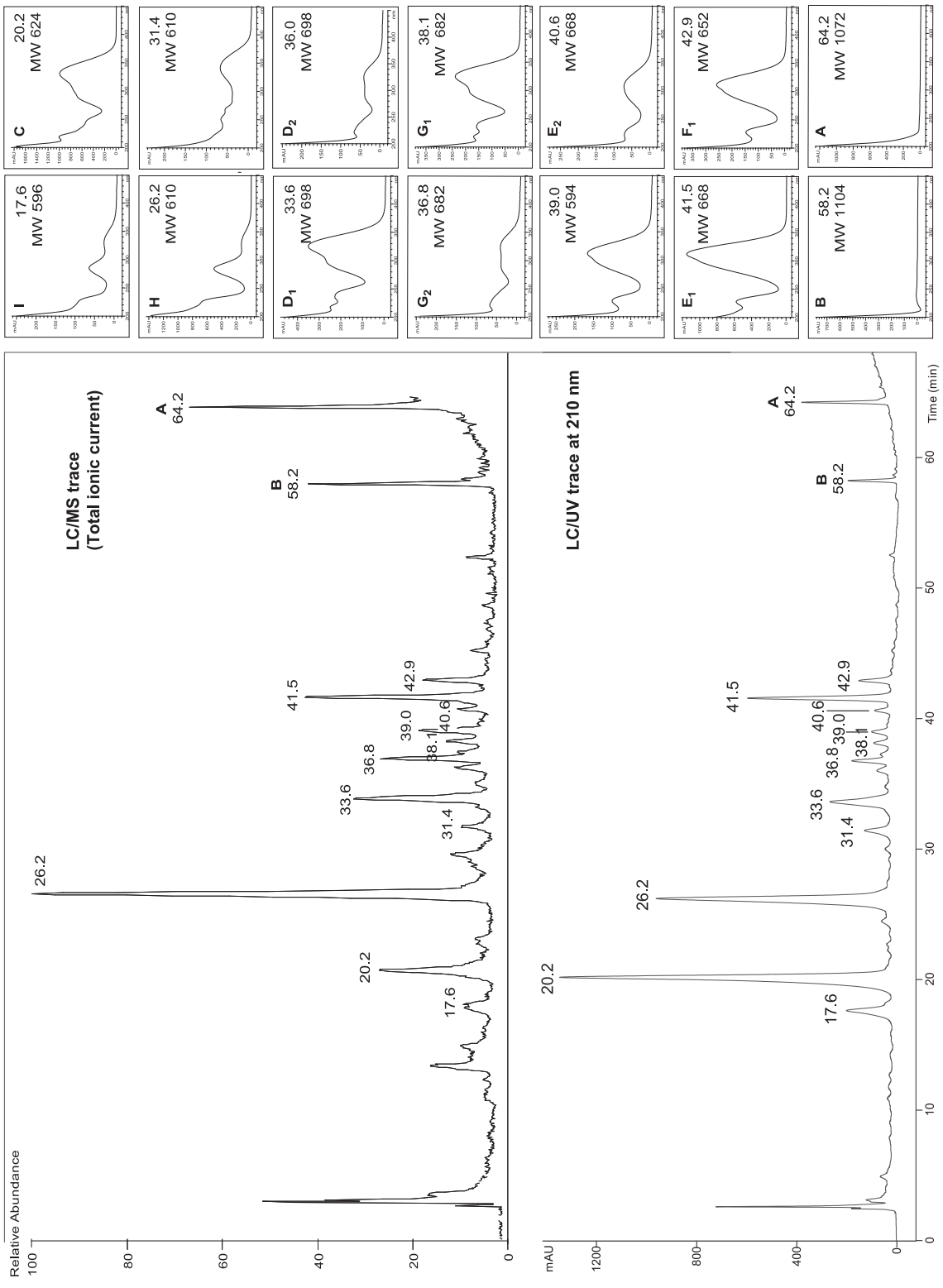


Figure 26: LC/DAD-UV/APCI-MS analysis of the methanol extract of *Jamesbrittenia fodina* in the postive ion mode. Chromatographic conditions: Symmetry C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of methanol-water (28:72 to 46:54 in 30 min, 46:54 to 62:38 in 16 min, 62:38 to 100:0 in 14 min), injection of 50 μg of the extract, flow rate 1 ml/min. UV spectra were recorded between 200 and 450 nm.

3.2.2.1 Identification of verbascoside

From the cinnamic acid derivatives detected by LC/DAD-UV, the compound eluting at R_t 20.2 minutes displayed pseudomolecular ions at *m/z* 642.0 $[M+H_2O]^+$ and *m/z* 624.9 $[M+H]^+$, which indicated the possible presence of verbascoside, a compound already described in some species of the Scrophulariaceae (Andary *et al*., 1981). This hypothesis was confirmed by coelution with a verbascoside standard.

Moreover, verbascoside was demonstrated to be responsible, at least in part, for the free radical scavenging activity in the TLC chemical screening assay with DPPH. This activity was previously reported by Potterat in his review on antioxidants of natural origin (1997).

3.2.2.2 Observation of cinnamic acid derivatives

Mass analyses brought additional information concerning the other cinnamic acid derivatives. The compounds with R_t 33.6 and 36 minutes displayed pseudomolecular ions at *m/z* 699.8 $[M+H]^+$ and 699.6 $[M+H]^+$, those with R_t 40.6 and 41.5 minutes at *m/z* 669.4 $[M+H]^+$ and 669.7 $[M+H]^+$, 36.8 and 38.1 minutes at *m/z* 682.5 $[M+H]^+$ and 682.6 $[M+H]^+$ and 42.9 minutes at *m/z* 652.5 $[M+H]^+$ (Figure 26). These relatively high molecular weights suggested that the present compounds were not simple cinnamic derivatives, but more complex molecules. They could not be identified directly based on the LC/UV/MS analysis. Their isolation was therefore undertaken as described in paragraphs 3.2.3 to 3.2.6.

3.2.2.3 Observation of flavonoid derivatives

The compound at R_t 26.2 minutes gave a pseudomolecular ion at *m/z* 610.9 $[M+H]^+$ with fragmentation showing the departure of two sugar units (ions at *m/z* 465.0 $[M+H-146]^+$ and *m/z* 303.2 $[M+H-146-162]^+$) suggesting the presence of a flavone diglycoside, but the UV spectrum with a maximum at λ 285 nm and a shoulder at about λ 330 nm provided evidence for the presence of a flavanone. The compound at R_t 17.6 minutes displayed a similar UV spectrum and its LC-MS analysis showed a pseudomolecular ion at 596.8 $[M+H]^+$. The isolation and structure elucidation of these polyphenols will be described in paragraphs 3.2.3 and 3.2.7.

3.2.3 Isolation scheme for compounds C to I

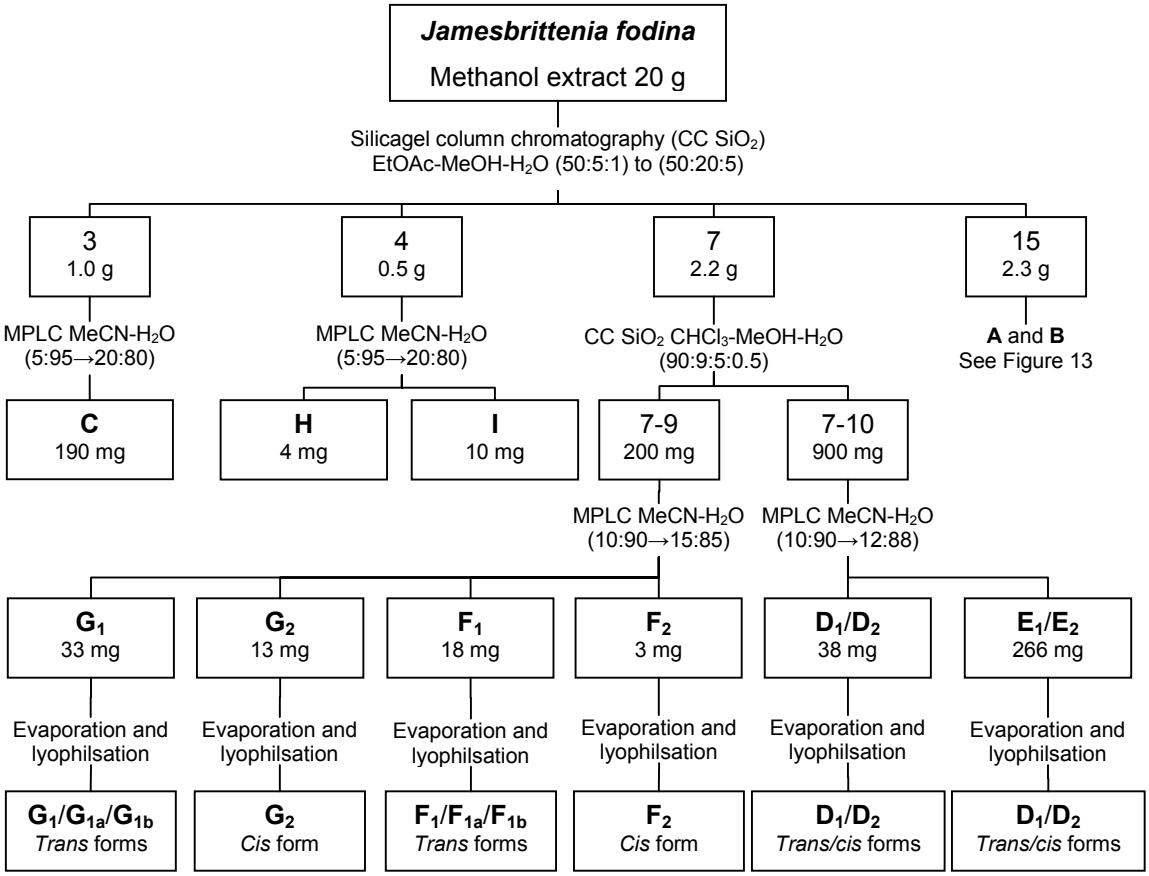
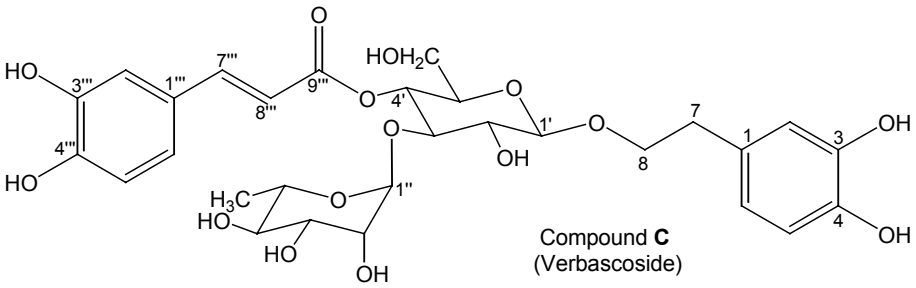


Figure 27: Isolation scheme of compounds **C** to **I**

Verbascoside (compound **C**) was easily isolated by means of a MPLC from fraction 3 of the first open column silicagel chromatography. This known compound was isolated in order to perform biological tests on the pure product. It gave negative results in both antifungal tests, in the antibacterial test, and in the inhibition of acetylcholinesterase. As mentioned above, verbascoside was responsible for the radical scavenging activity of the extract in the DPPH test.



Isolation of the other cinnamic acid derivatives, **D** to **G**, was then undertaken (see 3.2.4 and 3.2.5).

Finally, flavonoids **H** and **I** were isolated (see 3.2.7).

3.2.4 Instability problem and structure elucidation of compounds D_1/D_2 and E_1/E_2

3.2.4.1 Instability problem

Silicagel open column chromatography of fraction 7 followed by MPLC of fraction 7-10 were carried out in order to isolate the compounds with retention times 33.6 and 41.5 minutes (see Figure 26). But in spite of the good separation conditions, both products were obtained as mixtures of two compounds $\mathbf{D_1}/\mathbf{D_2}$, and $\mathbf{E_1}/\mathbf{E_2}$.

The first mixture ($\mathbf{D_1}$ and $\mathbf{D_2}$) could be easily separated by HPLC on a semi-preparative Symmetry C-18 column with isocratic conditions of 40 % methanol. $\mathbf{E_1}$ and $\mathbf{E_2}$ were also separated on a semi-preparative Lichrospher 100 C-18 column under isocratic conditions with 22 % acetonitrile. However, the LC-UV analysis of each collected peak after evaporation of the solvent and lyophilisation still indicated the presence of two constituents in the same proportions (Figure 28).

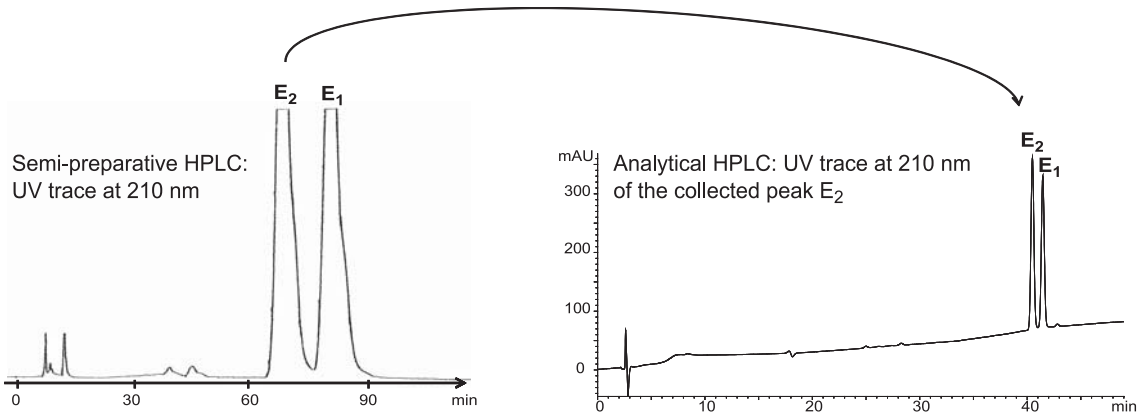


Figure 28: Instability of compound $\mathbf{E_2}$

LC/ESI-MS analysis in the positive ion mode of the mixtures revealed the presence of two isomeric pairs of constituents, pair **D_1/D_2** displaying a pseudomolecular ion at *m/z* 699 $[M+H]^+$ and pair **E_1/E_2** a pseudomolecular ion at *m/z* 669 $[M+H]^+$ (Figure 29).
These two pairs were also found in the total methanol extract, detected in APCI-MS in the negative ion mode and in ESI-MS in the positive ion mode (Figure 26 and Figure 29). Interestingly, these pairs were present in different proportions in the extract: 65:35 for **D_1**:**D_2** and 77:23 for **E_1**:**E_2**, compared to 50:50 observed in the mixtures obtained after fractionation. Their masses were confirmed by in-mixture DCI-MS measurements using methane as reagent gas in the negative ion mode, where the corresponding deprotonated molecular ions were observed.

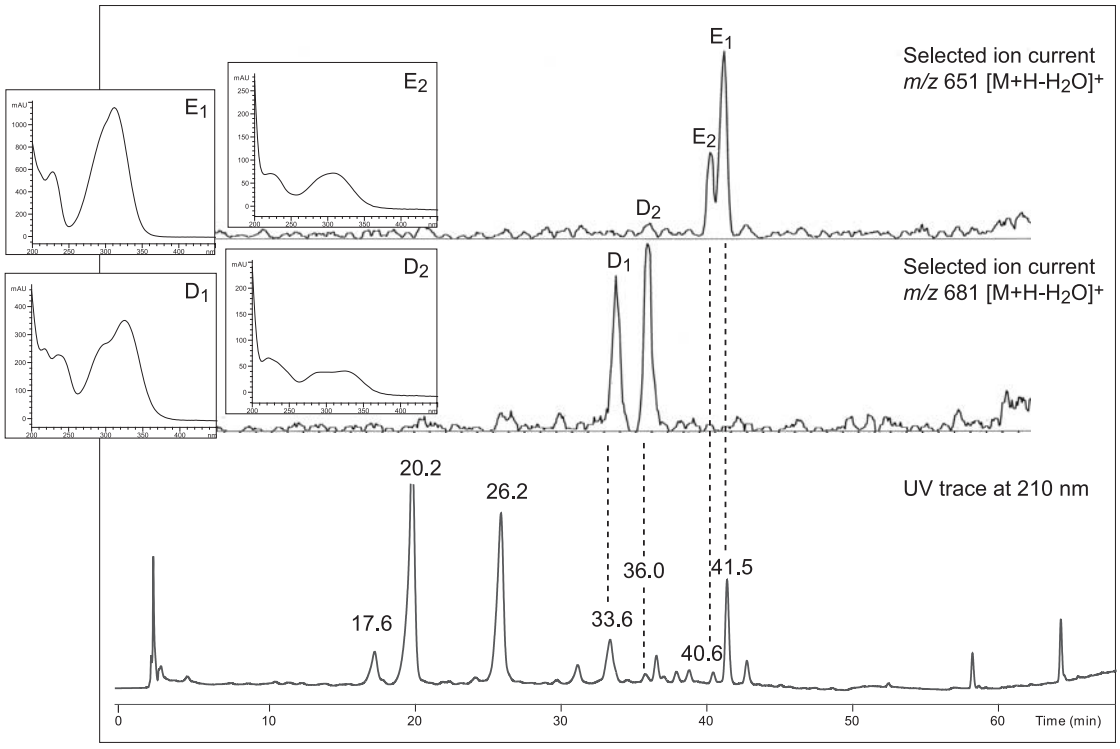


Figure 29: LC/DAD-UV/ESI-MS of the methanol extract of *Jamesbrittenia fodina* in the positive ion mode. Chromatographic conditions identical to Figure 26.

As the pure molecules could not be obtained, LC/NMR analyses of the mixtures **D_1/D_2** and **E_1/E_2** were performed in order to obtain more information about these unstable compounds.

3.2.4.2 LC/NMR analyses in the stop-flow mode

LC/NMR analyses were performed in the stop-flow mode.
For the analysis of **E_1/E_2**, acetonitrile was used instead of methanol since NMR signal resolution was better in this solvent. A good separation was obtained with a gradient of acetonitrile-deuterated water (25:75 to 30:70 in 30 minutes) on a Symmetry C-18 column. A relatively high loading was needed for adequate sensitivity: 240 µg of the mixture were injected. Satisfactory LC/^{1}H-NMR spectra were obtained for each individual compound (Wolfender *et al*., 2001).

LC/^{1}H-NMR spectra of compounds **E_1** and **E_2** showed many similarities (Figure 30).

Both compounds displayed two multiplets between δ 2.20 and 2.60, and a number of signals from δ 3.20 to 5.10, suggesting the presence of one or two sugars. Moreover, the presence of a methyl group at about δ 1.1 suggested that one of the sugars might be a rhamnose (Andary *et al*., 1981). Integration of the signal at δ 3.82 for **E_2** showed the existence of three protons and thus suggested the presence of a methoxyl group. Seven aromatic protons were observed between δ 5.8 and 7.8. Six of these were attributed to a cinnamic acid moiety, the presence of which had already been suggested by the DAD-UV spectra: two signals corresponding to four *ortho*-coupled protons at δ 7.01 (2H, d, *J*=8.8 Hz) and δ 7.61 (2H, d, *J*=8.2 Hz) in **E_1** and δ 6.89 (2H, d, *J*=8.8 Hz) and δ 7.50 (2H, d, *J*=8.8 Hz) in **E_2** could be attributed to a 1,4-disubstituted aromatic ring. The striking difference between the spectra of **E_1** and **E_2** concerned the signals attributed to the cinnamoyl double bond: δ 7.72 (1H, d, *J*=15.9 Hz) and δ 6.42 (1H, d, *J*=15.9 Hz) were characteristic of a *trans* double bond in the case of **E_1**, whereas in **E_2**, the two corresponding doublets at δ 7.04 (1H, d, *J*=12.6 Hz) and δ 5.84 (1H, d, *J*=12.6 Hz) were typical of a *cis* double bond (Figure 30) (Silverstein *et al*., 1998).

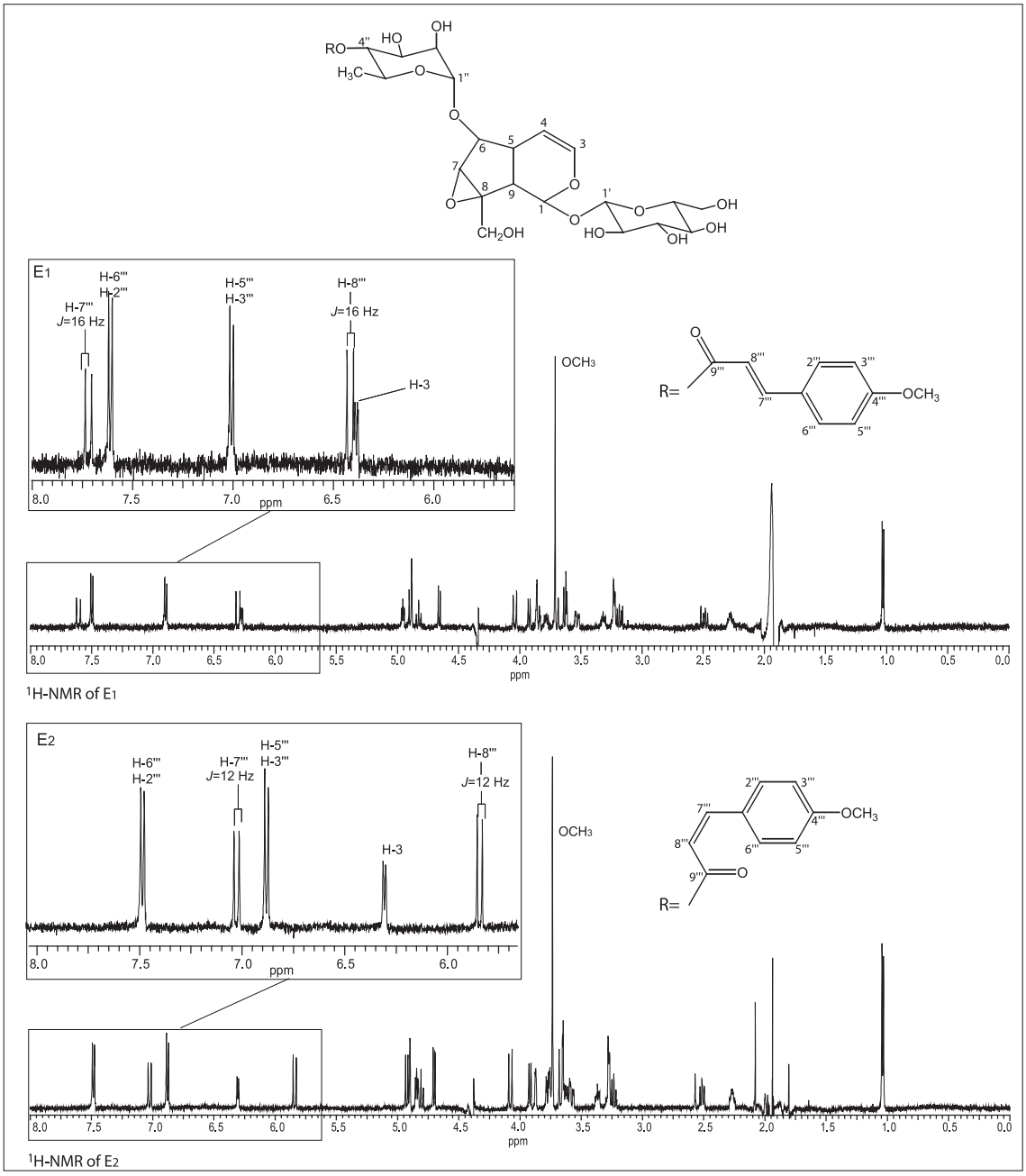


Figure 30: Stop flow LC/^{1}H-NMR analysis of **E_1** and **E_2**. Chromatographic conditions: Symmetry C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of acetonitrile-deuterated water (25:75 to 30:70 in 30 minutes), injection of 240 μg, flow rate 1 ml/min. LC/NMR conditions: NMR frequency 500 MHz, TMS internal standard, Varian ^{1}H[^{13}C] flow probe (60 μl)

The fraction containing **D_1** and **D_2** was submitted to the same analysis (Figure 31).

As a good separation could not be obtained with acetonitrile, stop-flow LC/NMR analysis was performed in methanol-deuterated water using the same conditions as for LC/UV/MS, with an injection amount of 500 µg.

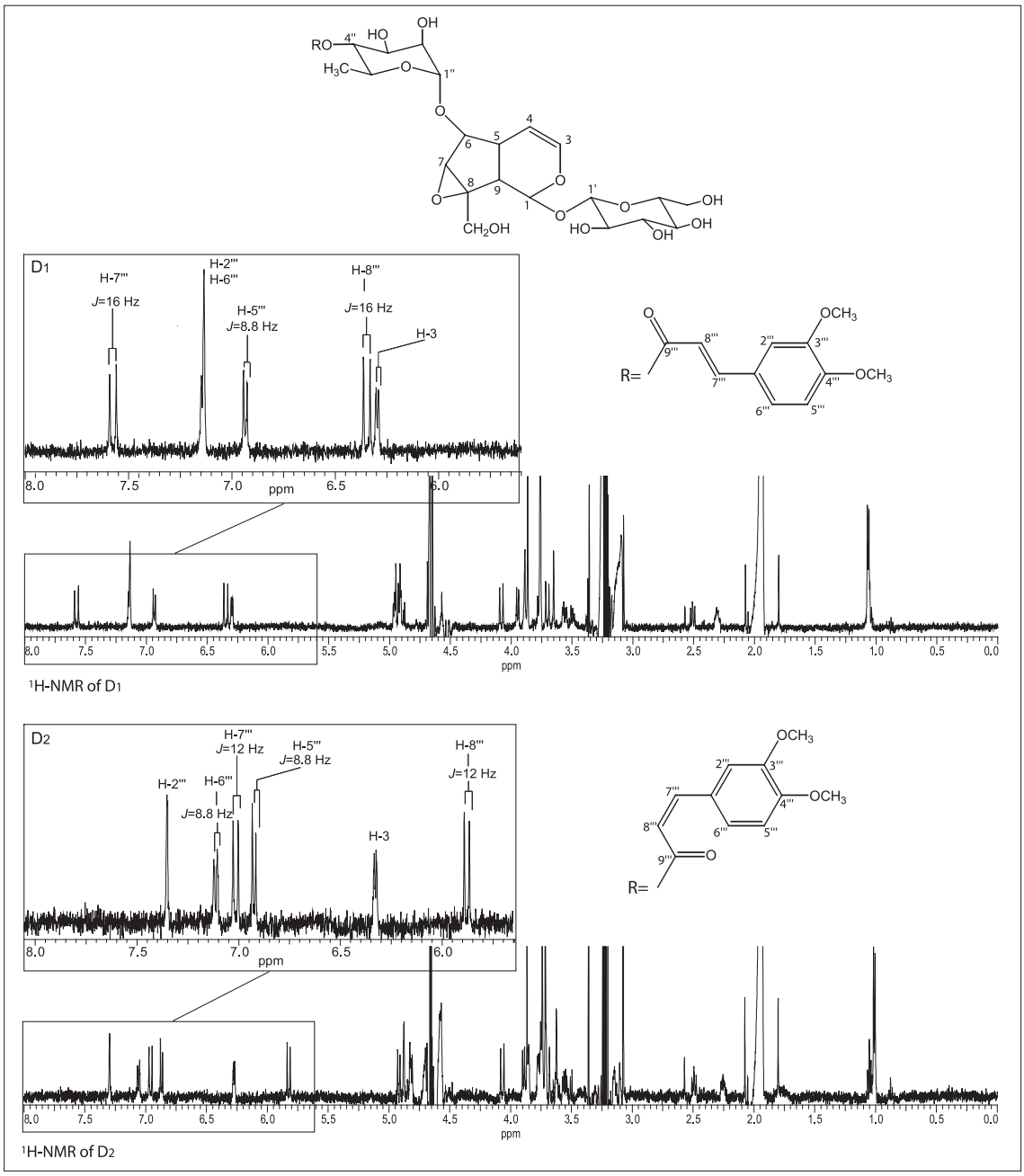


Figure 31: Stop flow LC/^{1}H-NMR analysis of **D_1** and **D_2**. Chromatographic conditions identical to Figure 26, injection of 500 µg of the mixture. LC/NMR conditions identical to Figure 30.

Resolution of the signals decreased, but the spectra demonstrated that **D_1** and **D_2** were also *trans* and *cis* isomers as shown by the two doublets of the double bond at δ 6.40 (d, *J*=15.9 Hz) and 7.64 (d, *J*=15.9 Hz) for **D_1**, and δ 5.88 (d, *J*=12.6 Hz) and 7.02 (d, *J*=12.1 Hz) for **D_2**.

The spectra could not be exactly compared with those of **E_1** and **E_2** recorded in acetonitrile-deuterated water, but it was clear that both LC/^{1}H-NMR spectra showed many similarities with those of **E_1** and **E_2**: a methyl group at about δ 1.1, two multiplets between δ 2.30 and δ 2.60 and a number of signals from δ 3.20 to δ 5.20. The signals at around δ 3.80, however, represented six protons, suggesting the presence of two methoxyl groups.

The presence of a second methoxyl group was confirmed by the mass difference of 30 between compounds **D_1**/**D_2** (MW 698) an **E_1**/**E_2** (MW 668).

Moreover, the aromatic moiety of **D_1**/**D_2** displayed significant differences compared to **E_1**/**E_2** with only 6 protons detected between δ 5.80 and 7.70 instead of 7.

In the case of **D_2**, two *ortho*-coupled aromatic protons at δ 6.93 (1H, d, *J*=8.8 Hz) and δ 7.12 (1H, d, *J*=8.8 Hz) and another aromatic proton detected at δ 7.36 (1H, s) confirmed the presence of a supplementary methoxyl group on the cinnamic acid moiety.
The LC/NMR spectra thus suggested that the two fractions consisted of mixtures of *cis*- and *trans*- cinnamoyl glycoside derivatives. Based on these LC/^{1}H-NMR spectra it was not possible, however, to deduce the complete structure of both pairs of constituents, because information on the core nucleus of these molecules was lacking.

3.2.4.3 In-mixture NMR analyses

The mixtures of compounds **D₁**/**D₂** and **E₁**/**E₂** were thus submitted to classical 1D and 2D NMR analyses recorded in methanol-*d4* (Figure 32, Figure 33).

In the case of **E₁**/**E₂**, 2D NMR experiments (gCOSY, gHMBC and gHSQC) confirmed the presence of the cinnamoyl moiety, as well as two sugar units, identified as α-rhamnose and β-glucose. The β configuration of glucose was determined through the LC/^{1}H-NMR shift of H-1' at δ 4.77 (d, $J_{1',2'}$=8.2 Hz) for **E₁** and δ 4.70 (d, $J_{1',2'}$=7.7 Hz) for **E₂**. The configuration of rhamnose was deduced from the chemical shifts of the carbons, which differ between α and β configurations, as the coupling of the anomeric proton was not clear (Agrawal, 1992).

The ^{13}C NMR spectrum exhibited a signal at δ 55.77 for **E₁** and δ 55.89 for **E₂**, attributed to a methoxyl group (Figure 32). The gHMBC spectrum correlations between the methoxyl protons at δ 3.83 (3.81 for **E₂**) and the aromatic carbon C-4''' at δ 163.20 (162.05 for **E₂**) confirmed a *para* substitution for compounds **E₁** and **E₂**.

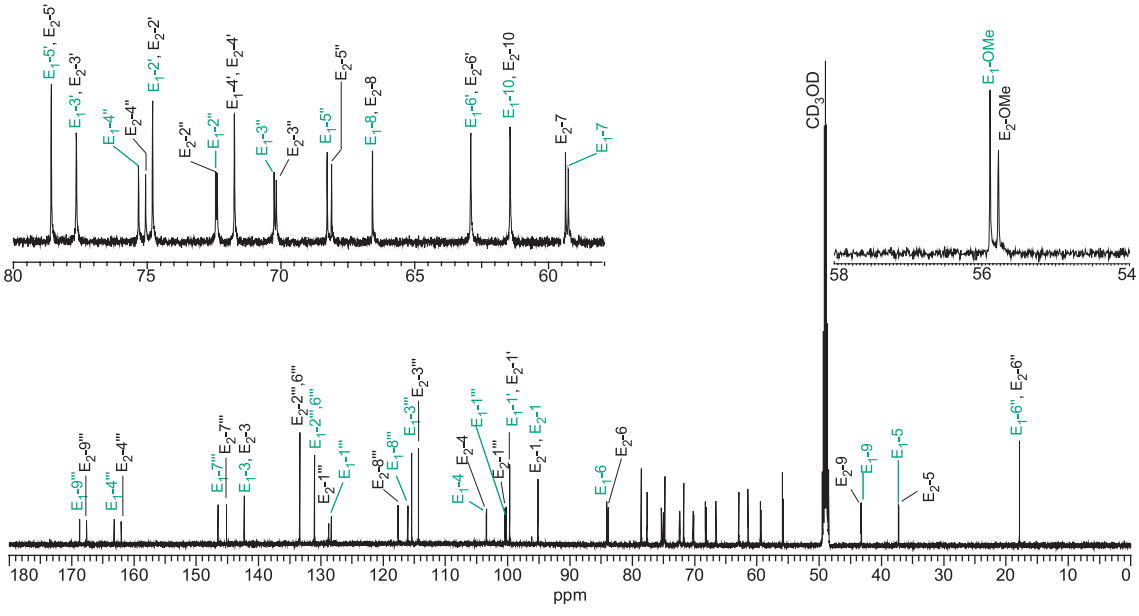


Figure 32: ^{13}C-NMR spectrum of compounds **E₁** and **E₂** in-mixture (125 MHz, methanol-*d4*)

In the case of **D_1**/**D_2**, a second substitution on the phenyl moiety was confirmed. The ^{13}C NMR spectrum exhibited two methoxyl group signals at δ 56.54 and δ 56.44 for **D_1** and δ 56.36 and δ 56.51 for **D_2** (Figure 33). 2D NMR experiments indicated substitutions at C-3''' and C-4'''.

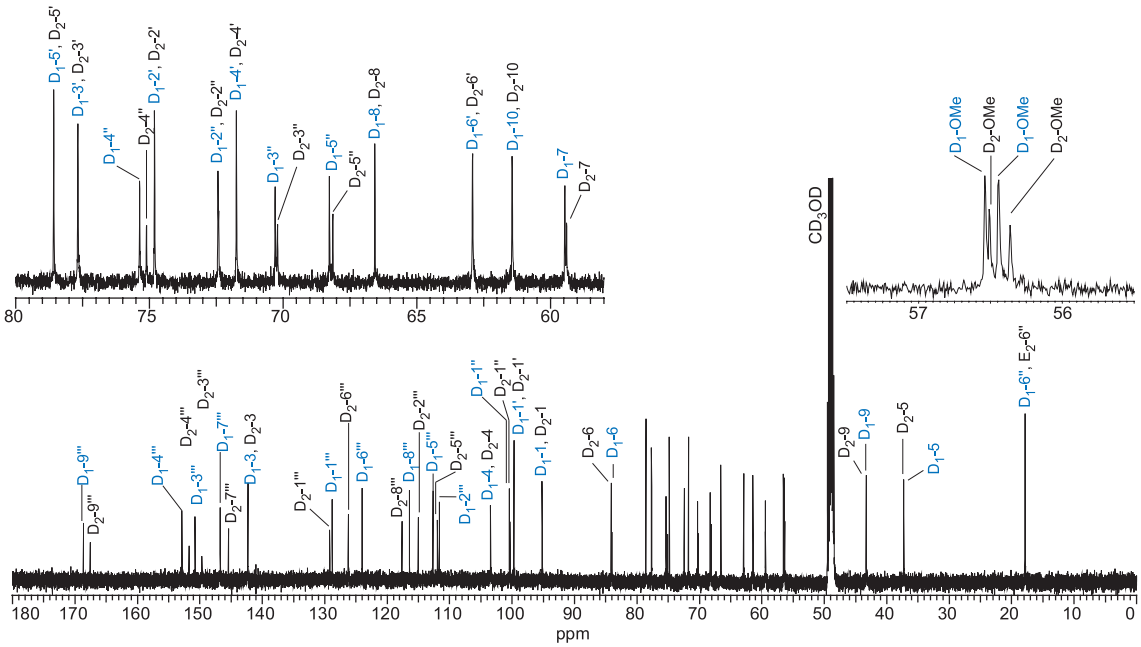


Figure 33: ^{13}C-NMR spectrum of compounds **D_1** and **D_2** in-mixture (125 MHz, methanol-*d4*)

For both **D_1**/**D_2** and **E_1**/**E_2**, the remaining signals consisted of 9 carbons and 10 protons, which were found from extensive 2D NMR experiments to belong to a catalpol aglycone (Agababyan *et al*., 1982).

In the case of **E_1**/**E_2**, attachment of the glucosyl moiety to C-1 of the iridoid moiety was easily verified with gHMBC correlations between the anomeric proton H-1' at δ 4.78 and carbon C-1 at 95.16. It also showed the correlations between the anomeric proton of rhamnose H-1''at δ 5.01 and the carbon C-6 at δ 84.12 (83.89 for **E_2**).

Moreover, gHMBC showed correlations between H-4'' at δ 5.09 (5.02 for **E_2**) and the signal of the carbonyl δ 168.7 (167.6 for **E_2**), indicating the attachment of the cinnamoyl unit to C-4'' in the rhamnopyranosyl moiety (Figure 34).

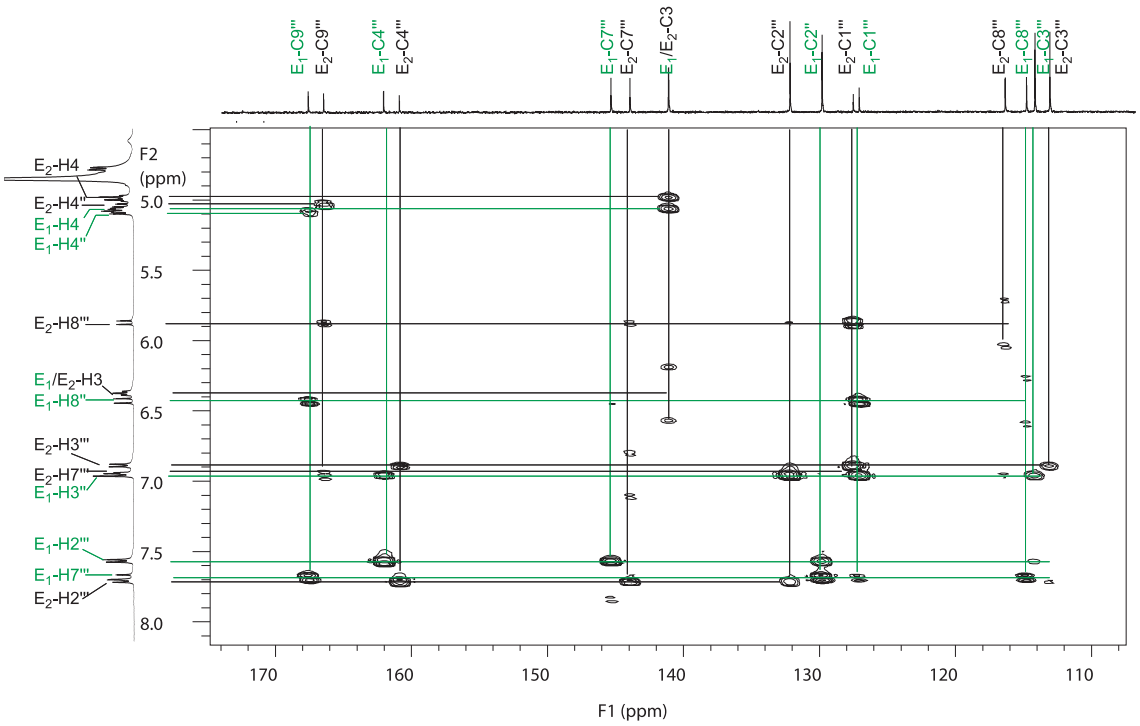


Figure 34: gHMBC-NMR of **E_1** and **E_2** in-mixture (500 MHz, in methanol-*d4*, TMS as internal standard).

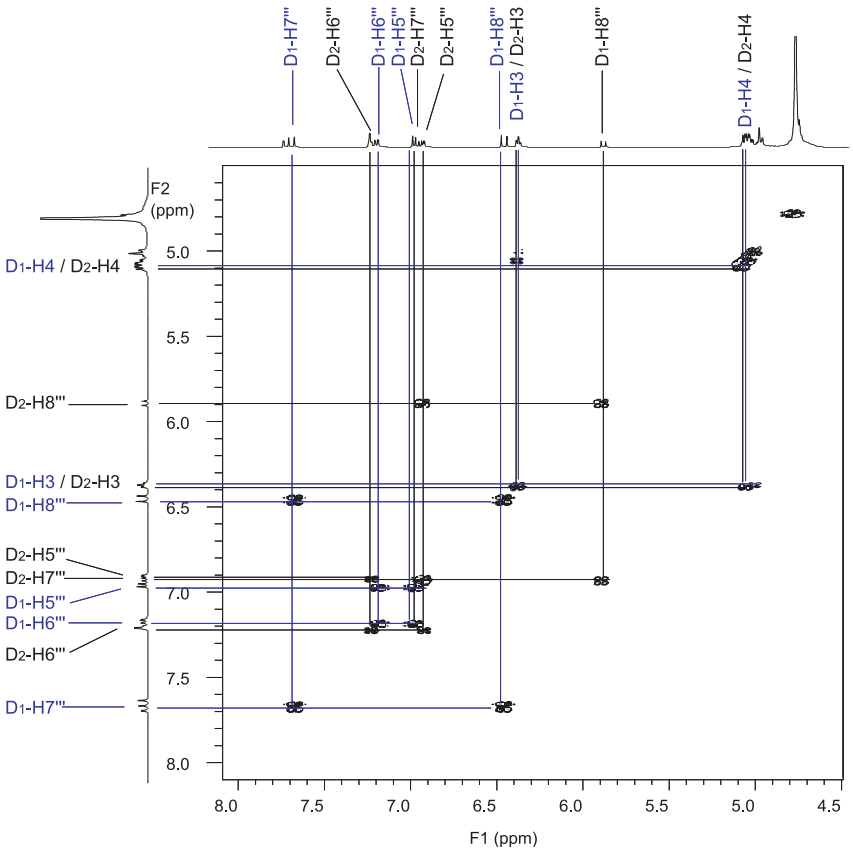


Figure 35: dqCOSY-NMR spectrum of **D_1** and **D_2** in-mixture (500 MHz, in methanol-*d4*, TMS as internal standard).

The structures of **E_1** and **E_2** were established to be the *trans* and *cis* forms of 6-*O*-[4–*O*-(4-methoxycinnamoyl)-α-rhamnopyranosyl]catalpol, respectively. The *trans* form has already been described by Agababyan *et al*. (1982) from *Verbascum georgicum* BENTH. (Scrophulariaceae).

Compounds **D_1**/**D_2** were similar to **E_1**/**E_2** except for the substitution on the cinnamoyl moiety. **D_1** and **D_2** were identified as the *trans* and *cis* forms of 6-*O*-[4–*O*-(3,4-dimethoxycinnamoyl)-α-rhamnopyranosyl]catalpol, respectively. The *trans* form has already been described by Myase *et al*. (1991) from *Buddleja japonica* HEMSL. (Buddlejaceae).

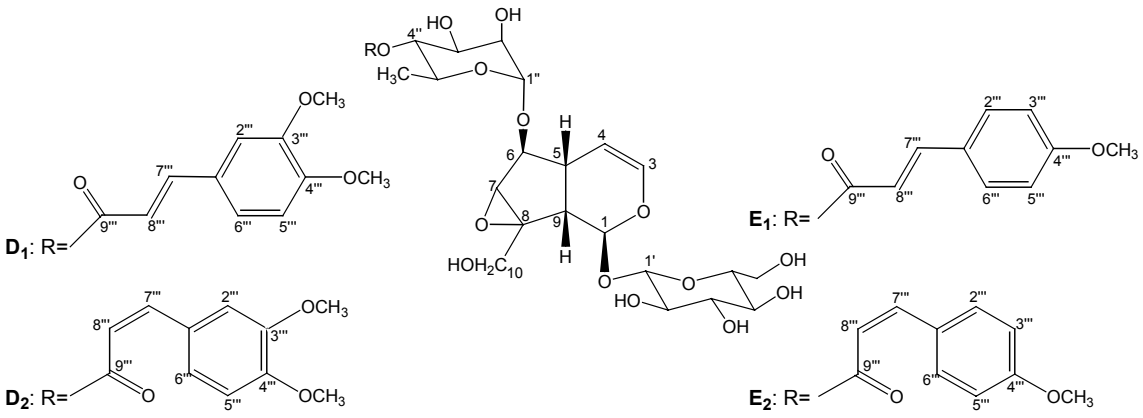


Figure 36: Structure of componds **D_1**/**D_2** and **E_1**/**E_2**

3.2.4.4 Discussion

The combined analysis of the isomeric mixtures **D_1**/**D_2** and **E_1**/**E_2** from a methanol extract of the aerial parts of *Jamesbrittenia fodina* by LC/UV/NMR, LC/UV/MS and classical 2D NMR permitted the identification of four unstable natural products. LC-NMR was very valuable for the attribution of all resonances obtained in standard NMR experiments in-mixture.

These compounds were finally identified as *cis* and *trans* catalpol derivatives. The *trans* forms of these compounds have been described in other members of the Scrophulariaceae and Buddlejaceae families (Agababyan *et al*., 1982; Myase *et al.*, 1991), but there has been no mention of the *cis* forms.

The chemical instability of *p*-coumaric acid derivatives was previously described by Rodriguez *et al.* (1998) and by Bergman *et al*. (2001); the latter authors attributed a light-induced isomerisation for the *cis*/*trans* mixture obtained after purification of some compounds. Other cases of transformation of a *trans* to a *cis* form of cinnamoyl iridoid glycoside esters showed a proportion of 20 to 30 % of *cis* form (Helfrich and Rimpler, 1998; Otsuka *et al*., 1990, 1991). The light-induced isomerisation could explain the fact that the proportion of *cis* form was 46 % for **E_1/E_2** and 40% for **D_1/D_2** at the time of the in-mixture NMR experiment whereas these proportions were only 35 % and 23 %, respectively, in the preliminary LC/UV analysis of the crude extract. The products were isolated in summertime when light was especially bright. It was noticed that during stop-flow LC-NMR analyses, the products were stable; the NMR cell was protected from light. When a mixture of **E_1/E_2** containing 50% of the *cis* form was exposed to halogen light for 48 hours, the proportion reached equilibrium at 60 % of the *cis* form **E_2**, which was stable after 10 days storage in darkness (Figure 37).

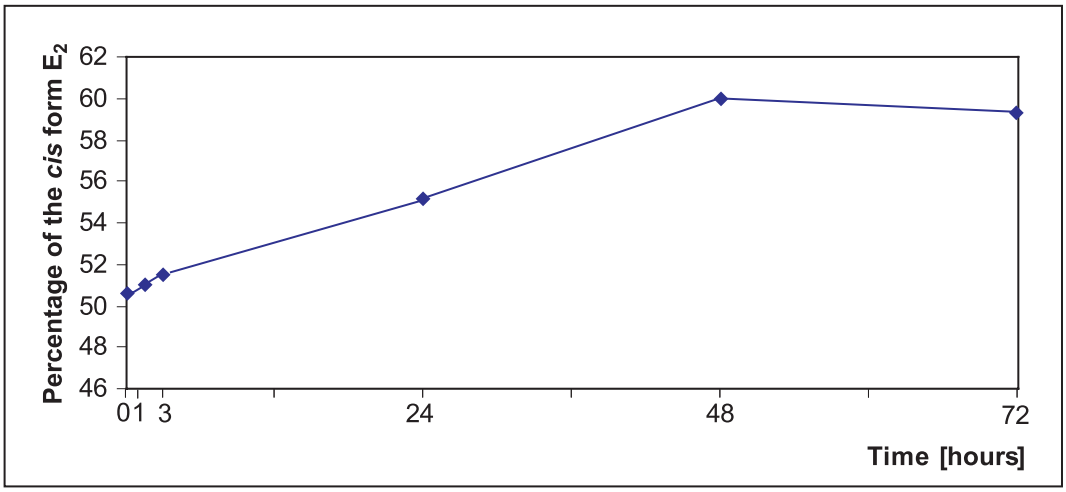


Figure 37: Light-induced isomerisation in the *cis* form **E_2** of the mixture **E_1/E_2**

It is, however, difficult to determine if the *cis* forms are biosynthesised in the plant, or if they are artefacts formed during processing. The plant extract was stored in darkness, but it might have been exposed to light during drying or extraction. Rapid analysis of the fresh plant tissue extracted in the dark might give an answer to this question.

3.2.5 Structure elucidation of compounds F_2/G_2

LC/UV/MS analysis of the extract revealed the presence of further cinnamic ester derivatives. In the continuation of our work, their isolation was tentatively undertaken. All further manipulations were performed in darkness to avoid a *cis*/*trans* isomerisation.

MPLC, performed on Fraction 7-9, obtained after two successive open column chromatographies, yielded four pure compounds **F_1**, **F_2**, **G_1** and **G_2** (Figure 27).
A comparison of their UV spectra with those of the previously isolated **D_1**/**D_2** and **E_1**/**E_2** suggested that **F_1**/**F_2** and **G_1**/**G_2** also consisted of two *cis*/*trans* isomeric pairs (Figure 38).

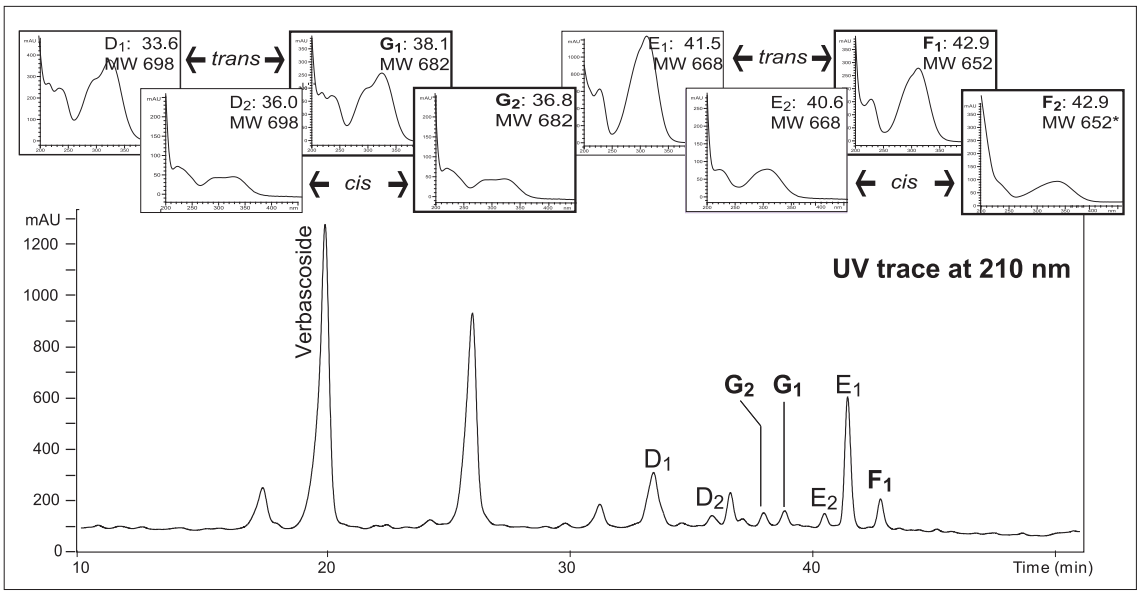


Figure 38: LC/DAD-UV analysis of the methanol extract of *Jamesbrittenia fodina*. Chromatographic conditions identical to Figure 26.
* Data taken from analysis of fraction 7-9.

After evaporation and lyophilisation, only **F_2** and **G_2** were still pure, as **F_1** and **G_1** were degraded. Before searching the cause of the degradation, structures of the isolated compounds **F_2** and **G_2** were elucidated.

3.2.5.1 Structure elucidation of F_2

Compound **F_2** displayed molecular ions at *m/z* 652.2 $[M]^+$ and *m/z* 669.9 $[M+NH_4]^+$ in DCI-MS, with ammonia used as a reagent gas.

Moreover, high resolution mass spectrometry displayed a molecular ion at *m/z* 675.22427 $[M + Na]^+$, indicating a molecular formula $C_{31}O_{15}H_{40}Na$.

^{1}H-, ^{13}C-, and 2D NMR analyses were performed on the pure compound **F_2** in order to determine its structure.

As expected from the DAD-UV spectrum, typical resonances of a cinnamoyl moiety were observed in the ^{1}H-NMR spectrum, with two protons corresponding to a *cis* double bond: δ 6.96 (1H, d, *J*=12.7 Hz) and δ 5.88 (1H, d, *J*=12.7 Hz), and two signals corresponding to four *ortho*-coupled protons at δ 7.72 (2H, d, *J*=8.8 Hz) and δ 6.90 (2H, d, *J*=8.8 Hz) that could be attributed to a 1,4-disubstituted aromatic ring (Figure 39).

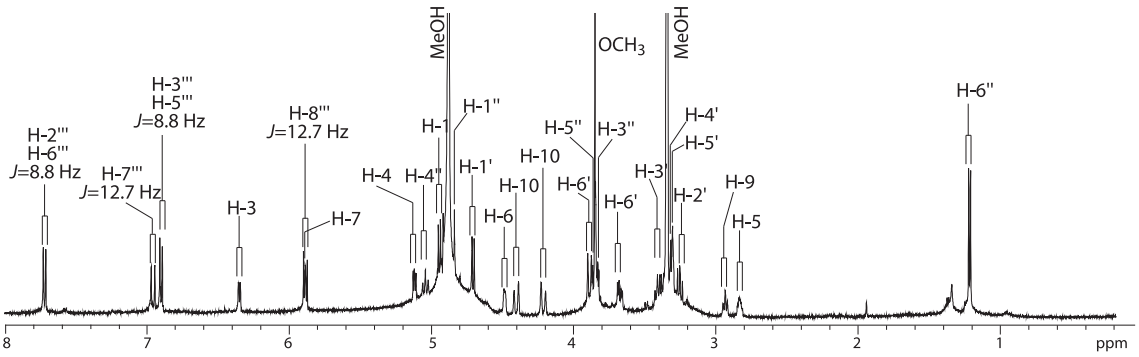


Figure 39: ^{1}H-NMR of **F_2** (500 MHz, in methanol-*d4*, TMS as internal standard).

The ^{13}C NMR spectrum exhibited a signal at δ 55.77, attributed to a methoxyl group and corresponding to a signal at δ 3.82 integrating for 3 protons in the ^{1}H-NMR spectrum (Figure 40).

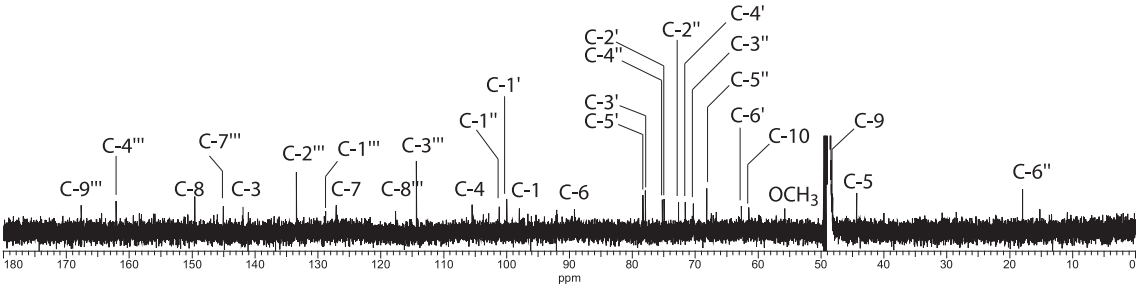


Figure 40: ^{13}C-NMR of **F_2** (125 MHz, in methanol-*d4*).

The gHMBC spectral correlations between the methoxyl protons at δ 3.82 and the aromatic carbon C-4''' at δ 162.10 confirmed a *para* substitution (red in Figure 41). Like compound **E₂**, **F₂** possesses a *cis*-*p*-methoxycinnamoyl moiety.

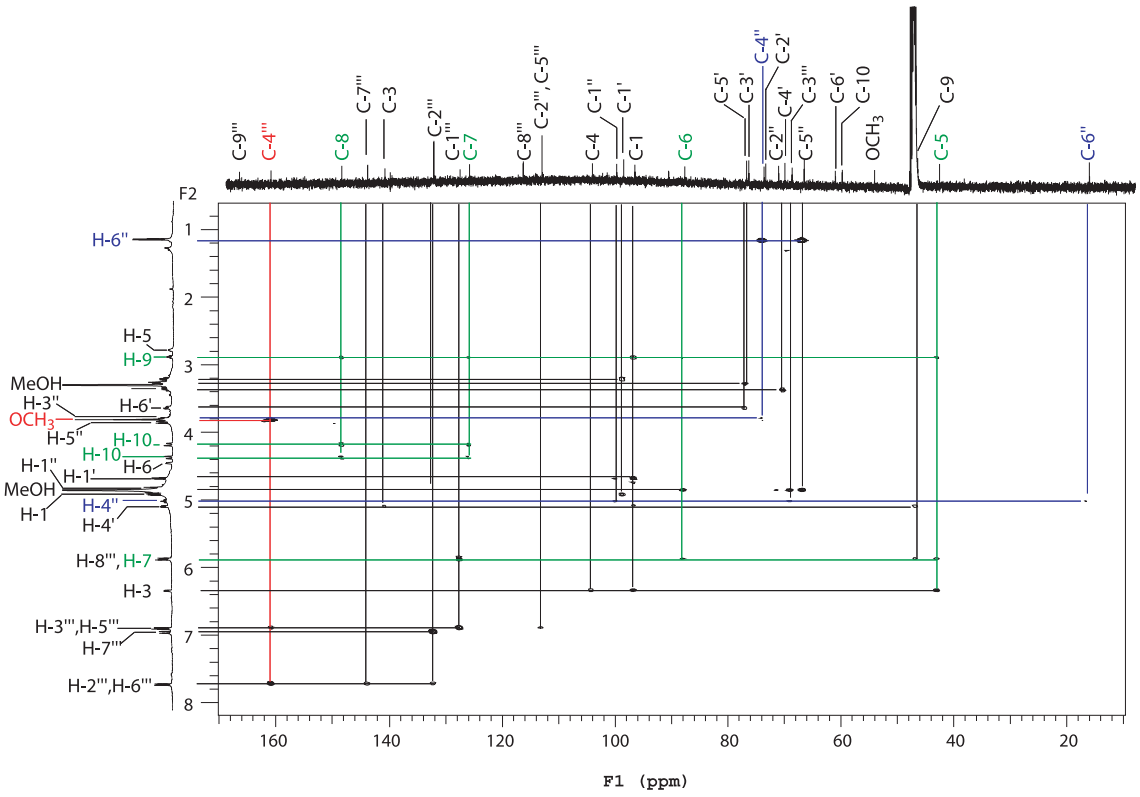


Figure 41: gHMBC NMR of **F₂** (500 MHz, in methanol-*d4*, TMS as internal standard).

As for **E₂**, the presence of numerous signals between δ 3.20 and 5.20 in the ^{1}H spectrum (between δ 70 and 80 in the ^{13}C spectrum), and the existence of a signal at δ 1.16 corresponding to 3 protons (δ 17.93 for the carbon) suggested the presence of two sugars, one of them a rhamnose. The configuration of the sugar units was determined through the coupling of the anomeric protons and the chemical shifts of the carbons (Agrawal, 1992). This indicated the presence of α-rhamnose and β-glucose.

However, the core nucleus of the molecule displayed differences relative to **E_2**: two carbon signals at δ 127.12 and 149.62 corresponding to a double bond replaced the epoxy group in position 7, 8. The position of the double bond was confirmed by the gHMBC correlations (green in Figure 41), and by the downfield shifts observed for the other carbons and protons of the iridoid moiety. Comparison of the ^{1}H and ^{13}C spectra of **F_2** with those of 6-*O*-(α-L-rhamnopyranosyl)aucubin or sinuatol (Vesper and Seifert, 1994) indicated that **F_2** is a methoxycinnamoyl ester of sinuatol (Table 4).

Table 4: Comparative ^{13}C-NMR spectral data of sinuatol and **F_2** (methanol-*d4*)

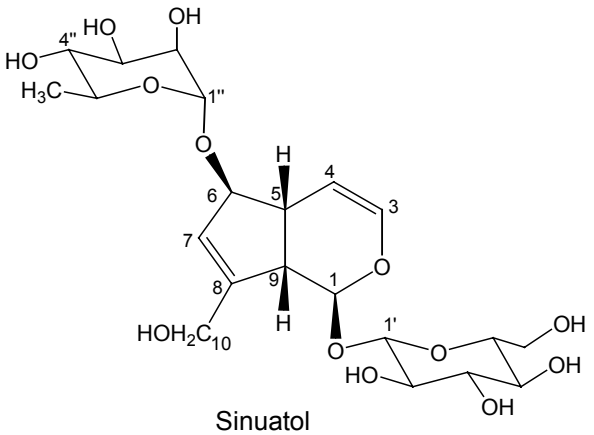
Position	**F_2**	Sinuatol*	Position	**F_2**	Sinuatol*
1	98.0	98.1	**1''**	101.2	101.2
3	142.0	141.9	**2''**	72.7	72.6
4	105.5	105.6	**3''**	70.3	72.3
5	44.3	44.4	**4''**	75.2	74.0
6	89.2	89.0	**5''**	68.2	70.2
7	127.1	127.2	**6''**	17.9	18.0
8	149.6	149.4	**1'''**	128.8	
9	48.2	48.3	**2'''-6'''**	133.4	
10	61.5	61.5	**3'''-5'''**	114.4	
1'	100.0	99.9	**4'''**	162.1	
2'	74.9	74.9	**7'''**	145.1	
3'	77.9	77.9	**8'''**	117.7	
4'	71.6	71.5	**9'''**	167.5	
5'	78.3	78.3	**MeO**	55.8	
6'	62.7	62.6			

* Data taken from Vesper and Seifert, 1994.

No connectivity could be observed with the signal of the carbonyl C-9''' at δ 167.54 in the gHMBC spectrum. The position of the methoxycinnamoyl group on the rhamnose moiety could, however, be determined by other means.

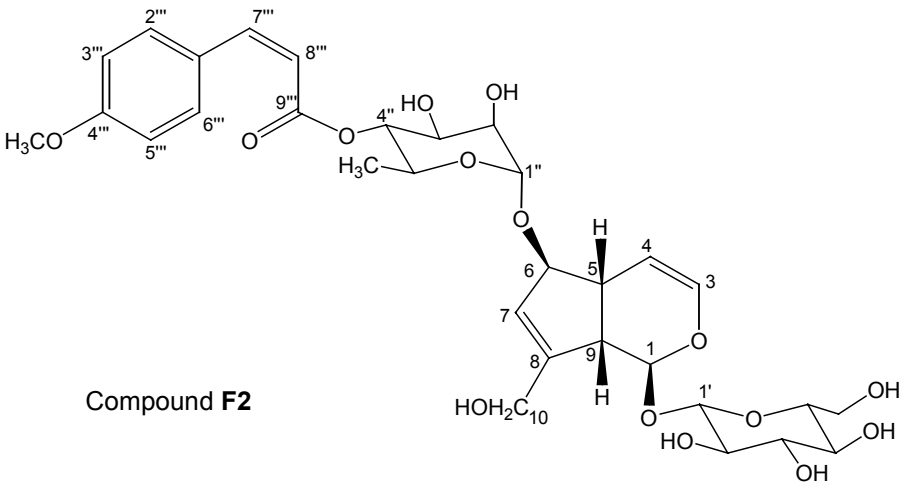
Proton δ 5.01 attached to the carbon at δ 75.24 displayed a downfield shift compared to the non-substituted rhamnosyl of sinuatol (Vesper and Seifert, 1994), indicating the attachment of the carbonyl to this carbon. gHMBC correlations between H-6'' at δ 1.16 and the signal at δ 75.24, and between the proton at δ 5.01 and C-6'' at δ 17.93 indicated the position of the ester in C-4'' (blue in Figure 41).

Moreover, a downfield shift of C-4'' (Δδ=+1.2) and upfield shifts of C-3'' and C-5'' (Δδ=-2) were observed in comparison with sinuatol (Seifert *et al*., 1982), which is in agreement with acylation in position 4'' (Table 4).



Sinuatol

Compound **F_2** was identified as the *cis* form of 6-*O*-[4–*O*-(4-methoxycinnamoyl)-rhamnopyranosyl]aucubin. This product has not been reported previously.



Compound **F2**

3.2.5.2 Structure elucidation of compound G_2

Compound **G_2** displayed pseudomolecular ions at *m/z* 699.9 $[M+H_2O]^+$ and 682.7 $[M+H]^+$ in LC/APCI-MS in the positive ion mode.

Moreover, high resolution mass spectrometry displayed a molecular ion at *m/z* 705.23527 $[M + Na]^+$, corresponding to the molecular formula $C_{32}O_{16}H_{42}Na$.

The compound was submitted to complete NMR analyses.

Spectra were very similar to those of **F_2**. As for **D_2**, the difference laid in the presence of two methoxyl groups at δ 3.84 and δ 3.85, each representing 3 protons. Two *ortho*-coupled aromatic protons at δ 6.94 (1H, d, *J*=8.3 Hz) and δ 7.23 (1H, d, *J*=8.3 Hz) and another aromatic proton detected at δ 7.73 (1H, s), replacing the two signals corresponding to four *ortho*-coupled protons in **F_2**, confirmed the presence of a 3, 4-dimethoxycinnamoyl moiety. This is in agreement with the mass difference of 30 between compounds **F_2** (MW 652) and **G_2** (MW 682).

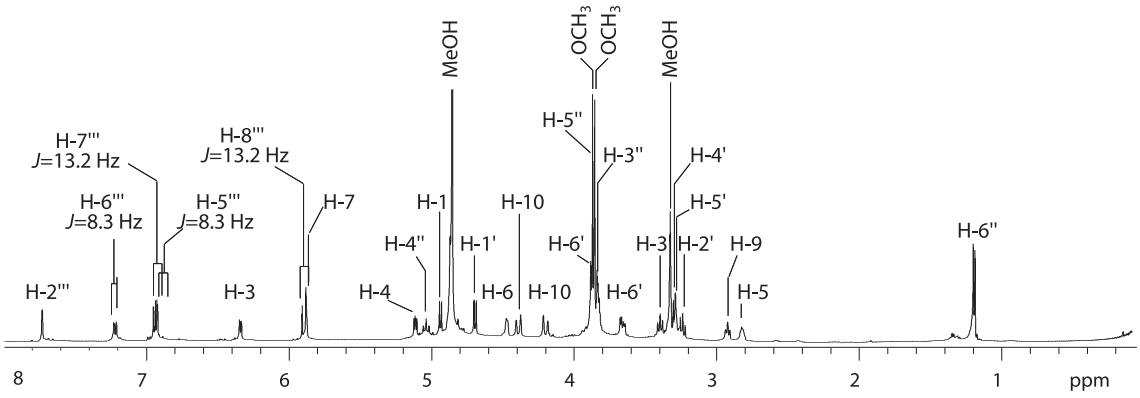
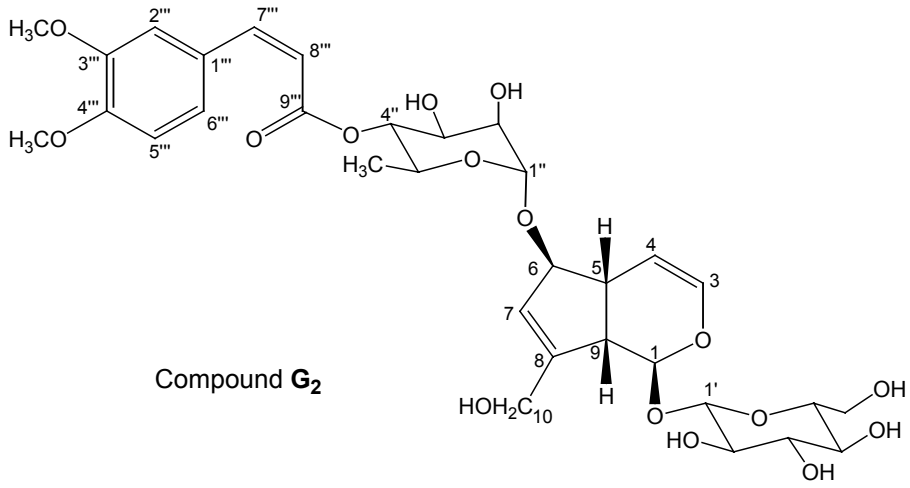


Figure 42: ^{1}H-NMR of **G_2** (in methanol-*d4*, TMS as internal standard).

The remaining signals were similar to those of **F_2**, and the structure of sinuatol could be established.

The attachment position of the dimethoxycinnamoyl moiety on the rhamnose unit was determined with the gHMBC correlations. As for **F_2**, the ester was determined to be on position 4'', considering the correlations between the proton H-4'' at δ 5.03 and the signal of the carbonyl C-9''' at δ 167.60, between the proton H-6'' at δ 1.17 and the signal at δ 75.27, and between the proton at δ 5.03 and C-6'' at δ 17.95.

Therefore, compound **G_2** was identified as the *cis* form of 6-*O*-[4–*O*-(3,4-dimethoxycinnamoyl)-rhamnopyranosyl]aucubin. This product has not been isolated previously.



Compound **G_2**

3.2.6 Second instability problem and structure elucidation of compounds F_1/G_1

3.2.6.1 Second instability problem

The MPLC performed on 7-9, that permitted the isolation of **F_2** and **G_2**, also led to their supposed *trans* isomers (Figure 38), called **F_1** and **G_1**, detected by LC/DAD-UV analysis carried out directly on the fractions after the MPLC. After evaporation and lyophilisation, however, three peaks were detected for both **F_1** and **G_1**, suggesting another problem of instability or degradation. Compound **F_1** gave **F_1**, **F_{1a}** and **F_{1b}**, and **G_1** gave **G_1**, **G_{1a}** and **G_{1b}**. All degradation products displayed the same molecular weight and UV spectra identical to the original compounds **F_1** and **G_1** (Figure 43).

This suggested that the instability was this time not related to a *cis*/*trans* isomerisation, as UV spectra of the *cis*/*trans* forms **D_1**/**D_2** and **E_1**/**E_2** displayed significant differences (Figure 29, Figure 38).

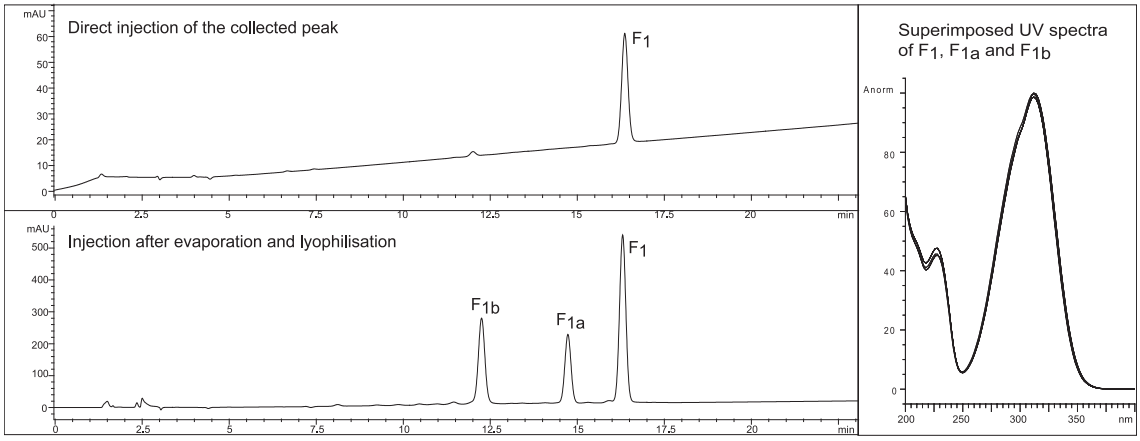


Figure 43: LC/DAD-UV analyses showing the transformation of **F_1** into **F_{1a}** and **F_{1b}** during the evaporation and lyophilisation process. Chromatographic conditions: Lichrosorb C-18 column (250x3.9 mm, i.d., 7 μm, Waters), gradient of acetonitrile-water (20:80 to 36:64 in 20 min), flow rate 1 ml/min. UV spectra were recorded between 200 and 400 nm.

The forms **a** and **b** must be artefacts, as they were not detected in the total methanol extract. **F_1** corresponds to the peak at retention time 42.9 min and **G_1** at 38.1 min (Figure 26).

The related compounds **F_1** and **G_1** could not be obtained as pure compounds, and their instability was probably due to the same mechanism in both cases. LC/^{1}H-NMR analyses were thus performed on **F_1**, **F_{1a}** and **F_{1b}** in order to elucidate the origin of the instability.

3.2.6.2 LC/NMR analyses in the stop-flow mode

LC/NMR analyses were performed in the stop-flow mode.

Acetonitrile was used for a better resolution. A good separation was obtained with a gradient of acetonitrile-deuterated water (20:80 to 36:44 in 20 minutes) on a Nucleosil C-18 column. 200 μg of the mixture were injected. Satisfactory LC/^{1}H-NMR spectra were obtained for each individual compound.

Comparison of the ^{1}H-NMR spectra obtained for the three forms **F_1**, **F_{1a}** and **F_{1b}** indicated a great resemblance (Figure 44).

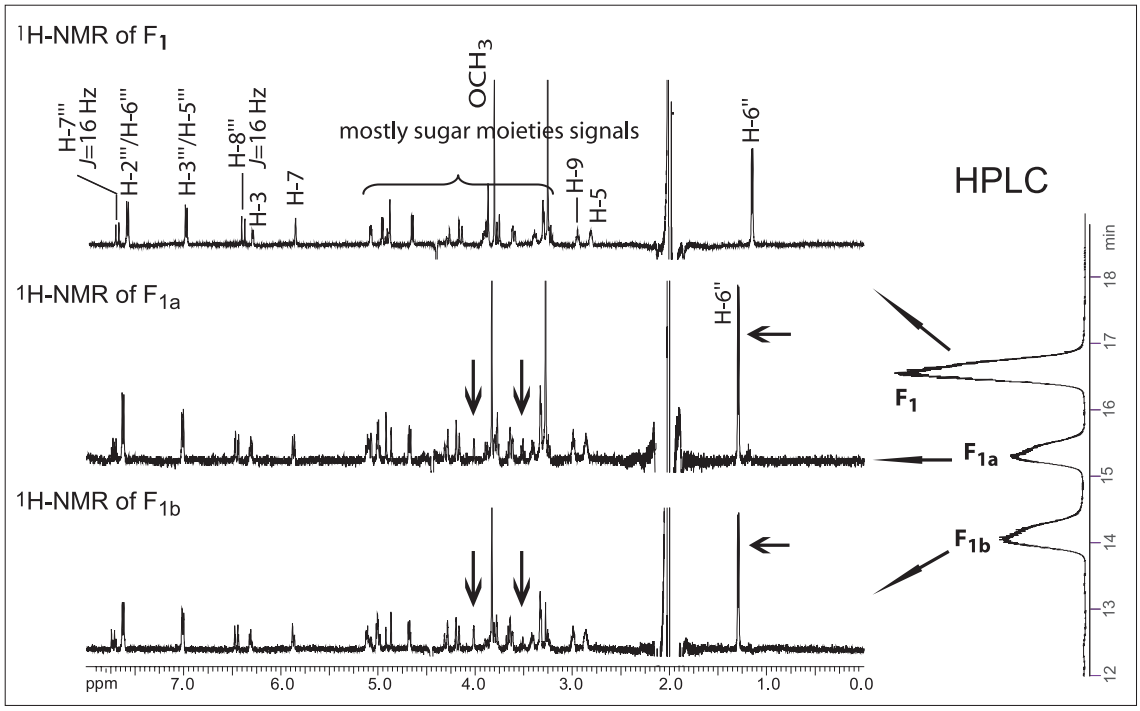


Figure 44: Stop flow LC/^{1}H-NMR analysis of **F_1**, **F_{1a}** and **F_{1b}**. Chromatographic conditions: Nucleosil C-18 column (250x8 mm, i.d., 5 μm, Macherey-Nagel), gradient of acetonitrile-deuterated water (20:80 to 36:44 in 20 minutes), injection of 200 μg, flow rate 1 ml/min, UV detection 210 nm.
LC/NMR conditions: NMR frequency 500 MHz, TMS internal standard, Varian ^{1}H[^{13}C] flow probe (60 μl).
Assignments of **F_1** signals were determined by comparison with the *cis* form **F_2**.

A downfield shift (Δδ=+0.13) was observed for the peak corresponding to H-6'' in **F_{1a}** and **F_{1b}** compared to **F_1**. Moreover, differences were noticed between δ 3 and δ 5: two signals at δ 3.5 and δ 4 were present in **F_{1a}** and **F_{1b}** but absent in **F_1** (indicated with an arrow in Figure 44).

Spectra corresponding to **F_{1a}** and **F_{1b}** were very similar. Some aromatic signals were split compared to **F_1** (Figure 45).

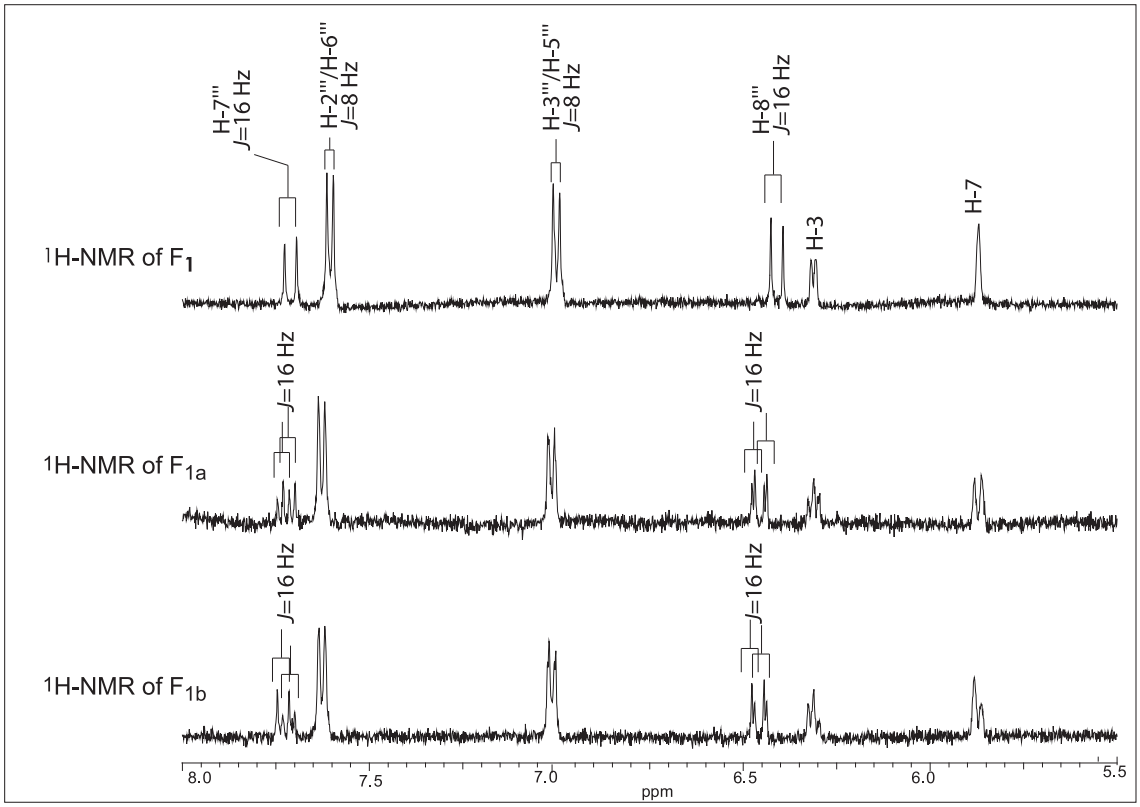


Figure 45: Detail of the aromatic signals of the LC/^{1}H-NMR analysis of **F_1**, **F_{1a}** and **F_{1b}** (expansion of Figure 44).

Signals corresponding to the double bond (H-7''' and H-8''') resulted in two superposed doublets with coupling constants *J*=16 Hz. Proportions between the doublets varied between **F_{1a}** and **F_{1b}**. The signal corresponding to H-7 was also split, and the proportion of the resulting peaks varied between **F_{1a}** and **F_{1b}**. This suggested instability in the flow probe cell.

To follow this degradation, the peak corresponding to **F_{1b}** was analysed at different time intervals, the flow being stopped during the whole operation (Figure 46). Progressive splitting of the doublets corresponding to the double bond (H-7''' and H-8''') and of the signal corresponding to H-7 in the aucubin moiety were observed, giving signals of the same importance as the original peak after 17 hours in the flow probe cell. This clearly confirmed the instability of **F_{1b}** in the probe cell which appeared to transform into **F_{1a}**.

However, the evolution of the spectrum with time also included the apparition of a second peak corresponding to H-6'' in $\mathbf{F_1}$ at δ 1.20, indicating at the same time a slow transformation into $\mathbf{F_1}$. The small proportion of this signal in comparison with the signal present initially at δ 1.30 demonstrated a first transformation of $\mathbf{F_{1b}}$ into $\mathbf{F_{1a}}$ followed by a slow transformation into $\mathbf{F_1}$.

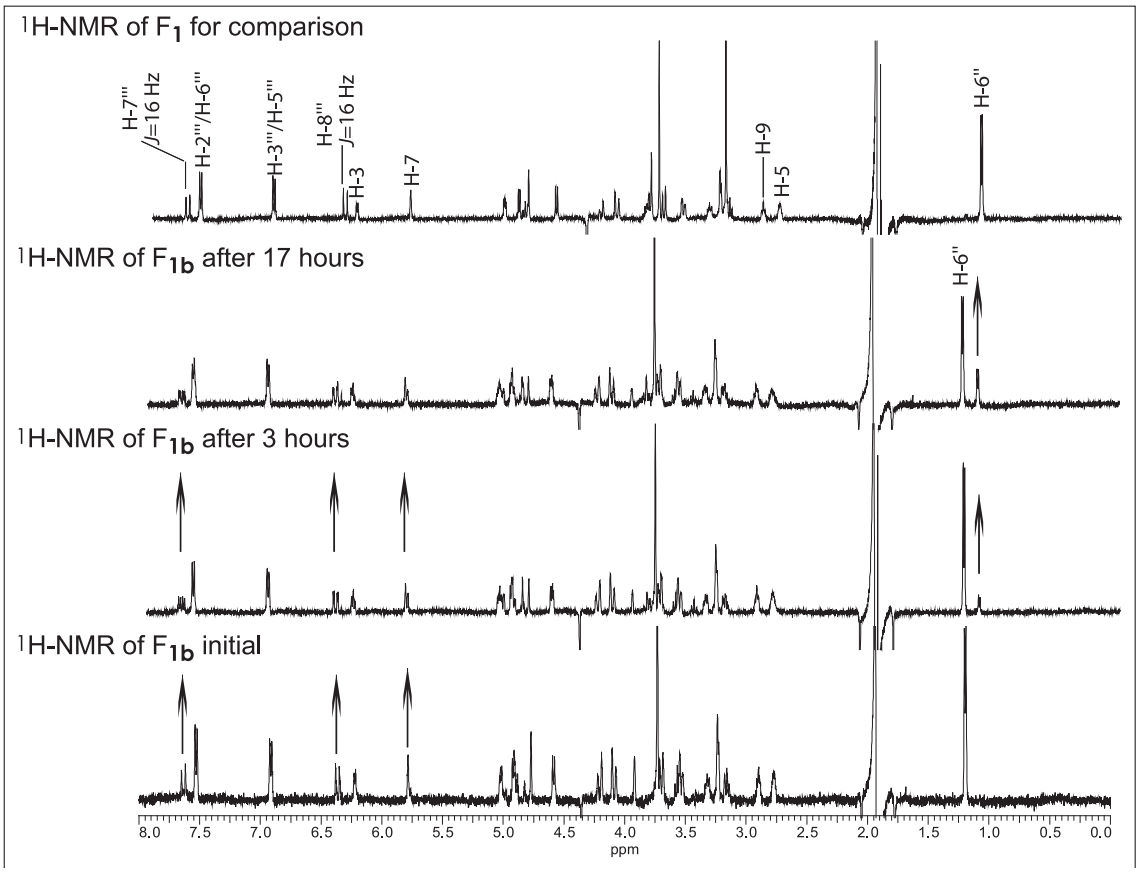


Figure 46: Analyses of the peak $\mathbf{F_{1b}}$ initially and after 3 and 17 hours in the probe

These experiments led to the conclusion that the transformations were related to the rhamnose moiety: a shift of the H-6'' signal, a slight shift of the signals corresponding to the neighbouring protons of the cinnamoyl moiety double bond (Figure 45), and the apparition of signals at δ 3.5 and 4.0.

To confirm this, a LC/WET-TOCSY irradiation (see 5.4.5) was thus carried out selectively on the peak corresponding to H-6''of the rhamnose, that is δ 1.20 in $\mathbf{F_1}$ and δ 1.30 in $\mathbf{F_{1b}}$ (Figure 47).

As previous LC/^{1}H-RMN measurements had demonstrated that the compounds corresponding to the peaks $\mathbf{F_{1a}}$ and $\mathbf{F_{1b}}$ were unstable and interchanged in the cell flow probe (Figure 45 and Figure 46), the WET-TOCSY selective irradiation on δ 1.30 from peak $\mathbf{F_{1b}}$ gave signals from both peaks $\mathbf{F_{1a}}$ and $\mathbf{F_{1b}}$.

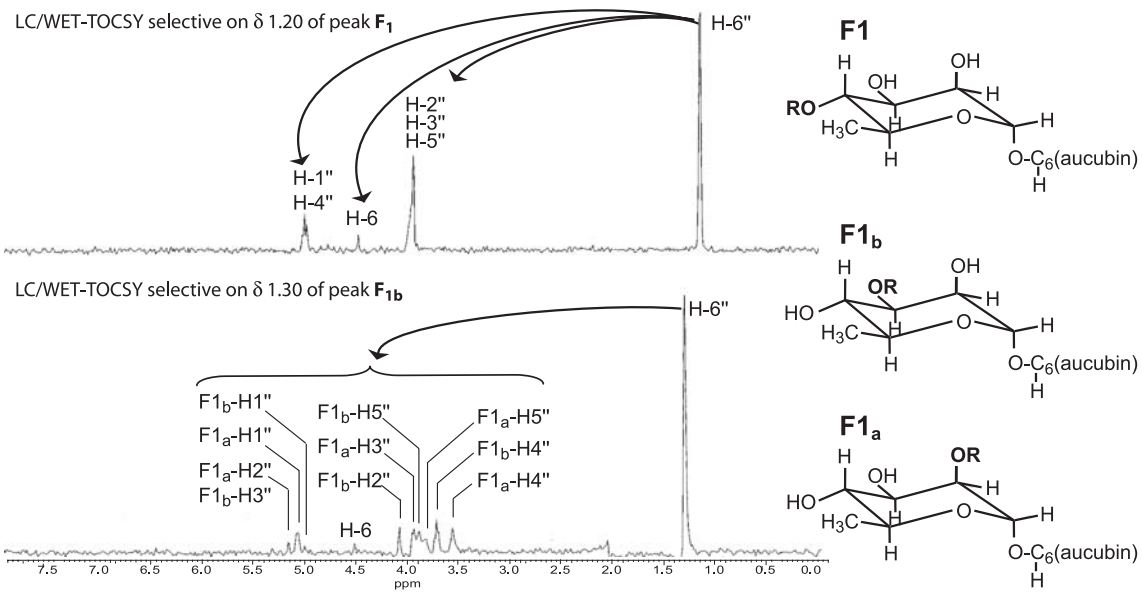


Figure 47: LC/WET-TOCSY selective irradiations of δ 1.20 of $\mathbf{F_1}$ and δ 1.30 of $\mathbf{F_{1b}}$

Irradiation of the signal at δ 1.20 in $\mathbf{F_1}$ indicated correlations with signals at δ 3.8-4.0 (H-2'', H-3'', H-5'') and 4.9-5.0 (H-1'', H-4'') of the rhamnose, and with δ 4.50 (H-6) of the iridoid moiety, which is in accordance with the shifts observed for a 4-acylated rhamnose unit (Otsuka et al., 1990, 1991b). Irradiation of the signal at δ 1.30 in $\mathbf{F_{1b}}$ indicated much more correlations with signals at δ 3.55, 3.72, 3.84, 3.89, 3.94, 4.09, 4.50, 5.00, 5.09 and 5.16. These signals could be attributed to two rhamnose moieties esterified in 2'' and 3'' (Otsuka et al., 1990, 1991b). The instability would thus consist of a transacylation on the three hydroxyls of the rhamnose at C-2'', C-3'' and C-4''.

Abdallah *et al.* (1994) reported that migration and scission of acyl groups of partially acylated carbohydrates often occurs under acidic and basic conditions, or by heating the compounds.

This would explain the downfield shift of H-6'' in the 2'' and 3'' esters. Several examples of cinnamic derivatives esterified on carbon 2'' and 3'' of a rhamnose moiety display such a deshielding effect on H-6'' (Otsuka *et al*., 1990).

3.2.6.3 In-mixture NMR analyses

F_1, **F_{1a}**, and **F_{1b}** were submitted to complete in-mixture NMR analyses recorded in methanol-*d4* in order to confirm this hypothesis (Figure 48 and Figure 49).

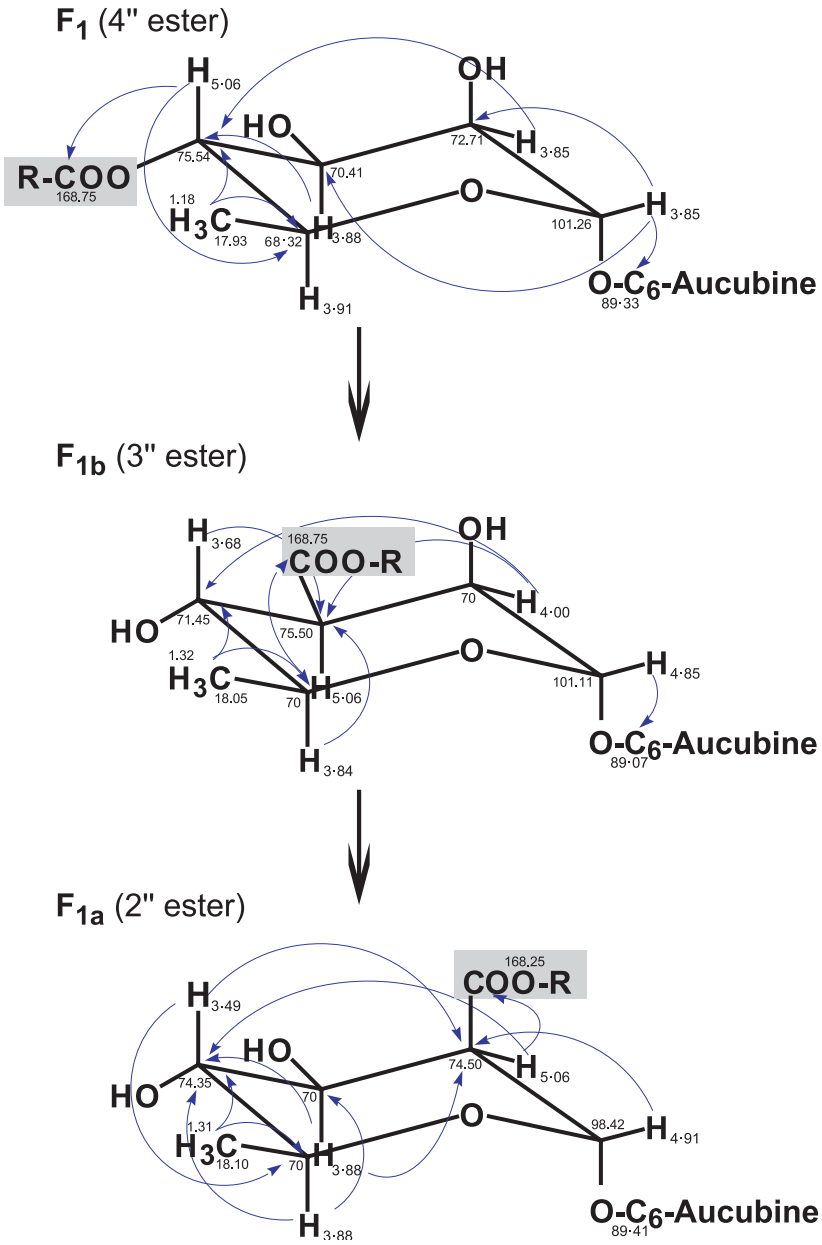


Figure 48: gHMBC correlations between the rhamnose moiety signals for **F_1**, **F_{1a}** and **F_{1b}**

2D-NMR analyses, particularly gHMBC-NMR correlations (Figure 48) and dqCOSY-NMR (Figure 49), confirmed the hypothesis of the presence of three esters at positions 2'', 3'' and 4'' of the rhamnose moiety.

Even if not all correlations could be observed, there was enough evidence to attribute carbon and proton signals to the rhamnose moieties for the three esters **F_1**, **F_{1a}** and **F_{1b}**.

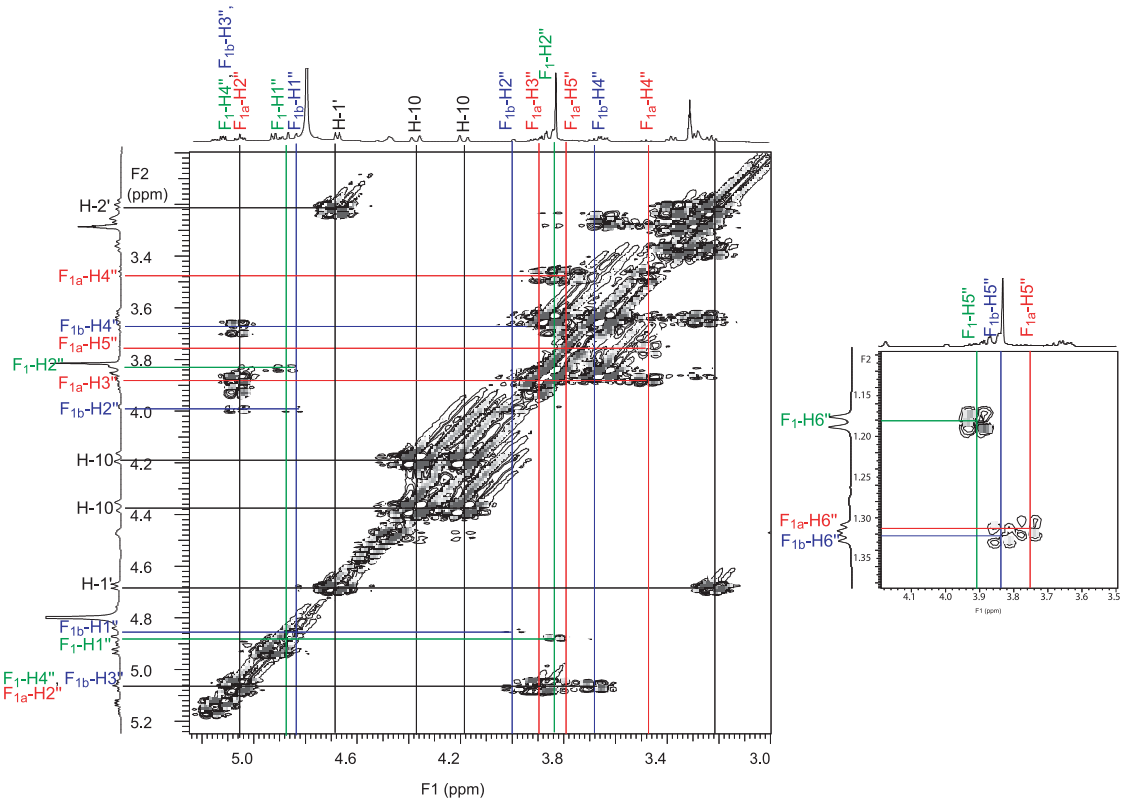


Figure 49: dqCOSY-NMR spectrum of **F_1**, **F_{1a}** and **F_{1b}** in-mixture (in methanol-*d4*, TMS as internal standard).

G_1, **G_{1a}** and **G_{1b}** were also submitted to complete in-mixture NMR analyses. As expected from its mass and UV spectra, the structure of **G_1** was confirmed to be the *trans* form corresponding to **G_2**.

Moreover, gHMBC and dqCOSY correlations enabled attribution of the ^{1}H and ^{13}C signals of the three different esters **G_1/G_{1a}/G_{1b}.**

3.2.6.4 Structure confirmation

Hydrolysis was performed on $\mathbf{F_1}$ and $\mathbf{G_1}$ in order to confirm that they were derivatives of aucubin. Considering the instability of the aucubigenin, two-step hydrolysis leading to aucubin was preferred to direct acidic hydrolysis that would lead to an unstable aglycone. Alkaline hydrolysis performed on the mixtures of $\mathbf{F_1/F_{1a}/F_{1b}}$ and $\mathbf{G_1/G_{1a}/G_{1b}}$ was followed by enzymatic hydrolysis with hesperidinase, leading first to the elimination of the methoxycinnamoyl moiety and then to the loss of the rhamnose unit (complete procedure detailed in 5.6.2.2 and 5.6.2.3).

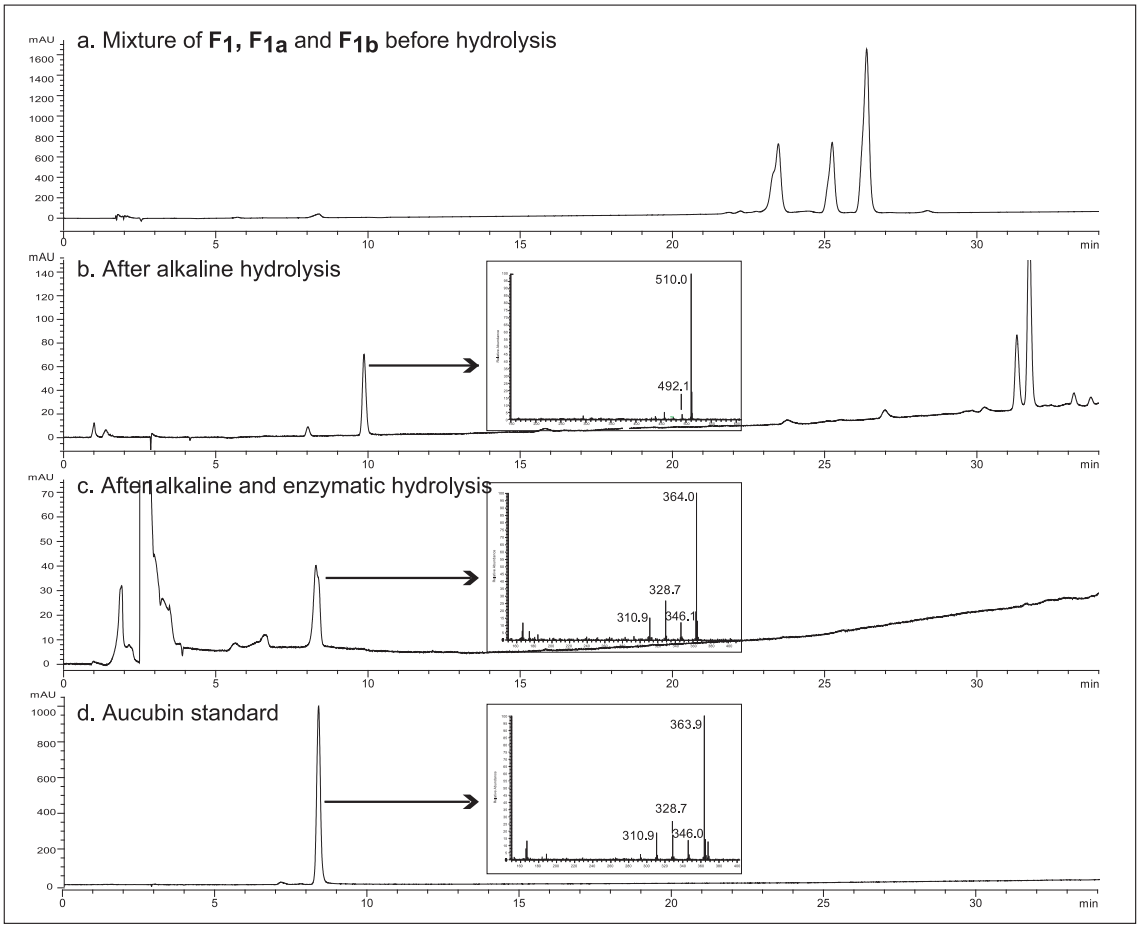


Figure 50: Comparative LC/APCI-MS of $\mathbf{F_1}$ in the positive ion mode with ammonium acetate **a.** before hydrolysis, **b.** after alkaline hydrolysis, **c.** after alkaline and enzymatic hydrolysis, **d.** comparison with aucubin standard. Chromatographic conditions: Lichrosorb C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of acetonitrile-water (0.5:99.5 to 30:70 in 20 min, to 100:0 at 40 min), flow rate 1 ml/min.

The products obtained were compared with aucubin standard by LC/APCI-MS in the positive ion mode with ammonium acetate (illustrated for **F_1** in Figure 50). For both **F_1** and **G_1**, mass spectra were identical for the hydrolysis products (Figure 50c) and the aucubin standard (Figure 50d), with molecular ions at *m/z* 364 $[M+NH_4]^+$ and *m/z* 346 $[M+NH_4-H_2O]^+$. Moreover, molecular ions at *m/z* 510 $[M+NH_4]^+$ and *m/z* 492 $[M+NH_4-H_2O]^+$ were observed for the intermediate product of the alkaline hydrolysis (Figure 50b), which is in accordance with the structure of the 6-*O*-α-L-rhamnopyranosyl aucubin or sinuatol (Vesper and Seifert, 1994).

Finally, acetylation was performed on **G_1**, **G_{1a}** and **G_{1b}** by stirring the mixture with acetic anhydride and pyridine during 48 hours under nitrogen atmosphere.
The acetylated **G_1** was isolated by semi-preparative liquid chromatography on a Bondapack C-18 column in a radial compression Waters PrepLC 25 mm module with an acetonitrile-water gradient (30:70 to 100:0 in 45 minutes).
It was then analysed by LC/ESI-MS in the positive ion mode: a molecular ion was observed at *m/z* 994.1 $[M+H_2O]^+$, corresponding to an addition of 7 acetyl groups, which is in concordance with the structure determined previously (Figure 51).

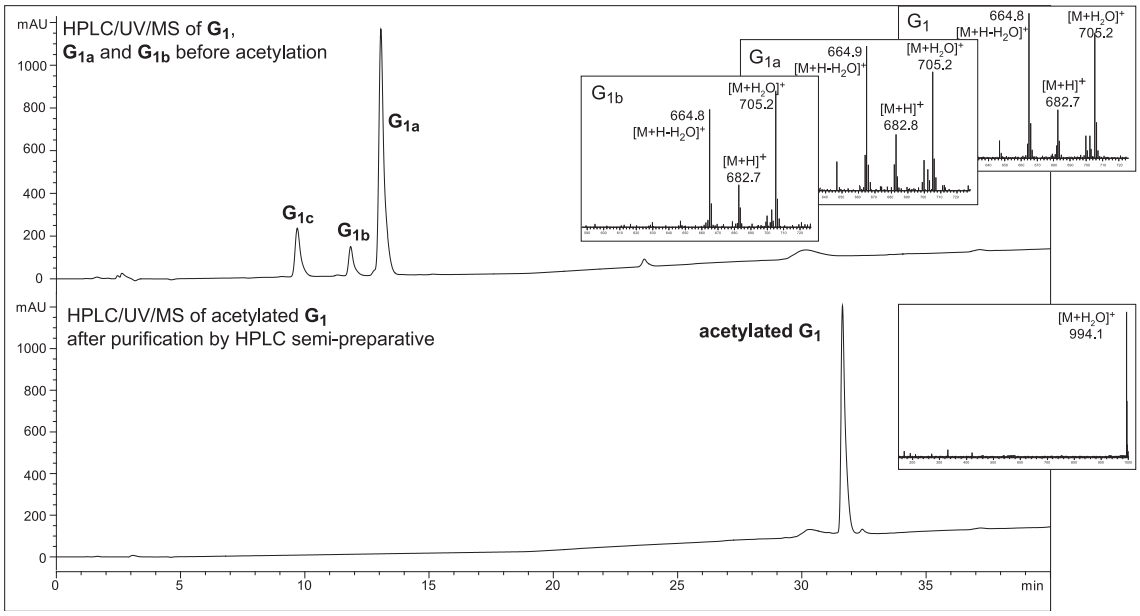
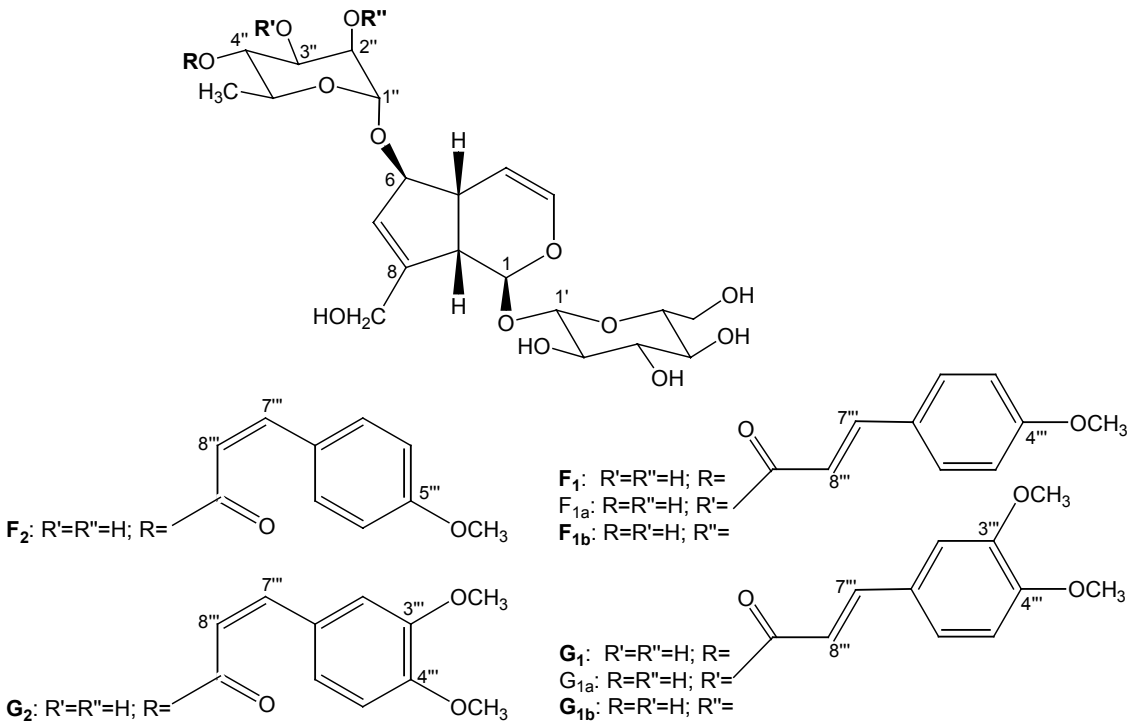


Figure 51: LC/UV/ESI-MS of **G_1** before and after acetylation. Chromatographic conditions: Lichrosorb C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of acetonitrile-water (20:80 to 32:68 in 15 min, to 100:0 at 40 min), flow rate 1 ml/min.

Acetylated G_1 was also analysed by NMR, confirming the structure of **G_1**.

In conclusion, eight aucubin derivatives could be characterized.



F_{1a}, **F_{1b}**, **G_{1a}** and **G_{1b}** must be artifacts, as they were not detected in the total extract, and "appeared" in the fractions during processing.

F_{1a} and **G_{1a}** have already been described in *Verbascum undulatum* (Magiatis *et al*., 2000). To our knowledge, the other compounds are new natural products.

3.2.6.5 Discussion

Once the origin of the light induced instability of the cinnamoyl moiety was determined, the *cis* and *trans* forms of the derivatives could be isolated by performing all further manipulations in darkness: **F_1** was separated from **F_2**, and **G_1** from **G_2**.

The *cis* forms **F_2** and **G_2** could be purified. Another type of instability appeared, however, for the *trans* forms **F_1** and **G_1**.

LC/UV/NMR analysis of **F_1**, **F_{1a}** and **F_{1b}** permitted to assign the ^{1}H and ^{13}C NMR signals obtained by analyses of the mixture to the three forms and to attribute the instability to a transacylation of the cinnamoyl moiety on the rhamnose unit.

A search of the literature revealed a similar case: Helfrich and Rimpler (1999) could not separate the 2''- and 3''-*O*-*trans*-cinnamoyl esters of 6-*O*-α-L-rhamnopyranosyl-catalpol. The authors suggested that to avoid a transacylation, fractions should be either diluted with water and directly lyophilised, or placed in the rotary evaporator at a temperature inferior to 30°C.
As mentioned above, Abdallah *et al*. (1994), in turn, asserted that "facile migration and scission of acyl groups of partially acylated polyhydric alcohols or carbohydrates often occurs under acidic and basic conditions or merely by heating or melting the compounds".

In the case of our aucubin ester derivatives, this transacylation reaction seems to occur more easily than described above, as the transformation could be observed directly in the LC/NMR probe cell, at a temperature inferior to 30°C and in darkness.

Concerning the potential therapeutic interest of these compounds, several similar products have displayed interesting antiinflammatory properties in different models. A "(methoxycinnamoylrhamnosyl)catalpol mixture" displayed a significant inhibition of TPA-induced ear oedema (Recio *et al*., 1994). This undefined mixture might correspond to mixtures of *cis*/*trans* and transacylated derivatives. The authors determined the structural features increasing the anti-inflammatory activity such as acylation of the sugar moiety rather than directly on the iridoid skeleton, and the presence of a double bond between C-7 and C-8.
More recently, scrovalentinoside (6-*O*-[2,3-di-*O*-acetyl-4–*O*-(4-methoxy-cinnamoyl)-α-rhamnopyranosyl]catalpol) and scropolioside A (6-*O*-[2,4-di-*O*-acetyl-3–*O*-(4-methoxycinnamoyl)-α-rhamnopyranosyl]catalpol), closely related to verbascoside A (compound **E_1**), were shown to possess anti-inflammatory properties in an ear swelling delayed-type hypersensitivity model (Giner *et al*., 2000). In contrast to mimengoside A (compound **A**) submitted to the same analyses, and particularly efficient in the acute models of inflammations, the iridoids were more effective in the delayed-type hypersensitivity reaction.
Moreover, a recent study demonstrated a growth stimulating activity of the human dermal fibroblast for three acylated and acetylated catalpol glycosides, indicating wound healing properties (Stevenson *et al*., 2002).

3.2.7 Structure elucidation of compounds H and I

A MPLC performed on fraction 4 obtained after a silicagel column chromatography of the extract gave two pure compounds, corresponding to the peaks at 26.2 and 17.6 minutes on the LC/UV/MS chromatogram of the methanol extract, named **H** and **I** (Figure 26 and Figure 27).

3.2.7.1 Compound H

The LC/APCI-MS analysis in the positive ion mode displayed for **H** a protonated molecule at *m/z* 610.9 $[M+H]^+$, with fragmentation showing the departure of two sugar units (ions at *m/z* 465.0 $[M+H-146]^+$ and *m/z* 303.2 $[M+H-308]^+$) suggesting the presence of a flavone diglycoside.

The UV spectrum, with a maximum at λ 286 nm (band II) and a shoulder at about λ 330 nm (band I), rather indicated the presence of a flavanone (Figure 52).

Use of shift reagents permitted to determine the substitution scheme (Markham, 1982) (see detailed procedure in 5.4.3).

A shift of + 22 nm of Band II was observed after addition of $AlCl_3$, indicating the presence of a hydroxyl group in position C-5. Addition of a base (NaOAc or NaOMe) caused no effect, showing the absence of a free hydroxyl in C-7. The absence of *o*-dihydroxyl groups on A- or B-rings could also be established

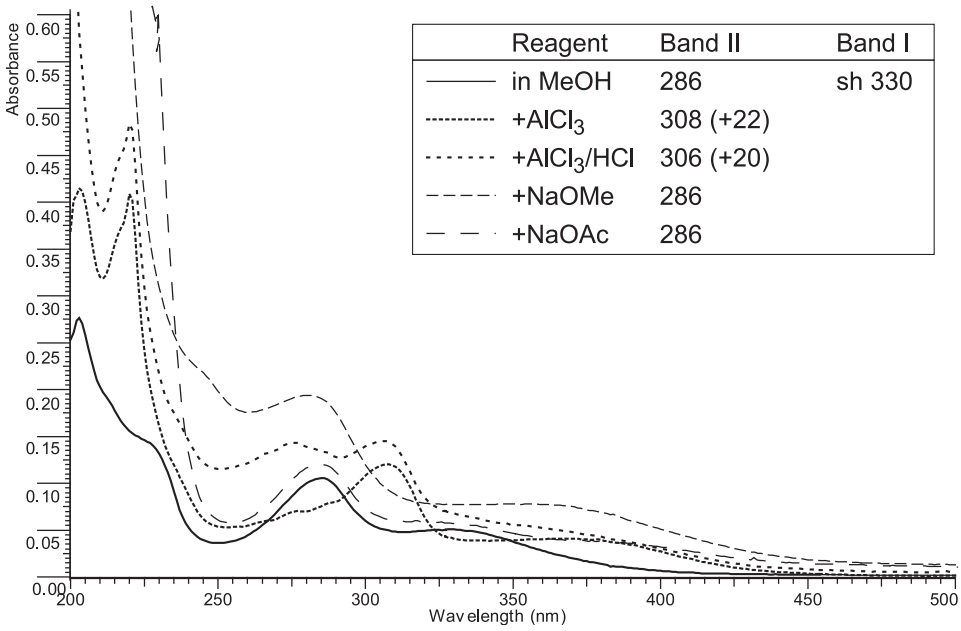


Figure 52: UV spectra of compound **H** with shift reagents

The NMR spectra were in accordance with the structure of a flavanone.

The ^{13}C spectrum (Figure 53) revealed a signal characteristic of a carbonyl at δ 198.00 and one aliphatic carbon at δ 42.21. Moreover, the presence of three quaternary carbons between δ 162 and δ 166 indicated the presence of a third oxygenated carbon on ring A, beside C-5 and C-9. The two quaternary carbons at δ 146.51 and δ 148.11 indicated two oxygenated carbons on ring B (Harborne, 1986). One methoxyl group was observed at δ 57.02.

The numerous signals between δ 66 and δ 80 and the two anomeric carbons at δ 99.61 and δ 100.75, correlating in gHSQC with protons at δ 4.97 and 4.52, confirmed the presence of two sugar units (Markham, 1982).

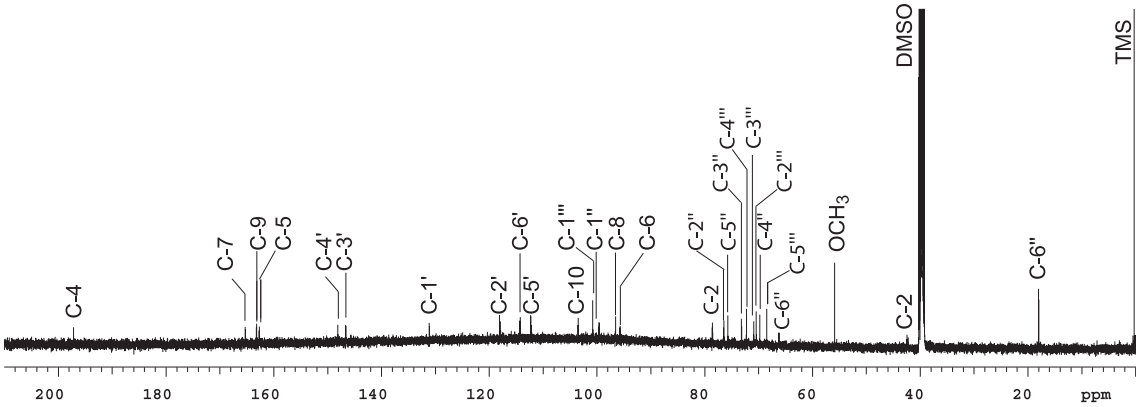


Figure 53: ^{13}C-NMR of **H** (125 MHz, in DMSO-*d6*). C-1'' to C-6'': Glc signals, C-1''' to C-6''': Rha signals.

The ^{1}H- NMR spectrum (Figure 54) revealed two geminal signals at δ 2.77 (1H, dd, *J*= 2.9, 17.0) and δ 3.28 (1H, m) corresponding to the two H-3 protons and to the proton H-2 at δ 5.50 (1H, dd, *J*= 2.9, 12.2).

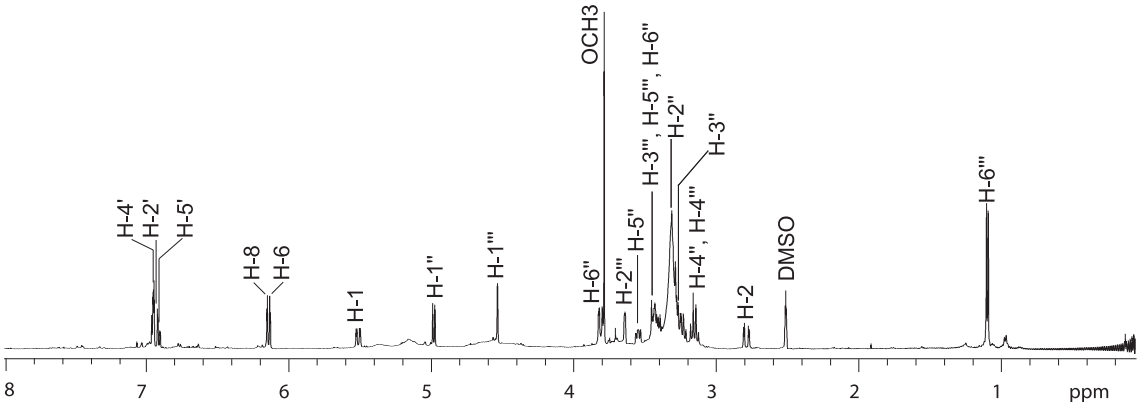


Figure 54: ^{1}H-NMR of **H** (500 MHz, in DMSO-*d6*). H-1'' to H-6'': Glc signals, H-1''' to H-6''': Rha signals.

The two *ortho*-coupled aromatic protons at δ 6.90 (1H, d, *J*=8.3 Hz) and δ 6.95 (1H, d, *J*=8.3 Hz) and the singlet aromatic proton detected at δ 6.93 (1H, s) formed an ABX system, and confirmed the disubstitution on the B-ring.
Observation of the two *meta*-coupled protons at δ 6.12 (1H, d, *J*=2.0 Hz, H-6) and δ 6.14 (1H, d, *J*=2.4 Hz, H-8) allowed attribution of the third oxygenation of the A-ring to C-7.

An acidic hydrolysis was performed. Observation of the shifts induced in the UV spectrum of the aglycone by addition of reagents enabled determination of the position of glycosylation (Figure 55). Unlike the glycoside, a bathochromic shift of 35 nm of band II was noted after addition of a strong (NaOMe) or weak base (NaOAc), indicating the presence of a free hydroxyl at C-7 (Bacon and Mabry, 1976). The sugar moiety was thus determined to be attached to C-7.

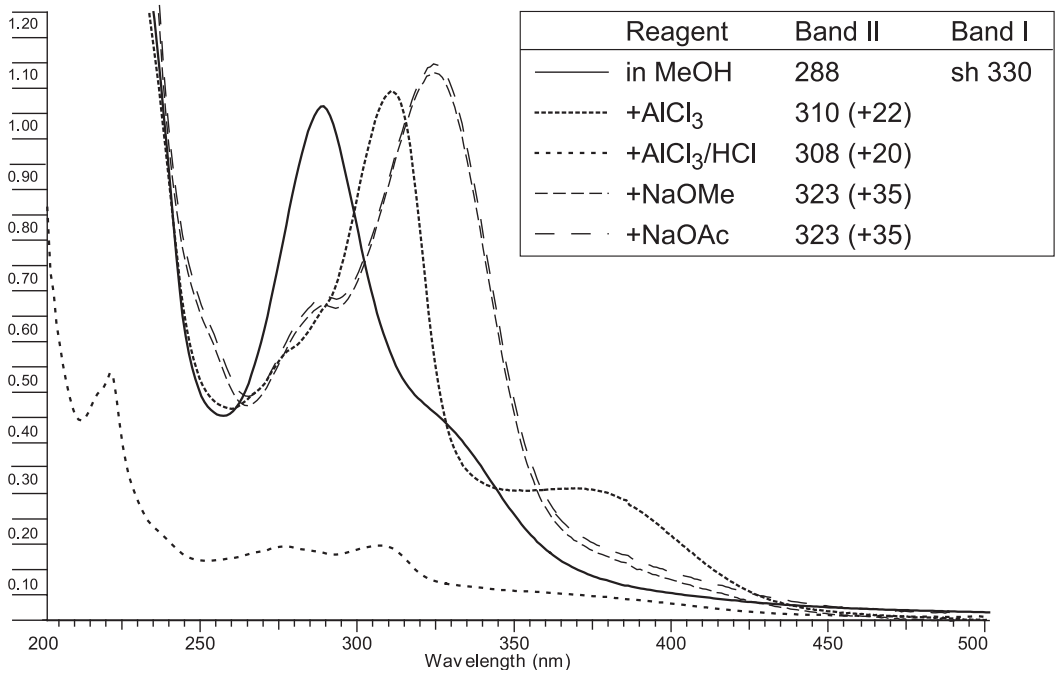


Figure 55: UV spectra of the aglycone of **H** with shift reagents

Finally, 2D NMR analyses, particularly gHMBC correlations, located the methoxyl group at C-4' (dashed lines in Figure 56). Moreover, the glycosidic part could be identified as rutinose [rhamnosyl-(α1→6)-glucose], as indicated by the gHMBC correlation between the proton at δ 4.52 (H-1''') and the carbon at δ 66.18 (C-6'') (dashed line in Figure 56).

A correlation between H-1'' and C-7 confirmed the position of the glycosylation (dashed line in Figure 56).

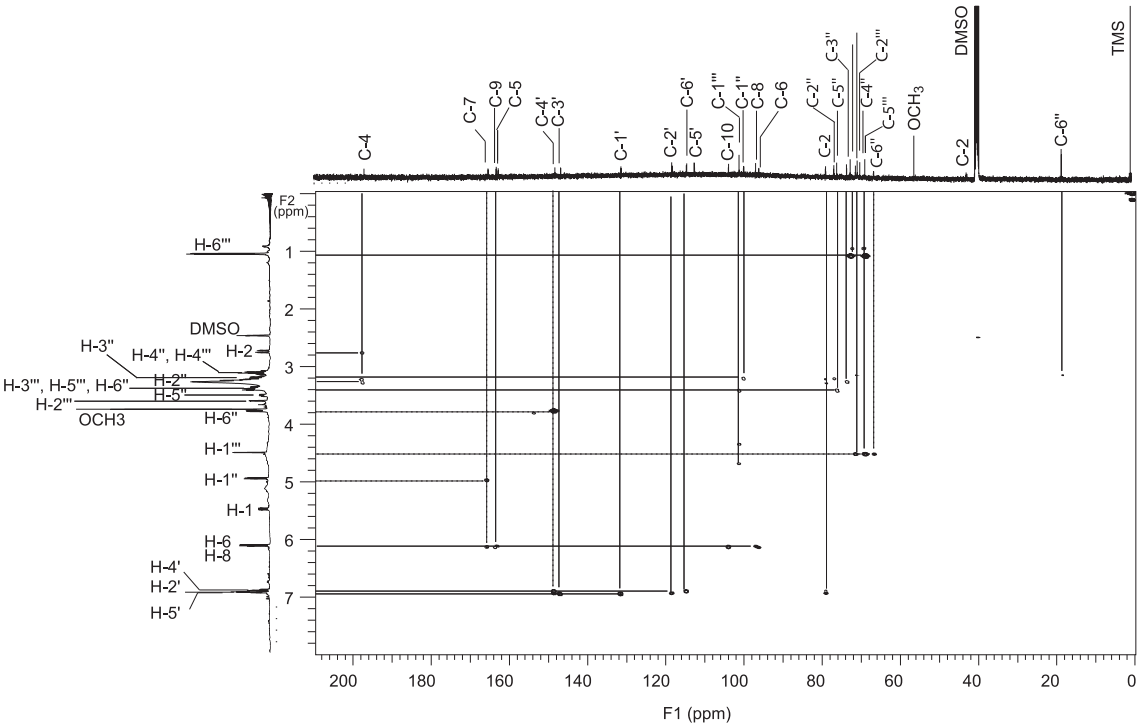
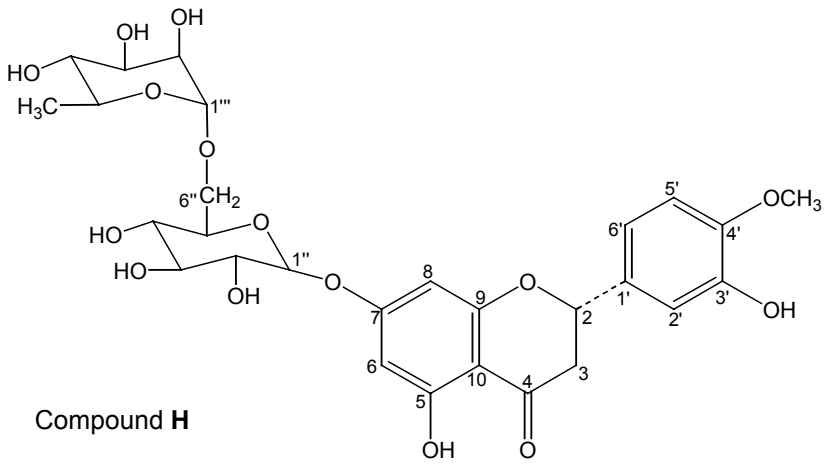


Figure 56: gHMBC NMR of **H** (500 MHz, in DMSO-*d6*, TMS as internal standard).

The coupling constant of the anomeric proton at δ 4.97 (1H, d, *J*=7.3Hz) indicated the β configuration of the glucose (Harborne, 1986). Resolution of the ^{1}H-NMR spectra was not good enough to reveal the coupling constant of the other anomeric proton. The α configuration of the rhamnose was determined by observing the chemical shifts of the carbons, which differ between α and β configuration (Agrawal *et al*., 1985, Hostettmann and Marston, 1995)

After comparison with the literature data, compound **H** was identified as the 7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl]-3', 5, 7-trihydroxy-4'-methoxyflavanone, or hesperidin. Its identity was confirmed by co-elution with hesperidin standard on a TLC plate.

Hesperidin is found in most citrus fruits and other members of the Rutaceae, but has never been isolated from a Scrophulariaceae (Rashid *et al*., 1995; Shen *et al*., 1993).



Compound **H**

3.2.7.2 Compound I

The LC/APCI-MS analysis in the positive ion mode displayed for compound **I** a protonated molecule at *m/z* 596.8 $[M+H]^+$, with fragmentation showing again the departure of two sugar units (ions at *m/z* 451.0 $[M+H-146]^+$ and *m/z* 289.1 $[M+H-308]^+$).

The UV spectrum was identical to that of **H**, showing **I** also to be a flavanone.
Observation of the UV spectra after addition of NaOMe revealed a slight intensity decrease of Band II, which might indicate the presence of two ortho hydroxyls on the B-ring. A bathochromic shift of 18 nm of Band II caused by $AlCl_3$ indicated the presence of a 5-OH (Figure 57).

Observation of the ^{1}H-NMR spectrum indicated a great similarity with compound **H**, the only difference being the absence of a methoxyl group at δ 3.80.
In the ^{13}C spectrum, the absence of the corresponding methoxylic carbon at about δ 55 was also noted. Two signals at δ 145.81 and δ 146.22 representing two oxygenated carbons indicated the presence of two hydroxyl groups on ring B. This was in accordance with the preliminary UV and mass analyses, with a mass difference of 14 between compounds **H** (MW of 610) and **I** (MW of 596).

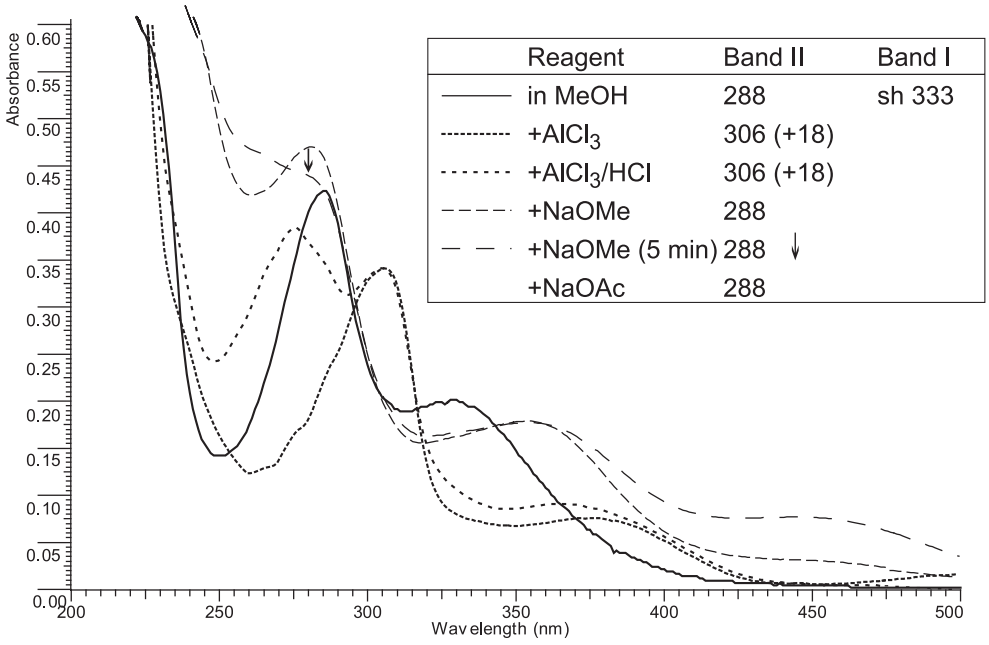
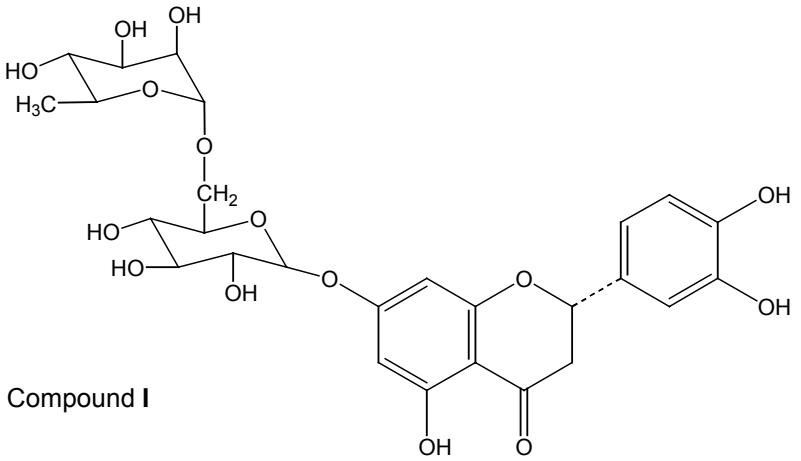


Figure 57: UV spectra of **I** with shift reagents (↓ indicates a decreased in intensity)

Compound **I** was thus identified as 7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl]-3', 4', 5, 7-tetrahydroxy-flavanone, commonly called eriocitrin. It has been isolated from *Citrus* spp. (Rutaceae), *Mentha piperita* (Lamiaceae) and *Myoporum tenuifolium* (Myoporaceae), but has never been found in a member of the Scrophulariaceae (Tomas *et al*., 1985).



Compound **I**

3.2.7.3 Discussion

Although flavones are frequently encountered in Scrophulariaceae, to our knowledge, no flavanone has been previously isolated in the family.

Both hesperidin and its aglycone hesperetin have been reported to possess a wide range of pharmacological properties, including antimicrobial, anticarcinogenic, antioxidant, anti-inflammatory, immunomodulatory effects and a protective action on the capillaries in case of vascular disorders (Garg *et al*., 2001).

A protective effect of hesperidin and eriocitrin has been observed in the oxidative stress by the diabetic rat (Miyake *et al*, 1998).

3.3 Phytochemical investigation of the methanol extract of Jamesbrittenia elegantissima

3.3.1 LC/UV/MS analysis

Jamesbrittenia elegantissima O.M. Hilliard presented in the biological screening the same activity as *J. fodina* O.M. Hilliard against *Cladosporium cucumerinum*, *Candida albicans* and *Bacillus subtilis* (Table 1).
Moreover, the reactive spot had the same R_f on TLC. The presence of the active mimengoside A was thus suspected in the methanol extract of *J. elegantissima*.
Comparative LC/DAD-UV/APCI-MS analyses of both polar extracts were undertaken in order to identify possible common compounds (Figure 58).
Different chromatographic conditions, with an acetonitrile-water gradient, were applied for a better resolution of *J. elegantissima* peaks.

Peaks at retention times 33.5 and 37.7 minutes were observed in the extracts of the two species. In both cases these peaks displayed pseudomolecular ions at *m/z* 1146.8 $[M-H+adduct]^-$, *m/z* 1103.9 $[M-H]^-$, and *m/z* 1114.7 $[M-H+adduct]^-$, *m/z* 1071.9 $[M-H]^-$ respectively. The nature of the adduct was not determined, but the mass corresponding to both peaks had been measured in other conditions from *J. fodina* extract (see paragraph 3.2.1.3.1) and corresponded to the data of mimengosides B and A. Mimengoside A was thus determined to be the active constituent of *J. elegantissima*.
Compound C (verbascoside), with a retention time of 11.1 minutes and a deprotonated molecule at *m/z* 623.5 $[M-H]^-$, was also detected in *J. elegantissima*.

The compounds with retention times of 10.4 (**L**), 11.4 (**K**) and 14.6 (**J**) minutes were detected in *J. elegantissima* but not *J. fodina* (Figure 58).

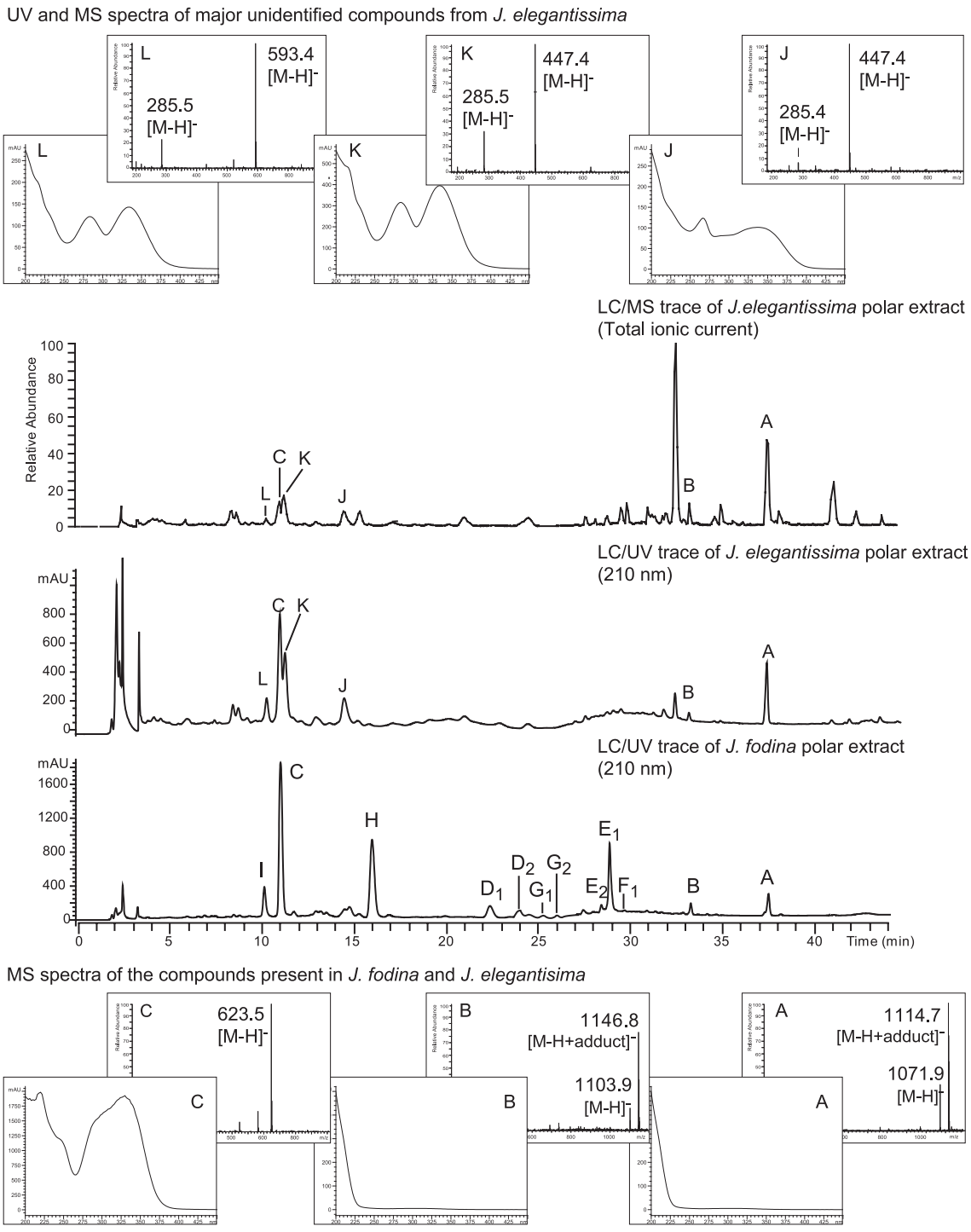


Figure 58: Comparative LC/DAD-UV/APCI-MS analysis of the methanol extract of *J. fodina* and *J. elegantissima* in the negative ion mode. Chromatographic conditions: Symmetry C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of acetonitrile:water (15:85 to 22:78 in 6 min, 22:78 to 24:76 in 16 min, 24:76 to 75:25 in 18 min, 75:25 to 100:0 in 5 min), injection of 50 μg of the extracts, flow rate 1 ml/min. UV spectra were recorded between 200 and 450 nm.

The UV spectrum of the compound **L** at R_t 10.4 minutes suggested the presence of a flavone or flavonol, with two maxima at λ 280 and 330 nm (Markham, 1982). Its mass spectrum displayed a pseudomolecular ion at *m/z* 593.4 $[M-H]^-$ with a fragment at *m/z* 285.5 $[M-H-308]^-$ representing the aglycone after the departure of two sugar units, suggesting the presence of a diglycoside.

A similar UV spectrum was observed for compound **K** at R_t 11.4 minutes and its LC-MS analysis showed pseudomolecular ions at *m/z* 447.3 $[M-H]^-$ and 285.4 $[M-H-162]^-$, indicating the departure of one sugar unit.

The compound **J** at R_t 14.6 minutes displayed a different UV spectrum with two maxima at λ 267 and 350 nm corresponding again to a flavone or a flavonol (Markham, 1982). A molecular ion at *m/z* 447.4 $[M-H]^-$ and a fragment at 285.5 $[M-H-162]^-$ identical to compound **K** were observed.

Isolation of these three products was undertaken.

3.3.2 Fractionation by CPC and isolation of the major compounds

3.3.2.1 Liquid-liquid partition of the polar extract of *Jamesbrittenia elegantissima*

A liquid-liquid partition of the extract was performed with butanol-water in order to separate the very polar products (peaks appearing before 5 minutes in Figure 58) from the compounds to be isolated. 12 g of the methanol extract gave 4.4 g of butanol fraction.

3.3.2.2 Adjustment of the CPC conditions

Centrifugal partition chromatography was chosen as the separation technique for products **J**, **K** and **L**. This all-liquid technique appeared preferable than a classical silicagel column that would retain the very polar flavonoid compounds.

A series of usual CPC solvent systems were tested by TLC (Hostettmann *et al*., 1998). 10 mg of the extract were partitioned between 1 ml of both phases of each system. Both phases were then spotted on a TLC and eluted in the organic phase. A suitable solvent system should have the following characteristics:

- Equal amounts of the products to be separated in both phases (partition coefficient approaching 1)
- R_f of the products between 0.2 and 0.5
- Roughly equal volumes of upper and lower phase and short settling time

The systems tested were adjusted to improve partition and solubility of the compounds. The partition coefficient was measured with more precision by comparative LC/UV analyses of the inferior and superior phases. The ratio of the area under the curve (AUC) for each peak in both solvents should be close to one.

The selected system was composed of $CHCl_3$-MeOH-^{i}PrOH-H_2O (7:6:3:5). The partition and R_f of the three compounds tested by TLC was good. The partition coefficient calculated by LC/UV measures was not ideal for compound **L** (7.7), but it was acceptable for **J** (2.1) and **K** (2.4) (Figure 59).

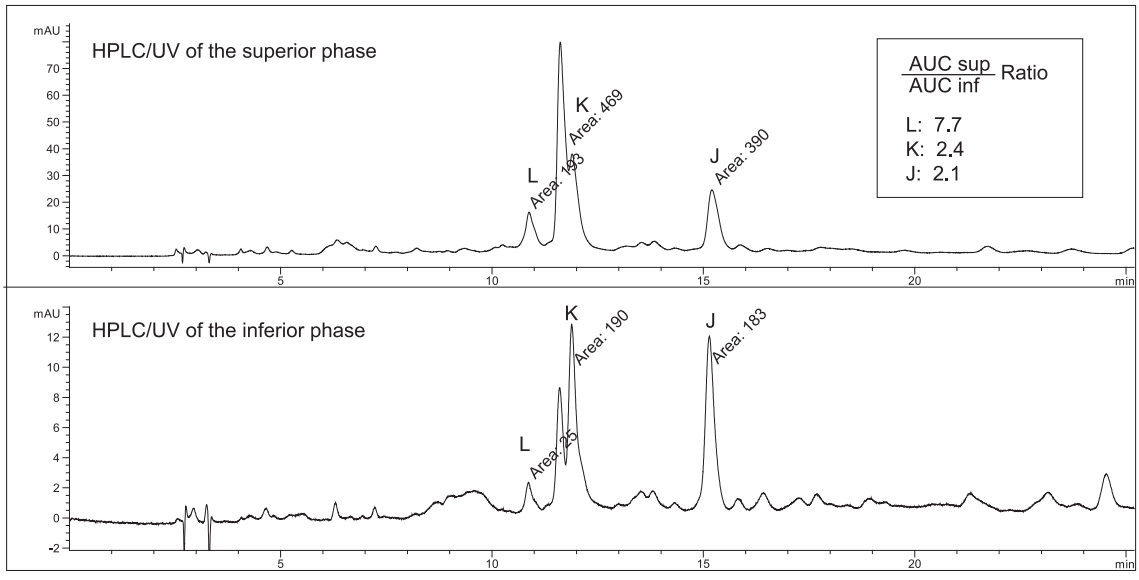


Figure 59: Comparative LC/UV of the superior and the inferior phases of the solvent system $CHCl_3$-MeOH-^{i}PrOH-H_2O (7:6:3:5) after solubilisation of the butanol fraction of *J. elegantissima*. Chromatographic conditions identical to Figure 58.

3.3.2.3 Isolation of compounds J, K, L

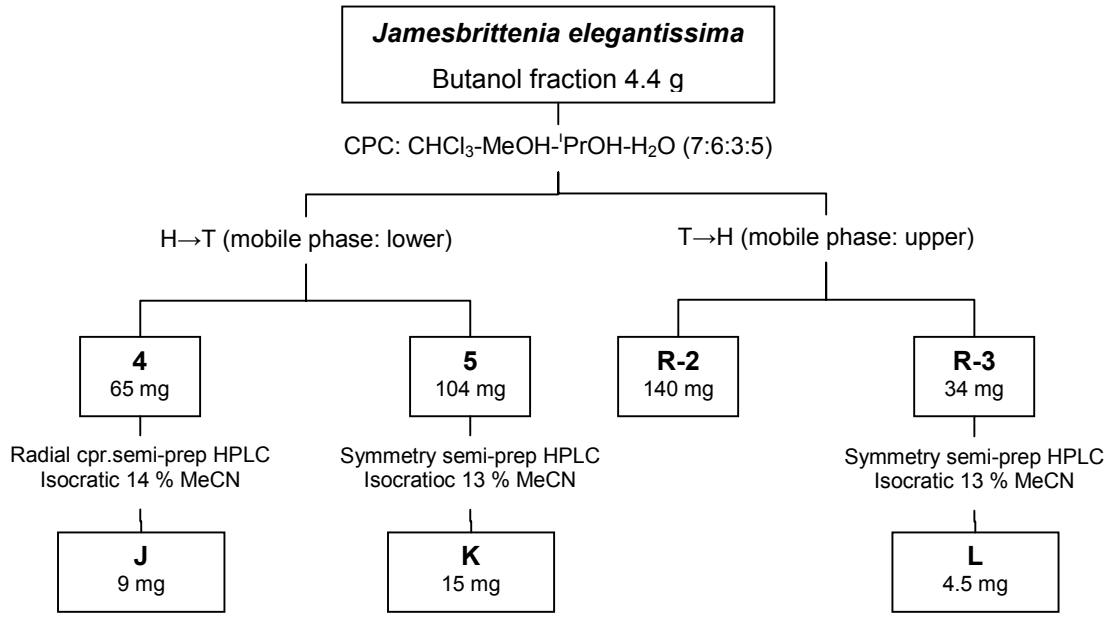


Figure 60: Isolation scheme of compounds **J**, **K**, **L**

The CPC was first run in the "head to tail" mode (H→T), with the lower phase as the mobile phase. Fractions 1 to 7 were collected. Then the mode was changed to "tail to head" (T→H), with the upper phase as the mobile phase. Fractions R-1 to R-3 were collected (Figure 61 and Figure 62). See 5.3.2 for details.

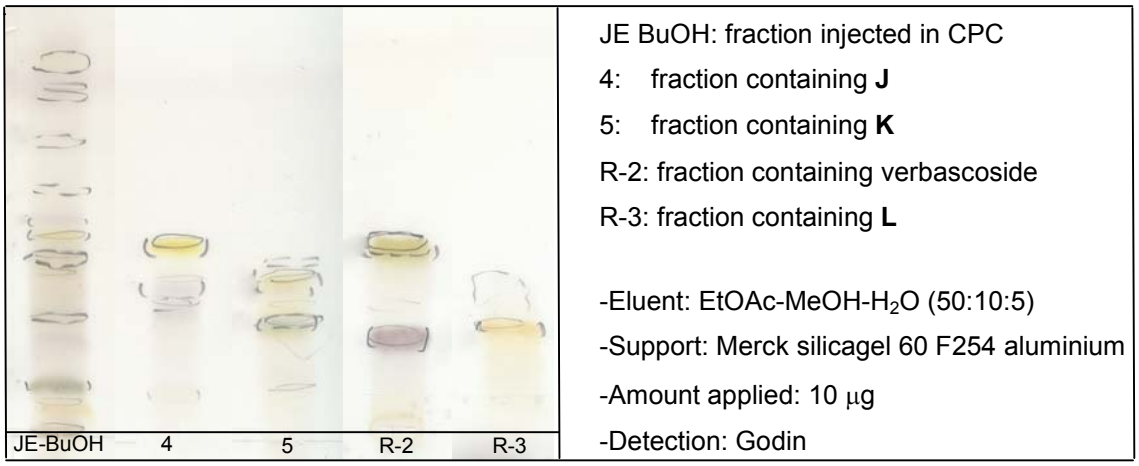


Figure 61: TLC of the interesting fractions of the CPC separation

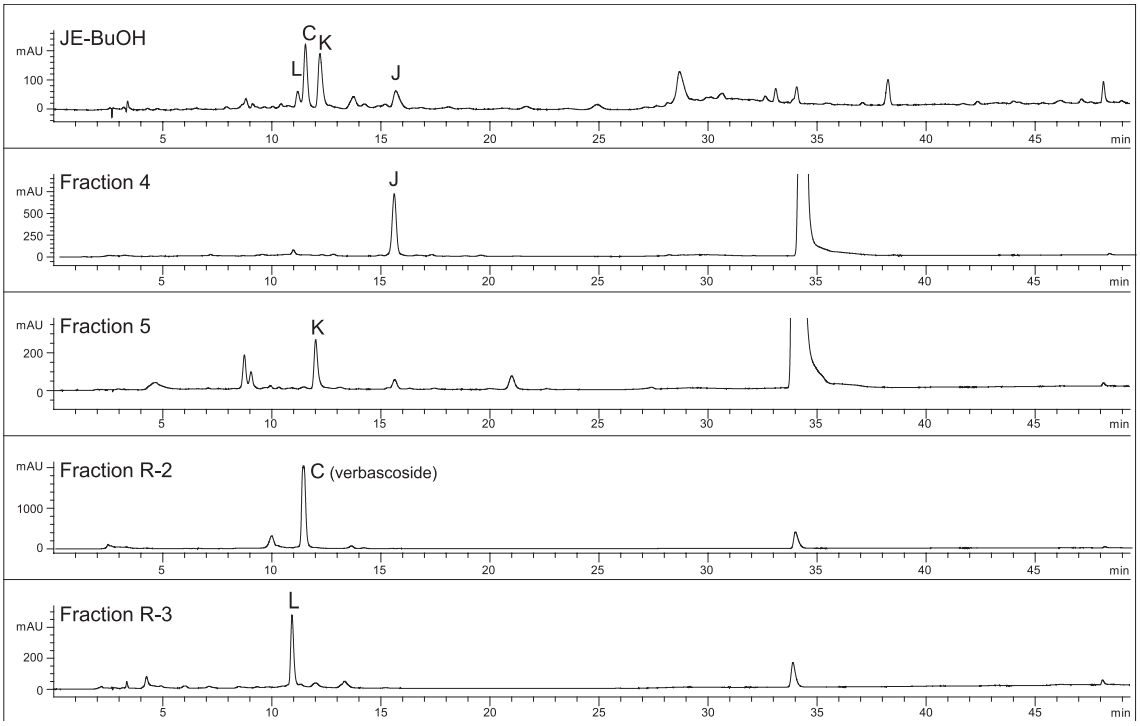


Figure 62: LC/UV of the interesting fractions of the CPC separation. Chromatographic conditions identical to Figure 58.

Semi-preparative LC of fraction 4 with a Bondapack C-18 column in a radial compression module led to the pure compound **J**.

Compound **K** and **L** were isolated by means of semi-preparative HPLC with a preparative Symmetry column of fractions 5 and R-3 (Figure 60).

3.3.2.4 Structure elucidation of compound J

The mass observed in LC/APCI-MS was confirmed by DCI-MS of the pure compound with ammonia as reagent gas, with pseudomolecular ions at *m/z* 476.1 $[M+NH_4]^+$, *m/z* 449.3 $[M+H]^+$ and *m/z* 286.9 $[M+H-162]^+$, indicating the loss of a hexose moiety.

As mentioned above, the UV spectrum indicated the presence of a flavone or a flavonol. Observation of the UV spectra with the use of shift reagents enabled determination of the flavonoid substitution scheme (Markham, 1982).

A shift of + 45 nm of Band I was observed after addition of $AlCl_3$, indicating the presence of a hydroxyl group in position C-5.

Addition of the strong base NaOMe caused a shift of + 50 nm, showing the presence of a hydroxyl in C-4', and the appearance of a third band at 325 nm indicated the presence of a 7-OH. The bathochromic shift of 22 nm caused by the weak base NaOAc confirmed substitution by a hydroxyl group at C-7 (Bacon and Mabry, 1976).

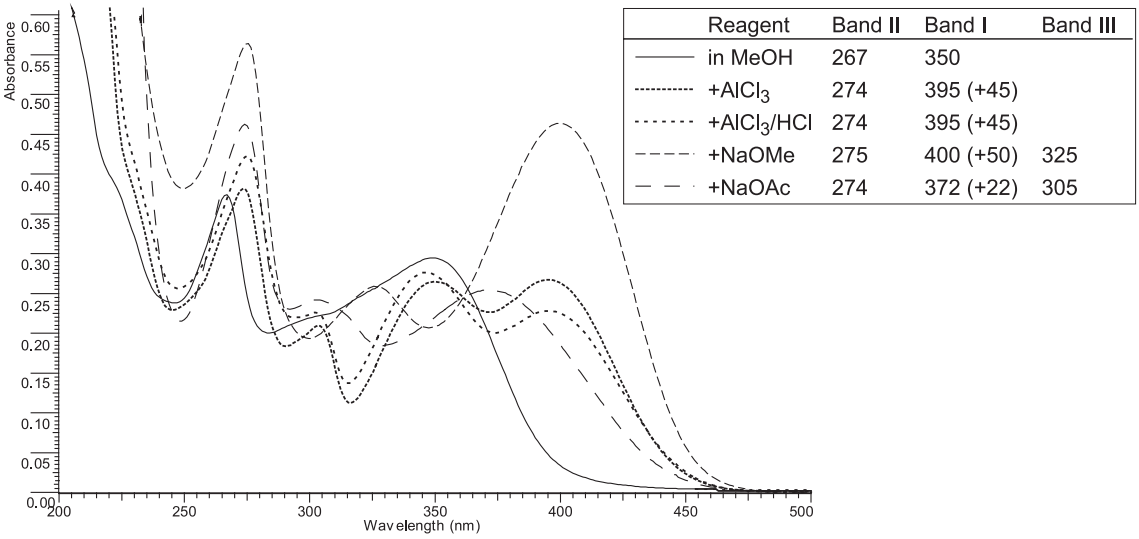


Figure 63: UV spectra of **J** with shift reagents

The NMR spectra were in accordance with the structure of a flavonol.

The ^{13}C spectrum (Figure 64) revealed a signal characteristic of a carbonyl at δ 179.40, five oxygenated aromatic carbons at δ 158.59, 158.98, 161.59, 163.01 and 167.11, signals at δ 132.26 and 135.47 representing non-oxygenated (or oxygenated with an *ortho* or *para* oxygenation (*o*/*p*)) aromatic signals, and two non oxygenated aromatic carbons with a *o*/*p* oxygenation at δ 105.43 and 116.09 (Harborne, 1986).

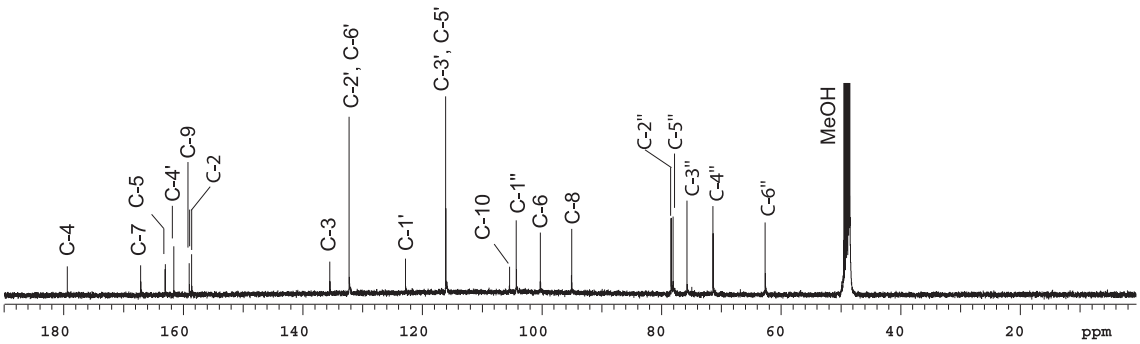


Figure 64: ^{13}C-NMR of **J** (125 MHz, in methanol-*d4*).

The five signals between δ 62 and δ 80, correlating with proton signals between δ 3.2 and 3.8 in the gHSQC spectrum, and the anomeric carbon at δ 104.29, correlating with proton at δ 5.22 (1H, d, *J*=7.7 Hz) confirmed the presence of one hexose. Observation of the exact ^{13}C and ^{1}H shifts and of the coupling of the anomeric proton indicated a β-glucose (Harborne, 1986).

In the ^{1}H-NMR spectrum (Figure 65), two signals corresponding to four *ortho*-coupled protons at δ 8.05 (2H, d, *J*=8.8 Hz) and δ 6.88 (2H, d, *J*=8.2 Hz), linked to carbons at δ 132.26 and 116.09, confirmed the 4'-hydroxylation of the B-ring. Observation of the two *meta*-coupled protons at δ 6.18 (1H, d, *J*=1.5 Hz, H-6) and δ 6.38 (1H, d, *J*=1.5 Hz, H-8) also confirmed the third oxygenation of the A-ring at C-7.

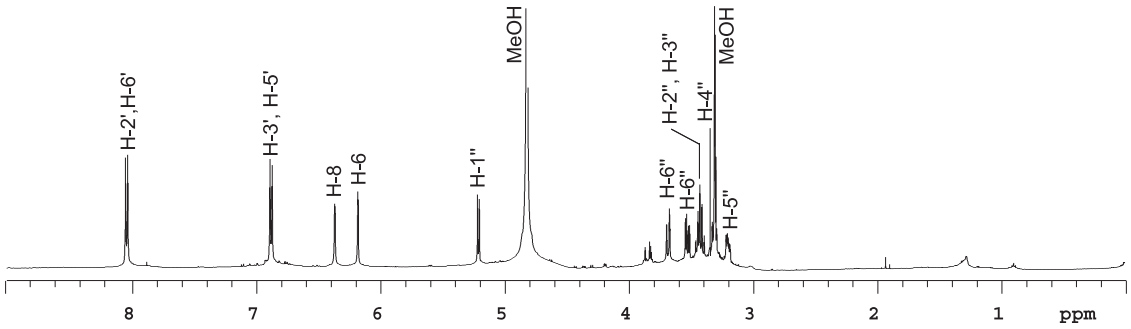
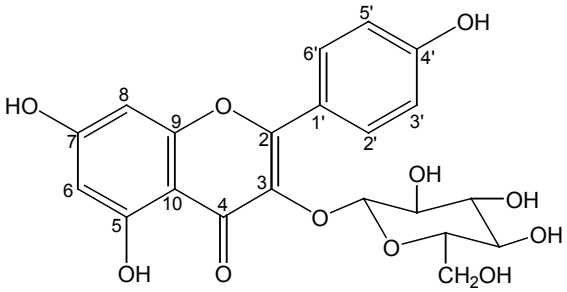


Figure 65: ^{1}H-NMR of **J** (500 MHz, in methanol-*d4*).

Considering that the hydroxyl groups at C-5, C-7 and C-4' were free, as indicated by the UV spectra in Figure 63, the sugar unit had to be attached to C-3. The attachment position at C-3 was confirmed by observation of the gHMBC correlation between the anomeric proton at δ 5.22 and the carbon C-3 at δ 135.47.

Enzymatic hydrolysis was performed with β-glucosidase, leading to the aglycone kaempferol, identified by LC/DAD-UV comparison with a standard.

Compound **J** was therefore identified as 3-*O*-*β*-glucopyranosyl -4', 5, 7-trihydroxyflavone, or kaempferol 3-glucoside, also called astragalin. This flavonol and its derivatives have been isolated from *Astragalus* spp. and plants from different families. One of the first records dates from 1955 when it was isolated from the bracken fern *Pteridium aquilinium* (Dennstaedtiaceae) by Nakabayashi. It has been recently reported to possess antihistaminic properties (Kotani *et al*., 2000).



Compound **J**

3.3.2.5 Structure elucidation of compound K

The mass observed in LC/APCI-MS was confirmed by DCI-MS of the pure compound with ammonia as reagent gas, with pseudomolecular ions at *m/z* 449.0 $[M+H]^+$ and *m/z* 287.0 $[M+H-162]^+$, indicating the loss of a hexose moiety.

As for compound **J**, the substitution scheme was determined by observation of the UV spectra with the use of shift reagents (Markham, 1982).

A shift of + 33 nm of Band I was observed after addition of $AlCl_3$, indicating the presence of a hydroxyl group in position C-5. Moreover, the shift of only + 26 nm after addition of HCl to $AlCl_3$ indicated the presence of an *o*-dihydroxyl A-ring. Addition of the strong base NaOMe caused a shift of + 44 nm, showing the presence of 4'-OH; the decrease in intensity indicated two *o*-hydroxyls on the A- or B-ring. The absence of a free 7-OH was demonstrated by the absence of a third band between 320 and 335 nm.

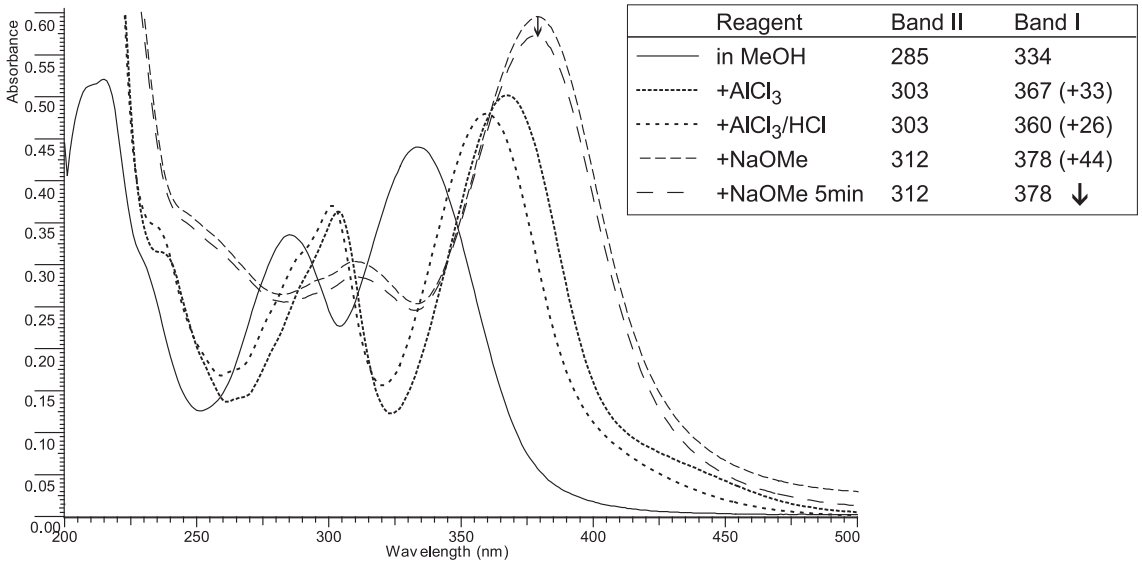


Figure 66: UV spectra of **K** with shift reagents (↓ indicates a decreased in intensity)

The NMR spectra were in accordance with the structure of a flavone.

The ^{13}C spectrum (Figure 67) revealed a signal characteristic of a carbonyl at δ 184.39, two oxygenated aromatic carbons at δ 166.77 and 162.78, five non-oxygenated (or oxygenated with an *o*/*p* oxygenation) aromatic signals at δ 152.75, 151.75, 147.92, 131.86 and 129.56, and four non-oxygenated aromatic carbons with an *o*/*p* oxygenation at δ 117.00, 107.47, 103.49 and 95.82.

The five signals between δ 62 and δ 80, correlating with proton signals between δ 3.4 and 4.0 in the gHSQC spectrum, and the anomeric carbon at δ 102.66, correlating with proton at δ 5.09 (1H, d, *J*=5.9 Hz) indicated again the presence of β-glucose (Harborne, 1986).

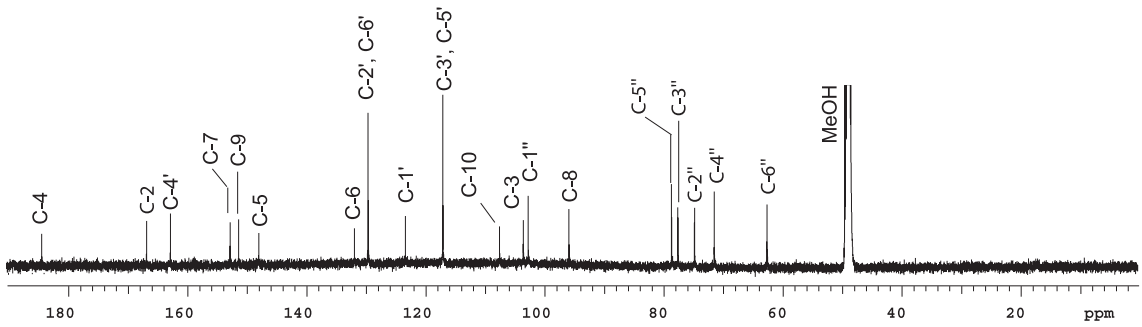


Figure 67: ^{13}C-NMR of **K** (125 MHz, in methanol-*d4*).

In the ^{1}H-NMR signal (Figure 68), two signals corresponding to four *ortho*-coupled protons at δ 7.84 (2H, d, *J*=6.8 Hz) and δ 6.91 (2H, d, *J*=7.3 Hz), linked to carbons at 129.56 and 117.00, confirmed the 4'-hydroxylation of the B-ring.
Instead of the two meta-coupling protons at H-6 and H-8 of compound **J**, two singlet protons were observed at δ 6.59 (1H, s, H-3) and δ 6.98 (1H, s, H-8).

The UV analysis had demonstrated the presence of a hydroxyl group at C-5, therefore one of these non coupling protons had to be in position C-3, the other being either in position 6, 7 or 8, and the sugar moiety had to be positioned on the hydroxyl at 6, 7, or 8. Considering the presence of a *o*-dihydroxyl on the A-ring (determined with shift reagents), the second free hydroxyl had to be in C-6 (in *ortho* with 5-OH) and the glycosylated hydroxyl in C-7 or C-8.

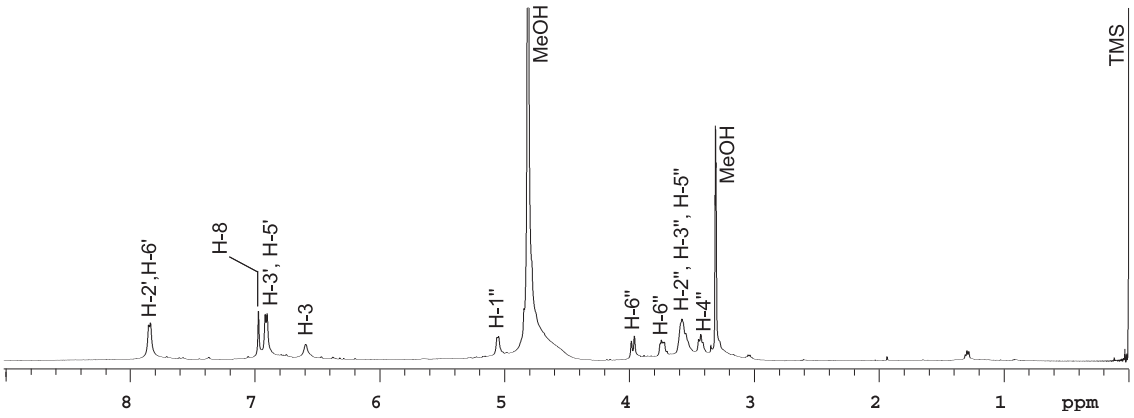


Figure 68: ^{1}H-NMR of **K** (500 MHz, in methanol-*d4*).

An acidic hydrolysis was performed in order to confirm the site of glycosylation by observation of the shifts induced in the UV spectrum of the aglycone by addition of reagents (Figure 69). Unlike the glycoside, addition of the strong base NaOMe caused the appearance of a third band at λ 330 nm, indicating the presence of a free hydroxyl at C-7; this was supported by a bathochromic shift of 11 nm after the addition of the weak base NaOAc (Bacon and Mabry, 1976).

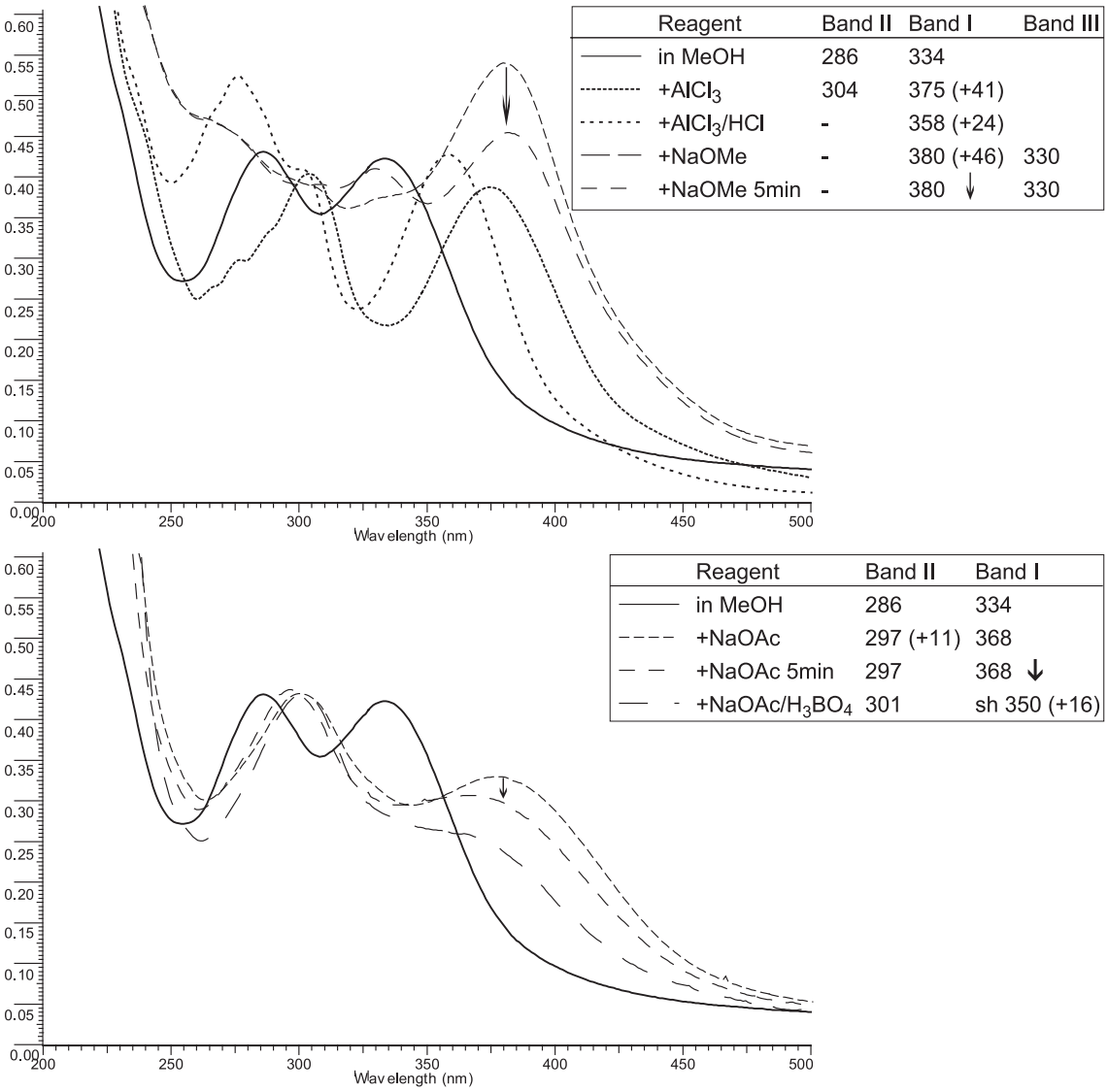
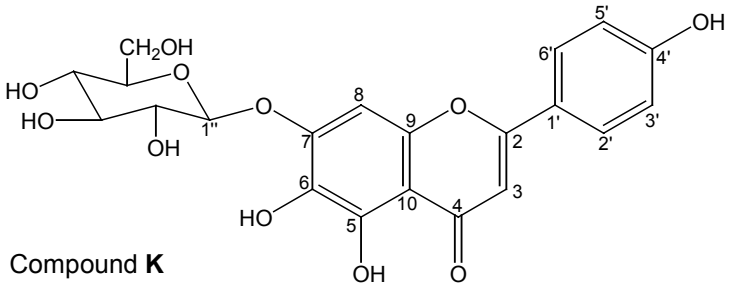


Figure 69: UV spectra of the aglycone of **K** with shift reagents (↓ indicates a decreased in intensity)

Compound **K** was therefore identified as 7-*O*-*β*-glucopyranosyl- 4', 5, 6, 7-tetrahydroxyflavone, or scutellarein 7-glucoside, also called plantaginin.



Compound **K**

It has been isolated from different families in genera such as *Plantago* (Plantaginaceae), *Scutellaria* (Lamiaceae), *Buddleja* (Buddlejaceae), *Crocus* (Iridaceae), *Centaurea* and *Erigeron* (Asteraceae). One of the first records dates from 1969 when it was isolated from the moss *Bryum weigelii* by Nilsson. Recent studies demonstrated a protective activity of this flavone on the renal cellular membrane (Yokozawa *et al*., 1999), and an inhibitory effect on HIV reverse transcriptase (Nishibe *et al*., 1997).

3.3.2.6 Structure elucidation of compound L

Comparison of the NMR data with those of compound **K** indicated a high correspondance. The structure of the aglycone appeared to be identical. Six supplementary signals were observed in the ^{13}C-NMR spectra (Figure 70), corresponding to α-rhamnose, with the C-6''' signal at δ 17.85 and the anomeric signal at δ 102.03.

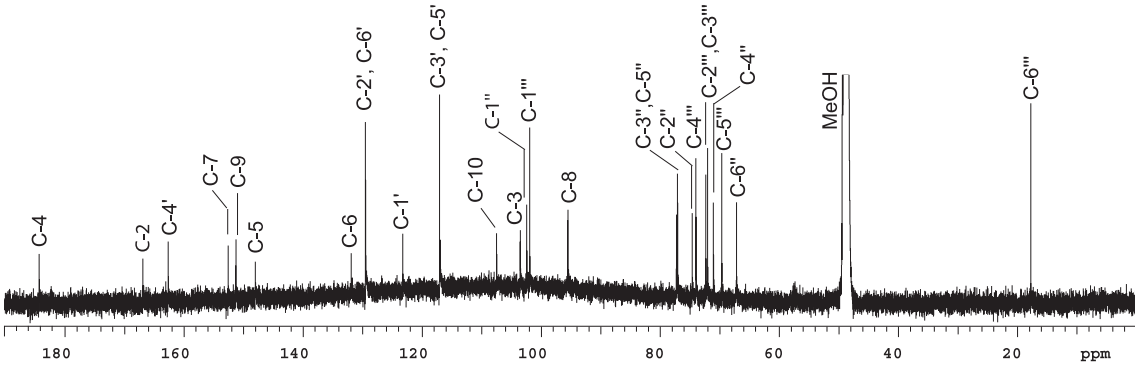
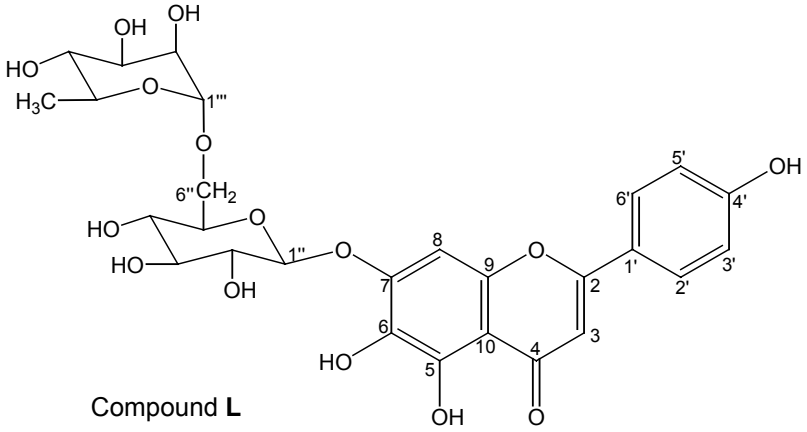


Figure 70: ^{13}C-NMR of **L** (125 MHz, in methanol-*d4*), C-1'' to C-6'': Glc signals, C-1''' to C-6''': Rha signals.

The position of the rhamnose unit was determined by observing gHMBC cross-peaks. The correlations between the signal corresponding to H-6''of the glucose unit at δ 3.70 and the anomeric carbon of the rhamnose C-1''' at δ 102.03, and between the anomeric proton of the rhamnose H-1''' at δ 4.72 and the carbon C-6'' of the glucose at δ 67.28 indicated the presence of rutinose, or [rhamnosyl-α(1→6)-glucose].

Compound **L** was identified as 7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl]-4', 5, 6, 7-tetrahydroxyflavone, or scutellarein 7-rutinoside.

It has been isolated twice previously from *Sempervivum ruthenicum* Koch. (Crassulaceae) (Gumenyuk, 1975) and *Parkinsonia aculeata* Linn. (Caesalpinaceae) (Shafiullah *et al*., 1994).



Compound **L**

3.3.2.7 Discussion

Three types of compounds could be isolated or identified from the aerial parts of *Jamesbrittenia elegantissima*: triterpene saponins, a phenylpropane derivative and flavonoids. Unlike *J. fodina*, no iridoid derivative was detected.

Moreover, the flavonoids isolated were one flavonol and two flavones, whereas in *J. fodina*, there were only flavanones.

4 Conclusion and perspectives

In the first part of this work concerning two plants used in traditional medicine, *Dioscorea sylvatica* (Dioscoreaceae) and *Urginea altissima* (Liliaceae), an explanation could be given for the inflammatory reaction caused by contact of the underground organs with the skin. The presence of sharp calcium oxalate needles grouped in raphides was observed by light microscopy in the tuber/bulb of both plants.
The cutaneous reaction might be increased by a simultaneous injection of chemical substances present in the plant. However, the sole irritating action of the calcium oxalate needles is sufficient to cause secondary effects associated with a long term use of the two plants, with the development of granulomatous lesions.

A future path to follow would be a study of the clinical, physiological and histological aspects of the inflammation caused by contact with the two plants. This would provide precious information on the type of reaction involved: pure mechanical irritation, non allergic contact urticaria or allergic reaction. Then, a search for the possible incriminating molecules could be efficiently performed, on the basis of their precise toxic effects.

The second part of this work consisted of the detection, isolation and characterisation of new natural compounds of potential therapeutic interest from African plants. For this purpose, a biological and chemical screening was performed on 70 extracts obtained from 28 higher plants of Zimbabwe. The different bioassays used consisted of antifungal, antibacterial, molluscicidal, radical scavenging tests and an inhibitory assay on the enzyme acetylcholinesterase.
The methanol extracts of the aerial parts of *Jamesbrittenia fodina* and *J. elegantissima* (Scrophulariaceae) were selected for their antifungal, antibacterial, molluscicidal and radical scavenging activities.

Phytochemical investigations of both extracts led to the characterisation of 20 compounds:

- 17 from *Jamesbrittenia fodina* (**A** to **I**) (Figure 71)
- 3 from *Jamesbrittenia elegantissima* (**J** to **L**) (Figure 72)

These compounds comprised of:

- 2 saponins (**A** and **B**)
- 1 phenylpropane derivative (**C**)
- 12 iridoids esterified with cinnamoyl moieties (**D_1**/**D_2** to **G_1**/**G_2**)
- 5 flavonoids: 2 flavanones (**H** and **I**), 1 flavonol (**J**) and 2 flavones (**K** and **L**)

A comparative LC/UV/MS study of the two polar extracts allowed the identification of compounds common to both extracts: **A**, **B** and **C**.

Saponins **A** and **B** were responsible for the antifungal, antibacterial and moluscicidal properties. These activities have already been described in the literature. They demonstrate that the mode of action is not very specific, as three different infectious agents are inhibited by the same molecule.
However, a different type of activity was discovered: for the first time, Mimengosides A (**A**) and B (**B**) were shown to inhibit the acetylcholinesterase. This activity, although weaker than that of the positive control galanthamine, was interesting, as to our knowledge, this inhibition has never been reported for this class of compounds, opening new research perspectives.
Moreover, recent studies demonstrated a significant anti-inflammatory activity of Mimengoside A in various acute and chronic experimental models.
This saponin appears to possess many biological activities. This may be due to its reactive epoxy group between C-13 and C-17. This reactivity was illustrated by the transformation of **A** into **B** and of **B** into **A** observed in this work and described in the literature.
Verbascoside (compound **C**) was determined to be responsible for the antiradical activity. It was identified by HPLC/UV/MS of the extract, and its isolation was performed in order to submit the molecule to different biological/chemical tests.

While the active compounds **A**, **B** and **C** have been reported previously in the literature, 8 new iridoid derivatives were also characterised. Although they were not active in our battery of tests, they could present interesting properties against other targets, particularly as anti-inflammatory and spasmolytic agents. The presence of such coumpounds could validate the traditional use of different *Jamesbrittenia* species for abdominal and menstrual pains in different African countries.

Finally, this study demonstrated the importance of modern hyphenated techniques, LC/UV/MS and LC/UV/NMR, in the screening and phytochemical investigation of plant extracts. In particular, LC/NMR enabled the characterisation of compounds that could not be isolated. Moreover, it allowed the understanding of two different mechanisms leading to the instability of plant constituents, attributed in one case to a light-induced *cis*/*trans* isomerisation, and in the other case to a transacylation of the cinnamoyl moiety on a sugar residue.
These cases of instability are rarely mentioned in the literature. The problem has sometimes been suggested by mentioning that two compounds could be characterised "in-mixture". But no formal explanation has previously been given for this phenomenon.

As an extension of this work, the iridoid derivatives could be submitted to different anti-inflammatory tests. Several similar compounds have indeed displayed significant anti-inflammatory properties in different models. The acylation at the sugar moiety rather than directly on the iridoid skeleton and the presence of a double bond between C-7 and C-8 seem to increase the anti-inflammatory activity.
As only two of the derivatives could be obtained pure (**F_2** and **G_2**), isolation of the other compounds should be attempted again from the raw extract, with precautions concerning exposition to light, heat, acids or bases.

Moreover, LC/UV/MS analysis of a second extract obtained from freshly collected plant material would indicate which of the characterised compounds are artefacts, and which are native to the plant. All operations, from the storage of the plant directly after its collection, to the drying, extraction and evaporation processes should be carefully performed, with precautions, as mentioned above, concerning exposition to light, heat, acids and bases, in order to prevent the *cis*/*trans* isomerisation and transacylation of the derivatives.

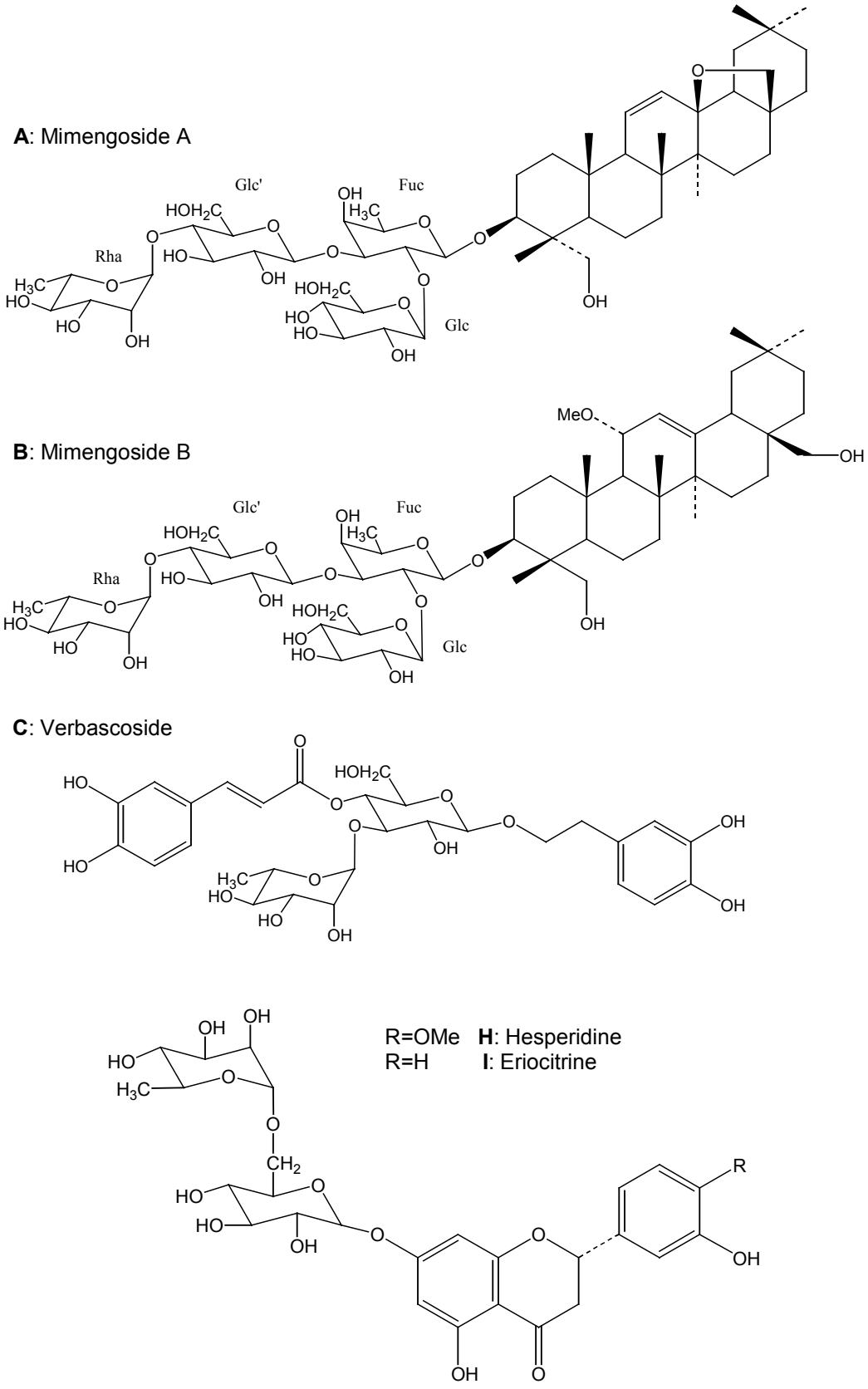
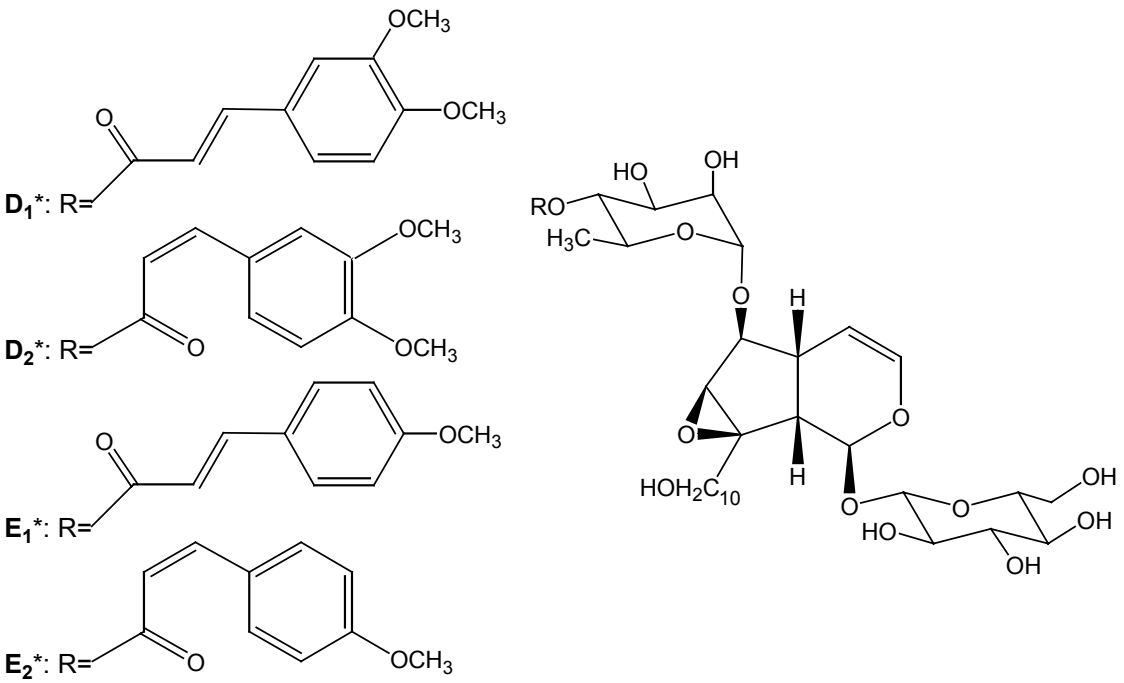
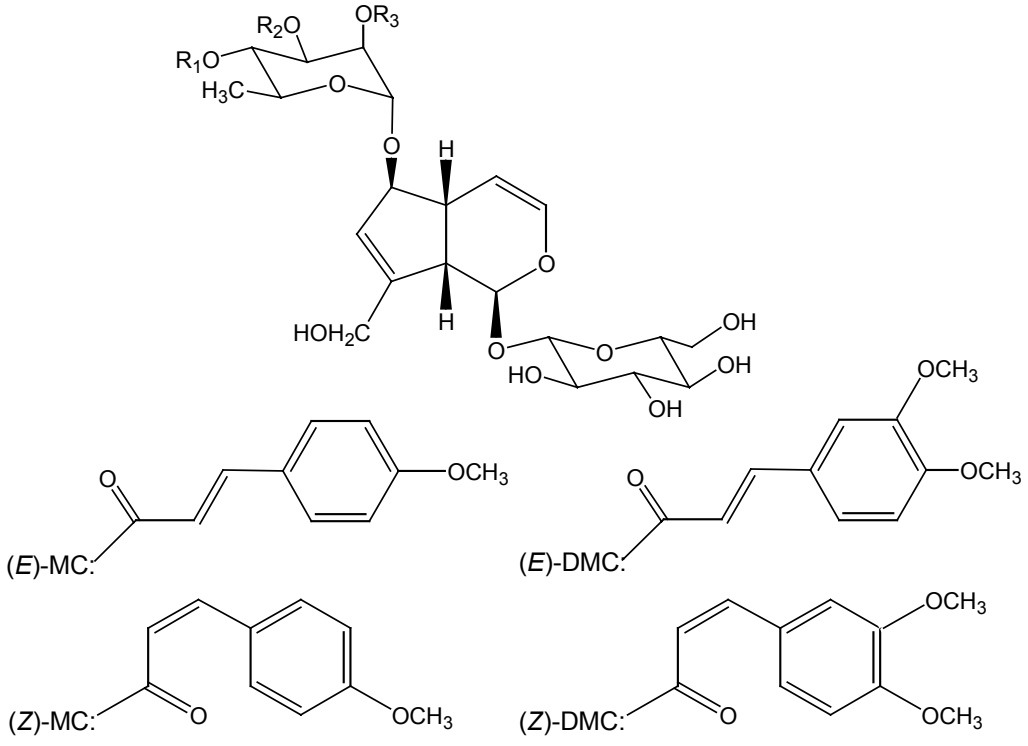


Figure 71: Structures of the compounds isolated from *Jamesbrittenia fodina*





F_1*: R_1=(***E***)-MC, R_2=R_3=H
F_{1a}*:R_3=(***E***)-MC, R_1=R_2=H
F_{1b}*:R_2=(***E***)-MC, R_1=R_3=H

F_2: R_1=(***Z***)-MC, R_2=R_3=H

G_1*: R_1=(***E***)-DMC, R_2=R_3=H
G_{1a}*:R_3=(***E***)-DMC,R_1=R_2=H
G_{1b}*:R_2=(***E***)-DMC,R_1=R_3=H

G_2: R_1=(***Z***)-DMC, R_2=R_3=H

Figure 71/cont: Structures of the compounds isolated from *Jamesbrittenia fodina* (* indicates the compounds that were characterised but not isolated)

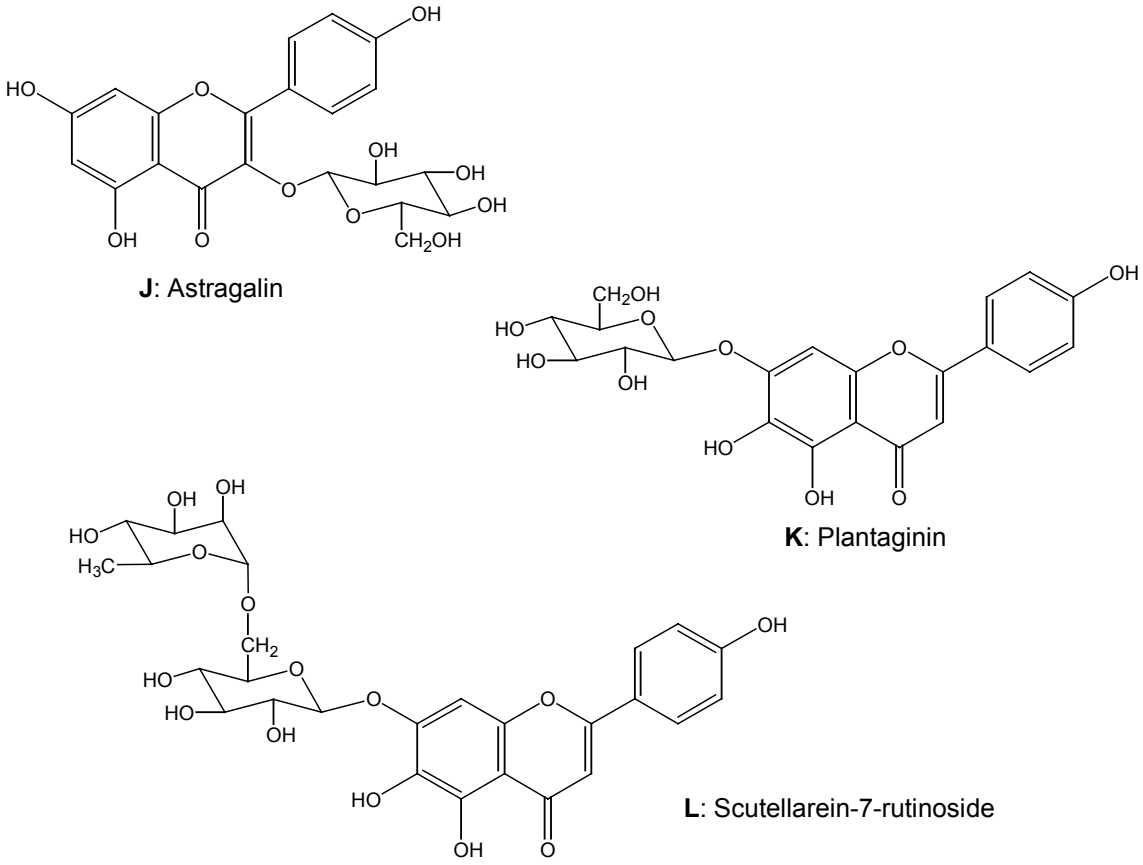


Figure 72: Structures of the compounds isolated from *Jamesbrittenia elegantissima*

5 Material and Methods

5.1 Plant material and extraction

The plants studied here were all collected in Zimbabwe. Table 5 presents the name of the collected plants, the organs extracted and the weights of extracts obtained. Botanical identification was carried out by Mr. S. Mavi from Zimbabwe. Specimen vouchers are available at the Institut de Phytochimie et Pharmacognosie, University of Lausanne, Switzerland.

Table 5: Enumeration of the plant material and the dichloromethane and methanol extracts weights

Family and plant name	Organ	Dry plant weight	DCM	MeOH	H_2O
Acanthaceae					
Dyschoriste alba S.Moore	Leaves & twigs	45 g	0.2 g	0.8 g	
Amaryllidaceae					
Buphane disticha Herb.	Bulbs	1013 g	24.0 g	67.0 g	
Asclepiadaceae					
Asclepias densiflora N.E.Br	Aerial parts	47 g	0.9 g	1.7 g	
Asteraceae					
Crassocephalum rubens Moore	Leaves	248 g	0.8 g	1.9 g	
Asteraceae					
Geigeria schinzii. O.Hoffm	Aerial parts	290 g	12.7 g	16.8 g	
Capparaceae		48 g			16.0 g
Cadaba termitaria N.E.Br.	Whole plant	247 g	6.0 g	24.4	
Celastraceae					
Hippocratea longipetiolata Oliver	Leaves & twigs	279 g	7.1 g	26.2 g	
Combretaceae					
Combretum apiculatum Sond	Leaves	46 g	2.0 g	3.9 g	
Dioscoreaceae		860 g (fresh			18.7 g
Dioscorea sylvatica Ecklon	Fresh bulb	374 g (fresh)		39.0 g	
Euphorbiaceae					
Pseudolachnostylis	Root	183 g	0.5 g	22.7 g	
maprouneifolia Pax	Bark	822 g	2.0 g	111 g	
Euphorbiaceae		900 g	3.8 g		
Acalypha petiolaris Sond	Root	200 g	2.0 g	60.0 g	

	Leaves			15.5 g	
Family and plant name	**Organ**	**Dry plant weight**	**DCM**	**MeOH**	**H_2O**
Leguminosae	Leaves	518 g	11.4 g	27.8 g	
Cordyla africana Lour.	Trunk bark	1125 g	2.1 g	23.4 g	
Leguminosae					
Macrotyloma densiflorum Verdc.	Leaves		0.9 g	2.1 g	
Leguminosae					
Tephrosia acaciaefolia Welw	Leaves & twigs	48 g	0.9 g	1.0 g	
Liliaceae		295 g (fresh)			14.5 g
Urginea altissima Baker	Fresh bulbs	560 g (fresh)		34.0 g	
Malvaceae					
Sida cordifolia L.	Aerial parts	225 g	2.4 g	10.3 g	
Meliaceae	Bark	192 g	7.2 g	37.0 g	
Ekebergia benguelensis Welw.	Leaves	130 g	3.4 g	32.0 g	
Moraceae					
Ficus capreaefolia Delile	Leaves & twigs	138 g	2.8 g	6.8 g	
Myrtaceae					
Syzygium huillense Engl.	Trunk bark	1104 g	1.8 g	41.0 g	
Nyctaginaceae					
Boerhaavia erecta Burm f.	Aerial parts	470 g	9.8 g	39.8 g	
Poaceae					
Brachiaria brizantha Stapf	Aerial parts	451 g	2.9 g	13.2 g	
Poaceae					
Panicum maximum Jacq.	Aerial parts	47 g	0.3 g	1.2 g	
Proteaceae					
Faurea speciosa Welw.	Leaves	427 g	0.7 g	6.2 g	
Scrophulariaceae					
Jamesbrittenia elegantissima Hilliard	Aerial parts	215 g	5.4 g	14.5 g	
Jamesbrittenia fodina Hilliard	Aerial parts	310 g	4.9 g	50.2 g	
Sellaginellaceae					
Sellaginella dregei Hieron	Whole plant	185 g	2.9 g	18.7 g	
Verbenaceae					
Clerodendrum glabrum f. pubescens R. Fernandes	Leaves	303 g	8.7 g	38.2 g	
Vitaceae	Aerial parts	433 g	7.1 g	20.8 g	

Cissus rotundifolia Vahl.					

The collected plant material was air dried and ground in liquid nitrogen to prevent heating that could alter its components. It was then macerated successively in dichloromethane and methanol, in about 1.5 l of solvent for 250 g of dry plant material. Maceration was performed 3 times for each solvent during 24 hours at room temperature and under constant agitation. Solvents were evaporated with a Büchi Rotavapor at a maximum temperature of 40 °C. Water extracts were performed in a similar way, with a second sample of ground material.

In the case of the fresh plant materials *Dioscorea sylvatica* and *Urginea altissima*, the peeled rhizomes and bulbs were extracted with 1000 ml of methanol and water for 400 g of material in an Ultra-Turrax mixer. The liquid was centrifuged at 3200 rpm for 10 minutes and the supernatant was filtered. The residue was extracted twice with the same quantity of solvent for 24 hours, centrifuged and filtered. The extracts were combined, evaporated and lyophilised.

5.2 Analytical separation techniques

5.2.1 Thin-layer chromatography (TLC)

TLC was the method of choice for routine phytochemical analyses of crude extracts, fractions and isolated pure compounds. It permitted to optimize the solvent composition for the centrifugal partition chromatography (see 5.3.2).

It was also used as a support for chemical and biological screening of extracts and pure compounds. These aspects will be developed in chapters 5.5 and 5.7.

Normal phase TLC was generally carried out on pre-coated Merck silicagel 60 F_{254} aluminium sheets. For reversed-phase chromatography, Merck RP-8 and RP-18 plates were employed. Some biological tests (*Candida albicans*, *Bacillus subtilis*) were performed on silicagel glass plates.

The TLC plates were developed in twin trough Camag chromatographic tanks saturated with an appropriate solvent system. The solvent system, often consisting of a binary or tertiary mixture, was adapted to the specific needs of an analysis. However, standard conditions were employed systematically on extracts and fractions:

	Composition	Proportions
DCM extracts Non polar fractions	Ligroin-Ethylacetate	1:1
MeOH extracts Polar fractions	Chloroform-Methanol-Water	65:35:5

The amounts spotted on the plates were usually 100 µg of extracts and 10 µg of pure compounds.

After migration, the plates were observed at 254 and 366 nm, and revealed with appropriate chemical reagents (see 5.5.1).

5.2.2 High performance liquid chromatography coupled with ultraviolet photodiode array (LC/DAD-UV)

Analytical high pressure liquid chromatography was used to:

- analyse raw extracts and fractions
- guide the separation and isolation processes
- optimise the conditions for MPLC, semi-preparative HPLC and CPC.
- check the purity of the isolated products
- determine the kinetics of a compound transformation

The three HPLC systems employed were the Hewlet Packard HP-1050, 1090 series II and 1100, all equipped with a diode-arrayed detector (DAD) for UV detection. The manipulation and data recording and analysis were done with the HP ChemStation software. These equipments allowed analyses in isocratic mode (constant composition of mobile phase) or gradient mode (variable composition of mobile phase during analysis).

The column routinely used was a Nova-Pak C-18 column (150 x 3.9 mm i.d., stationary phase particle size 4 μm, Waters). The flow rate was set to 1 ml/min. The mobile phase was composed of variable proportions of methanol (or acetonitrile) and water, sometimes acidified by 0,05 % of trifluoroacetic acid (TFA).

5.2.3 High performance liquid chromatography coupled with mass spectrometry (LC/MS)

High performance liquid chromatography (LC) coupled with mass spectrometry (MS) was used for:

- a preliminary study of the crude extracts
- a comparison of the two species extracts
- the determination of the mass of non separable compounds
- the comparison of hydrolysis products with the standard aucubin

Current LC/UV/MS analyses were made on the following apparatus:

- HPLC HP-1100 system (degasor, pump, injector and DAD-UV detector) equipped with an automatic sampler and controlled by HP ChemStations software.
- APCI (atmospheric pressure chemical ionization) or ESI (electrospray ionization) interface.
- LCQ (Finnigan MAT) ion trap mass spectrometer.

The chromatographic and ionisation conditions were adapted for each analysis. The ionisation conditions were generally as follow:

- APCI in the positive ion mode: source current 5 μA, vaporization temperature 450 °C, sheath gas flow (N_2) 80 psi, capillary temperature 150 to 200 °C ; in the negative ion mode : source current 5 μA, vaporization temperature 450 °C, sheath gas flow (N_2) 60 psi, capillary temperature 150 °C.
- ESI in the positive ion mode: source voltage 4.50 kV, sheath gas flow (N_2) 80 psi, capillary voltage 10 to 30 V, capillary temperature 200 to 270 °C.

Analyses were made in the full scan mode (180-1200 Da).

5.2.4 High performance liquid chromatography coupled with nuclear magnetic resonance (LC/NMR)

The high performance liquid chromatography (LC) coupled to nuclear magnetic resonance was used for the differentiation of compounds that could not be separated (*cis-trans* isomers **D_1/D_2** and **E_1/E_2** and transacylated esters **G_1/G_{1a}/G_{1b}**). This technique permitted the attribution of all resonances obtained in standard NMR experiments in-mixture.

The stop-flow mode was adapted to the analyses of these simple mixtures: the signal of the analyte of interest (detected by a UV detector) triggers a valve which stops the LC flow when the peak reaches the NMR cell. This mode allows long acquisition times and the measurement of 2D NMR experiments (Wolfender *et al*., 2001).

The analyses were performed with the following equipment:

- Varian (Palo Alto, Ca, USA) LC/UV modular system comprising a Varian 9012 pump, a Valco stop-flow valve and a Varian 9050 UV-visible detector, controlled by Varian LC Star software.
- Varian $^{1}H[^{13}C]$ flow probe (60 µl)
- Unity Inova 500 MHz NMR instrument

An amount of 10 to 50 µg of sample is necessary in the flow cell to get a good LC-NMR spectra quality, corresponding to a concentration of 100-500 µg/ml in the LC column if a flow cell volume of 100 µl is used, in opposition with the 10 µg/ml usually eluted in a LC/UV system. This overloading generates peak broadening. Therefore, a good resolution of the peaks has to be achieved in LC/UV (Wolfender *et al*., 2001).

Different chromatographic conditions were used for the analyses:

- Mixture of **D_1/D_2**: Symmetry C-18 column (250x3.9 mm, i.d., 5 µm, Waters), gradient of methanol:water (28:72 to 46:54 in 30 min, 46:54 to 62:38 in 16 min, 62:38 to 100:0 in 14 min), injection of 500 µg, flow rate 1 ml/min.

- Mixture of $\mathbf{E_1}/\mathbf{E_2}$: Symmetry C-18 column (250x3.9 mm, i.d., 5 μm, Waters), gradient of acetonitrile:deuterated water (25:75 to 30:70 in 30 minutes), injection of 240 μg, flow rate 1 ml/min.
- Mixture of $\mathbf{F_1}/\mathbf{F_{1a}}/\mathbf{F_{1b}}$: Nucleosyl C-18 column (250x8 mm, i.d., 5 μm, Macherey-Nagel), gradient of acetonitrile:deuterated water (20:80 to 36:44 in 20 minutes), injection of 200 μg, flow rate 1 ml/min

For the suppression of the solvent signal and its two ^{13}C satellites, as well as the residual HOD peak, a fast solvent suppression sequence WET was run before each acquisition (Smallcombe *et al*., 1995).

5.3 Preparative separation techniques

5.3.1 Open column chromatography on silica gel (CC)

The open column chromatography on silicagel was employed for the fractionation of the methanol extract of *Jamesbrittenia fodina* and of the complex fraction 7.

The size of the column and the granulometry of the silicagel were determined according to the amount of the sample and the degree of separation desired; Merck Silica gel with particle size ranges 63-200 μm was used for fractionation of the crude extract, whereas particle size ranges 40-63 μm was preferred for a better resolution for fractionation of fraction 7.

The composition of the mobile phase was determined by TLC analyses. It consisted of a mixture of 3 solvents (Hostettmann *et al*., 1998). Isocratic systems or stepwise gradient eluents were employed, depending on the nature of the components and of their separation. When the sample is soluble in the starting eluent, liquid introduction of the sample is the method of choice. As in our case the samples were insoluble, a solid introduction was performed: the sample was mixed with a quantity of silica gel corresponding to 2-3 times its weight and dried before application to the top of the column.

The fractions were collected with automatic fraction collectors (Retriever II ISCO, Büchi B-683).

5.3.2 Centrifugal partition chromatography (CPC)

Centrifugal partition chromatography was employed for the first fractionation of the polar extract of *Jamesbrittenia elegantissima*.

The stationary and mobile phases consist of the two phases of a mixture of immiscible solvents introduced in a coil. The rotation of the coil around two axes causes an agitation of the two solvent phases, and thus an efficient solute partitioning.
The head of the coil is the end to which the solvent travels under rotation; the tail is the opposite end. Head and tail are interchanged when direction of rotation is reversed (Hostettmann *et al*., 1998).

For this work, a rotating coil planet centrifuge Pharma-Tech Instrument, model CCC-1000 (Baltimore, USA) was used. Solvent was delivered via a S.S.I. 300 LLC pump. A Knauer UV detector and a Tarkan W+W recorder 600 were connected to the coil. The fractions were collected with an automatic Pharmacia LKB 2070 Ultrorac II fraction collector (Bromma, Sweden).
The rotation speed was set to 1000 rpm, the flow rate was 3 ml/min, and the mobile phase was the lower phase of the solvent system.
The selected solvent system was $CHCl_3$:MeOH:^{i}PrOH:H_2O (7:6:3:5), chosen on the base of TLC and LC/UV analyses (Hostettmann *et al*., 1998) (see 3.3.2.2).
The coils were first filled with the stationary phase and then mobile phase was pumped into the apparatus under rotation until conditions were stable and no more stationary phase went out. The sample was then dissolved in equal amounts of upper and lower phases and injected via a sample loop.

5.3.3 Medium pressure liquid chromatography (MPLC)

Medium pressure liquid chromatography was used for the separation of the polar fractions from *Jamesbrittenia fodina*.
The principle of this method is similar to the HPLC, but it allows the separation of several grams of a mixture with a good resolution.

The equipment used consisted of a Büchi B-681 pump, a Knauer K 2001 UV detector, a Pharmacia LKB Rec 1 recorder and an automatic Büchi B-684 fraction collector. The same stationary phase was used for all analyses, Lichroprep RP-18 (15-25 μm, Merck), packed in a pressure-resistant column of variable size depending on the sample amount. The choice of the solvent system was performed by analytical LC/UV. It consisted of a step gradient of water and acetonitrile or methanol. The pressure was set to 10-15 bars. The sample was mixed with 2-3 times its weight of stationary phase and placed in an introduction cartridge.

5.3.4 Low pressure liquid chromatography (Lobar)

The low pressure liquid chromatography was used in order to separate both saponins A and B from *Jamesbrittenia fodina* (See 3.2.1.2).

This system is similar to the MPLC, but the working pressure is only about 1-2 bars. It is used to separate smaller amounts of samples.
The system used consisted of a prepacked Lichroprep RP-18 (40-63 μm) Lobar column (B size, 310x25 mm i.d., Merck), a Duramat 80 (CfG ProMinent) solvent delivery pump, a LKB Uvicord SII UV detector, a Pharmacia LKB Rec 1 recorder and an automatic Isco Retriever II fraction collector. The mobile phase consisted of isocratic mixtures of acetonitrile-water. The sample was dissolved in the mobile phase before introduction.

5.3.5 Semi-preparative high performance liquid chromatography (SP-HPLC)

Semi-preparative HPLC was used in order to purify the compounds isolated by MPLC or CPC.
Three types of semi-preparative columns were used:

- a SymmetryPrep C-18 column (150x25 mm i.d., 7μm, Waters)
- a Lichrosorb 100 C-18 endcapped semi-preparative column (150x25 mm i.d., 5 μm, Waters)

- a Bondapack C-18 (10μm) column in a radial compression PrepLC 25 mm module (RCM) (Waters).

They were connected to a Shimadzu LC-8A pump equipped with a Pharmacia LKB UV detector and a Pharmacia LKB REC 1 recorder. The Gilson Fraction controller and collector (model 201) were used for some analyses. The flow rate was set to 10 ml/min and the UV detection to 210 nm. Injected amounts ranged from 5 to 10 mg.

Optimal separation conditions were first determined using analytical systems: for the SymmetryPrep and the Lichrosorb preparative columns, conditions were optimised with the classical LC/UV system using a Symmetry C-18 analytical column (150x3.9 mm i.d., 5 μm, Waters) and a Lichrosorb C-18 column (250x3.9 mm i.d., 7 μm, Waters) respectively.

For the determination of the conditions for the radial compression system, an analytical Bondapack C-18 column in a radial compression RCM 8x10 module (Waters) was used with a Shimadzu LC-10AD pump, at a flow of 1ml/min. Injected amounts ranged from 20 to 200 μg. Conditions were then adapted to the semi-preparative scale.

5.4 Physico-chemical methods

5.4.1 Melting point (M_p)

Melting points were measured on a Mettler FP 80/82 hot stage apparatus connected to a WILD M 11 microscope. The observed temperatures were not corrected.

5.4.2 Optical rotation ($[\alpha]_D$)

The optical rotation was measured on a Perkin-Elmer 241 polarimeter with sodium D line (589 nm) as the source of light. The measures were made at room temperature (25°C) in a 10 cm long cell.

The results were calculated with the following formula:

$$[\alpha]_D^T = \frac{1000 \cdot \alpha}{l \cdot c}$$

α = observed rotation

l = cell length in dm

c = concentration in g/l

The concentrations were expressed in g/100 ml, according to the conventions.

5.4.3 Ultraviolet spectrophotometry (UV)

The UV spectra of the isolated compounds were recorded in methanol on a Perkin-Elmer Lambda 20 spectrophotometer. A quartz cell was used.

The spectra maxima were presented as $\lambda_{\max}^{MeOH} nm$ (see 5.8).

Table 6 presents the shift reagents used for the structure determination of the isolated flavonoids.

Table 6: preparation and effect of shift reagents used for the structural determination of flavonoids (Markham, 1982)

Reagent	Preparation	Interpretation
$AlCl_3$	Add 6 drops of $AlCl_3$ 5% in MeOH	Complex formation between acid-stable hydroxyls and neighbouring ketones and acid-labile complexes with *o*-dihydroxy groups.
$AlCl_3$/ HCl	Add 3 drops of HCl 8% to the previous solution	Formation of hydroxyl-keto complexes only.
NaOMe	Add 3 drops of NaOMe solution (2.5 g Na in 100 ml MeOH)	Deprotonation of all phenolic hydroxyls. Degradation with time indicates the presence of alkali sensitive groupings.
NaOAc	Add powdered NaOAc (2 mm on the bottom)	Deprotonation of the most acid hydroxy groups (7-OH and 4'-OH).
NaOAc/ H_3BO_3	Add half this quantity of powdered H_3BO_3 to the previous solution	Bridge formation between *o*-dihydroxy groups.

5.4.4 Mass spectrometry (MS)

EI and D/CI mass spectra of the pure compounds were measured on a Finnigan-Mat TSQ-700 triple stage quadrupole mass spectrometer, with the following parameters:

- EI (electron impact ionization) spectra: power of 70 eV applied on the filament; vaporization of the samples by a linear increase of the temperature of the probe (50-300°C in 1 minute); temperatures of the source and quadrupoles maintained at 150°C and 70°C respectively.
- D/CI (desorption chemical ionization) spectra: linear increase of desorption filament temperature (50 to 1000°C in 1 minute); ammonia as reagent gas for measures in the positive ion mode, sometimes replaced by methane.

High resolution mass spectra were recorded for the new compounds in order to confirm their molecular formula. These measurements were carried out on a Bruker FTMS 4.7T mass spectrometer in the Laboratoire de Chimie Organique of Prof. Jenny in the University of Fribourg.

5.4.5 Nuclear magnetic resonance spectrometry (NMR)

^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 instrument at 500 and 125 MHz respectively, controlled by the Solaris VNMR software.
For advanced and two dimensional spectra including gdqCOSY, gHSQC, gHMBC and NOESY, standard pulse sequences provided in the original software were employed. DEPT spectra consisted of 90° pulse spectrum (CH signals positive) and 135° pulse spectrum (CH and CH_3 signals positive, CH_2 signals negative).
The TOCSY experiment gives cross-peaks with all of the protons compromising a sequence of coupled protons in individual monosaccharide units. The 1D TOCSY sequence allows a much better resolution than with 2D experiments (Reynolds and Enriquez, 2002).

Deuterated solvents were used to measure NMR spectra. The shifts are indicated in ppm, with tetramethylsilane (TMS) as internal standard for ^{1}H spectra, and the solvent shift as reference for ^{13}C spectra. The chemical shifts of the solvents employed and the operating temperatures are exposed below.

Solvent	Chemical shift in ppm (multiplicity)	Temperature
Chloroform-*d*	77.0 (3)	26°C
DMSO-*d6*	39.7 (7)	30°C
Methanol-*d4*	49.0 (7)	26°C
Pyridine-*d5*	149.2 (3), 135.5 (3), 123.5 (3)	30°C

5.5 Chemical and biochemical methods

5.5.1 TLC chemical spray reagents

The compounds were first detected by fluorescence extinction under UV light at 254 nm, by fluorescence under UV light at 365 nm, and subsequently sprayed with a chemical reagent. The reagents used in this work are presented below.

- Godin's reagent (Godin, 1954): polyvalent reagent. Equivalent volumes of 1% vanillin in ethanol and 3% perchloric acid in water are mixed; the plate is sprayed first with this mixture, then with an ethanolic solution of 10% sulphuric acid; it is then heated to about 100°C during 5 minutes. Different colorations are visible in day-light.

- NST-PEG (Naturstoff-Polyethylenglykol): detection of the flavonoids. The TLC plate is sprayed successively with a solution of 1% of diphenylboryloxyethylamine in methanol (NST) and with a solution of PEG 4000 5% in ethanol. Flavonoids appear as yellow, orange or blue fluorescent spots at 366 nm (Wagner and Bladt, 1996).

- Naphtoresocine: detection of the sugars. A solution of naphtoresocine 0.2 g heated in EtOH 100 ml and H_3PO_4 100 ml is sprayed on the plate, which is heated during 5 minutes. Sugar residues appear as blue or purple spots in day-light.

- Ninhydrine reagent: detection of the amines. 30 mg of ninhydrin are dissolved in 10 ml of butanol and acidified by 0.3 % of acetic acid 98%. The solution is sprayed on the plate, which is heated during 5 minutes. Amines appear as purple spots in day-light (Wagner and Bladt, 1996).

5.5.2 Free radical scavenging assay with DPPH

1,1-diphenyl-2-picrylhydrazyle (DPPH, figure 5.1) is a stable radical with a purple colour (maximum absorption at 517 nm). The assay is based on the decolouration of the compound when reduced by a free radical scavenger. The test is performed on TLC (Cuendet *et al.*, 1997).
A solution of 0.2% DPPH in methanol was sprayed on the developed TLC. After about 30 minutes, the active compounds clearly appeared as clear spots on the purple background. 100 μg of plant extracts and 10 μg of pure compounds were spotted on the plates.

5.5.3 Inhibitory test against the enzyme Acetylcholinesterase

The acetylcholinesterase (AChE, EC 3.1.1.7) is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) in cholinergic synapses. Inhibitors of this enzyme (iAChE) induce an increase of cholinergic transmission. Their clinical use includes symptomatic treatment of Alzheimer's disease.

A test was developed to detect inhibitors of the Acetylcholinesterase directly on TLC (Marston *et al.*, 2002). All crude extracts and isolated compounds were submitted to this test.

1000 unities of AChE were dissolved in 150 ml buffer pH 7.8 and 150 mg of albumin were added. This solution was sprayed on the developed and dry TLC plates, and the plates were incubated in humid atmosphere at 37°C for 20 minutes.

A mixture of 1 part of 1-naphtyle acetate (250 mg/ml in methanol) and four parts of « Fast Blue Salt B » (200mg / 80ml water) was then sprayed on the incubated plates. A purple coloration appeared after a few minutes: active compounds were localised by colourless spots. 40 µg of the extracts and 4 µg of pure compounds were spotted.
Galanthamine was used as a positive control.

5.6 Chemical reactions

5.6.1 Acetylation

This derivatisation was carried out on the mixture of compounds $\mathbf{G_1}$, $\mathbf{G_{1a}}$ and $\mathbf{G_{1b}}$ in order to stabilise the three esters and confirm the structural hypothesis.
15 mg of the mixture were dissolved in 2 ml of a mixture of acetic anhydride and pyridine (1:10), and the mixture was then agitated for 24 hours at room temperature, under nitrogen flow and in darkness.
The reaction was controlled by TLC. The pyridine was removed by evaporation. The acetylated esters were separated by semi-preparative HPLC (see 3.2.6.4).

5.6.2 Hydrolyses

5.6.2.1 Acidic hydrolysis

Acidic hydrolysis was performed on saponins **A** and **B**.
10 ml of HCl 2N were added to 10 mg of each saponin and heated during 3 hours.
The solution was neutralised with $NaHCO_3$. The aglycone was extracted with EtOAc. The sugars were extracted from the aqueous phase with anhydrous pyridine; the pyridine was evaporated and the residue dissolved in water for identification by TLC analysis and detection with naphtoresocine reagent.

5.6.2.2 Basic hydrolysis

Basic hydrolysis was performed on the mixtures of compounds **F_1/F_{1a}/F_{1b}**, and **G_1/G_{1a}/G_{1b}** in order to break only the ester bound with the cinnamoyl moiety, but not the ether bounds between the iridoid moiety and the two sugar units.

10 mg of the mixtures were agitated in 10 ml of the mixture Et_2N-MeOH-H_2O (1:8:1 v/v/v) at 35°C during 36 hours. The solvents were then removed by evaporation and the residue was dissolved in acetonitrile for LC/UV control.

5.6.2.3 Enzymatic hydrolysis

Enzymatic hydrolyses was performed on the previously hydrolysed (in basic conditions) mixtures **F_1/F_{1a}/F_{1b}**, and **G_1/G_{1a}/G_{1b}**. 75 mg (20 units) of hesperidinase (H-8510 Sigma chemicals) dissolved in 15 ml of acetate buffer pH 5.5 were added to the products obtained by basic hydrolysis and incubated at 37°C for 48 hours. The hydrolysis products were then compared with aucubin standard by LC/UV/MS.

5.7 Biological methods

5.7.1 Antifungal test against *Cladosporium cucumerinum*

Cladosporium cucumerinum is a microscopic phytopathogenic fungus, attacking particularly members of the Cucurbitaceae family.

Testing for antifungal activity was performed by direct bioautography on TLC (Homans and Fuchs, 1970). Aluminium-backed silica gel plates were developed according to 5.2.1.

After elution, the chromatograms were dried and sprayed with a conidial suspension of *Cladosporium cucumerinum* (provided by Novartis Agro, Basel, Switzerland) in Sabouraud medium. The fungus growth is accompanied by the production of a greenish grey pigment: active compounds appeared very clearly as white inhibition spots. Ethanol 94% was then sprayed on the plate to stop fungus growth.

The spotted amounts were 100 µg of extracts and fractions and 10 µg of pure compounds. Miconazole was used as positive control.

5.7.2 Antifungal test against *Candida albicans*

The yeast *Candida albicans* is frequently found in the gastrointestinal, buccal and vaginal human commensal flora. However, it can become pathogenic by immunodepressed patients. In the last decades, systemic candidosis has become a very serious infection concerning AIDS patients. The apparition of numerous resistances to the drugs available is in favour of a search for new drugs against *Candida albicans.*

The « agar overlay » method was developed by Rahalison *et al.* (1991).

The extracts were applied on a glass-backed silica gel TLC plate. The plates were developed as described in 5.2.1. After elution, the chromatograms were dried. A colony of *C. albicans* (obtained from the CHUV, Lausanne, Switzerland) was put in 50 ml of Sabouraud medium and kept for one night at room temperature with constant shaking. One ml of this solution was mixed with another 50 ml of Sabouraud medium. After 6 hours at room temperature with constant shaking, 5 ml of this solution was introduced in fractions of 50 ml of malt agar (maintained liquid at 45°C). 20 ml of this inoculums, containing approximately 10^5 cells/ml, were necessary for a 10 x 20 cm plate. It was laid on the plate in a layer of about 1-2 mm. The agar hardened while cooling. The plates were incubated overnight at 30°C in humid atmosphere, and then sprayed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) 2.5 mg/ml. This substance is metabolised by *C. albicans* in purple formazan, and after 4 hours incubation, inhibition spots were easily visualised as white spots on the purple background. Ethanol 94% was then sprayed on the plate to stop yeast growth.

Identical sample amounts as for *C .cucumerinum* test were spotted on the plates.

Miconazole was used as a positive control.

5.7.3 Antibacterial test against *Bacillus subtilis*

Bacillus subtilis is a Gram positif aerobic bacterium that is easily manipulated in a laboratory.

The « agar overlay » method used in our laboratories was adapted from Hamburger and Cordell (1987). The plates were prepared as described for *Candida albicans* (see 5.7.2).

Bacillus subtilis strain ATCC 6633 obtained from Sigma was used. A colony of the germ was grown in a liquid Luria-Bertani culture medium (10 g/l tryptone + 5 g/l autolytic yeast extract + 10 g/l NaCl). After one night at room temperature, 0.5 ml was used to inoculate 50 ml of a second culture medium of the same type. After 8 hours agitation at room temperature, 0.5 ml of this solution was introduced in fractions of 50 ml of Luria-Bertani agar (Luria-Bertani culture medium + 15 g/l type A bacteriologic agar; Biokar) kept at 45°C. 20 ml of this inoculum, containing approximately 10^8 cells/ml, were necessary to cover a 10 x 20 cm plate with a 1-2 mm layer. As for *C. albicans*, the plates were incubated overnight at 30°C in humid atmosphere, and then sprayed with MTT.

Active compounds could be detected as white spots on the purple formazan background. The same amounts were spotted on the TLC plates. Chloramphenicol was used as positive control.

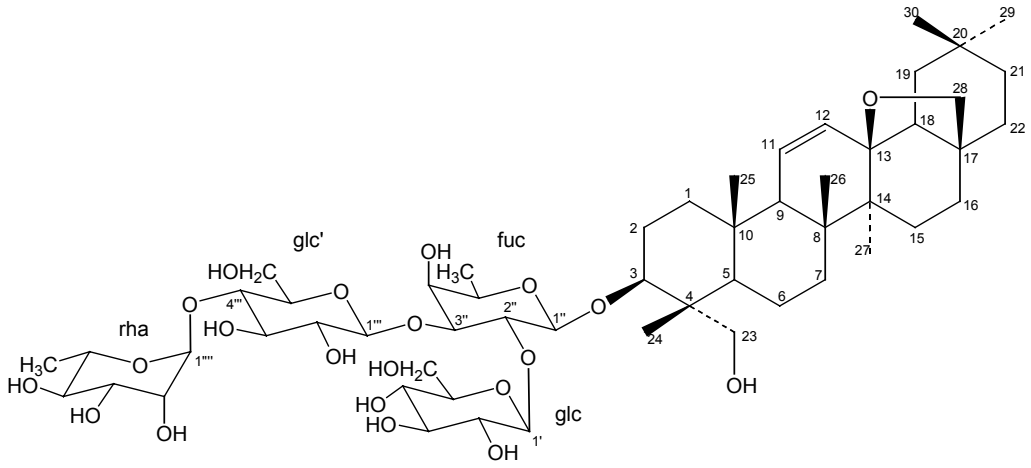
5.7.4 Molluscicidal test against *Biomphalaria glabrata*

Biomphalaria glabrata is a mollusc which constitutes one of the hosts of the vector of the parasite responsible for schistosomiasis (Billharzia). The aim of the test developed in our laboratories (Hostettmann *et al*., 1982) is to find molluscicidal compounds in order to eliminate the vector of the disease in the infected waters.

The tests were carried out by placing two snails in a 400 ppm aqueous solution of the extract. The mortality was checked after 24 hours by observing the heartbeats of the snails with a microscope.

5.8 Physical constants and spectral data of isolated compounds

Compound A



Mimengoside A, ilwensisaponin A or verbascosaponin.

13, 28 -epoxy-3-*O*- [α-rhamnopyranosyl- (1→4)-β-glucopyranosyl-(1→3)-[β-glucopyranosyl-(1→2)]-β-fucopyranosyl]-olean-11-ene-23-ol.

$C_{54}H_{88}O_{21}$

MW 1072

White amorphous powder

$[\alpha]_D^{20} =$ +31.7 (MeOH, c.0.1)

Mp 263-268 °C

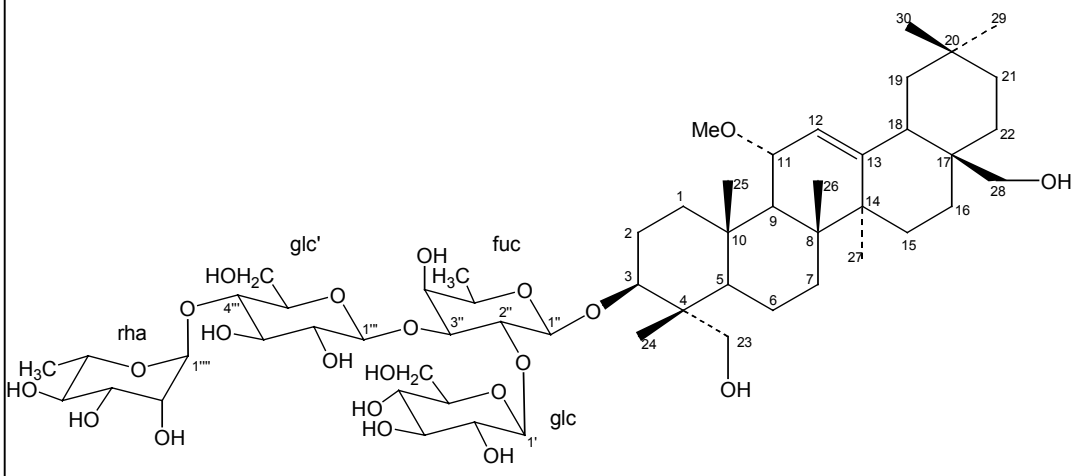
ESI-MS *m/z* (rel. Int.): 1073.0 $[M+H]^+$ (100), 927.1 (4), 911.1 (3), 765.3 (3), 603.3 (5), 577.2 (3), 457.0 (1), 439.3 (4).

APCI-MS *m/z* (rel. Int.): 1114.7 $[M-H+adduct]^-$ (100), 1071.9 (25).

^{1}H NMR (500 MHz, pyridine-*d5*): see Table 7

^{13}C NMR (125 MHz, pyridine-*d5*): see Table 8

Compound B



Mimengoside B, or ilwensisaponin C.

3-*O*-[α-rhamnopyranosyl-(1→4)-β-glucopyranosyl-(1→3)-[β-glucopyranosyl-(1→2)]-β-fucopyranosyl]-11-methoxy-olean-12-ene-3, 23, 28-triol.

$C_{55}H_{92}O_{22}$

MW 1104

White amorphous powder

$[\alpha]_D^{20} =$ +1.5 (MeOH, c.0.1)

Mp 279-282 °C

ESI-MS *m/z* (rel. Int.): 1105.3 $[M+H]^+$ (100), 957.2 (2), 941 (10), 795.4 (5).

APCI-MS *m/z* (rel. Int.): 1146.8 $[M-H+adduct]^-$ (100), 1103.9 (35).

^{1}H NMR (500 MHz, pyridine-*d5*): see Table 7

^{13}C NMR (125 MHz, pyridine-*d5*): see Table 8

Table 7: ^{1}H NMR signals of compounds **A** and **B** (500 MHz, in pyridin-*d5*, TMS as internal standard); chemical shifts in ppm.

^{1}H	A	B	^{1}H	A	B
1	1.04 u	1.51 m	T Glc		
	1.83 u	2.05 u	1'	5.59 d, *J*=7.8 Hz	5.60 s
2	1.06 u	2.03 u	2'	3.94 t, *J*=8.3 Hz	4.09 u
	1.78 u	2.26 m	3'	4.18 m	4.20 u
3	4.14 u	4.13 u	4'	4.12 m	4.20 u
5	1.58 u	1.65 u	5'	3.60 m	3.66 m
6	1.57 u	0.98 u	6'	4.26 u	4.30 m
	1.80 u	n.d.		4.33 u	4.34 u
7	1.18 m	1.18 m	Fuc		
	1.48 m	1.58 m	1''	4.90 d, *J*=7.3 Hz	4.90 d, *J*=7.8 Hz
9	2.01 s	1.97 d, *J*=8.8 Hz	2''	n.d.	4.64 u
11	5.55 dd, *J*=10.3; 2.4 Hz	3.81 u	3''	4.06 m	4.06 m
12	5.95 d, *J*=10.3 Hz	5.50 d, *J*=3.0 Hz	4''	4.20 m	4.15 u
15	1.34 u	0.96 u	5''	3.63 m	3.62 u
	1.41 d, *J*=5.86 Hz	n.d.	6''	1.38 d, *J*=6.3 Hz	1.36 d, *J*=6.3 Hz
16	1.03 u	1.45 u	I Glc		
	1.98 u	1.47 u	1'''	5.28 d, *J*=7.8 Hz	5.25 d, *J*= 7.8 Hz
18	1.73 u	2.38 dd, *J*=13.0;4.0Hz	2'''	3.94 u	3.93 m
19	1.25 m	1.26 u	3'''	4.16 u	4.16 u
	1.73 u	1.86 m	4'''	4.37 u	4.38 u
21	0.93 s	1.24 u	5'''	3.72 u	3.74 u
	n.d.	1.42 u	6'''	4.12 dd *J*=11.1;5.1Hz	4.08 u
22	2.20 m	1.67 u		4.22 m	4.18 u
	2.22 m	2.00 u	Rha		
23	3.74 u	3.74 u	1''''	5.85 s	5.85 m
	4.30 u	4.36 u	2''''	4.53 u	4.66 u
24	1.07 s	1.11 u	3''''	4.51 u	4.52 dd, *J*=9.3; 2.0 Hz
25	1.74 u	1.10 u	4''''	4.34 u	4.32 u
26	0.97 u	0.98 u	6''''	n.d.	4.92 m
27	1.32 u	1.31 s	5''''	1.74 d, *J*=6.4 Hz	1.72 d, *J*=6.4 Hz
28	3.33 d, *J*=6.4 Hz	3.60 u			
	n.d.	3.80 u	OMe		3.22 s
29	0.82 s	0.97 u			
30	1.32 s	0.90 s			

n.d.: not determined

Multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

T Glc: terminal glucose; I Glc: internal glucose

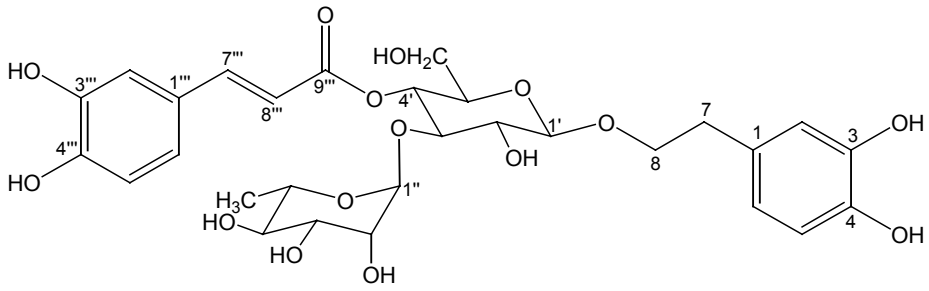
Table 8: ^{13}C NMR signals of compounds **A** and **B** (125 MHz, in pyridin-*d5*); chemical shifts in ppm.

^{13}C	**A**	**B**	**^{13}C**	**A**	**B**
1	38.40	40.07	Term Glc		
2	25.51	26.39	1’	103.94 [a)]	104.03 [a)]
3	82.50	82.92	2’	76.40	76.25
4	43.79	43.92	3’	78.30	78.83
5	47.80	48.24	4’	71.98	72.18
6	17.50	18.39	5’	77.15	77.53
7	31.47	33.21	6’	63.10	63.15
8	41.96	42.04	Fuc		
9	53.67	52.74	1’’	104.03 [a)]	103.97 [a)]
10	36.26	38.16	2’’	77.20	77.16
11	131.67	76.05	3’’	84.65	84.80
12	131.96	122.55	4’’	72.19	72.03
13	84.82	149.38	5’’	70.43	70.41
14	44.07	43.45	6’’	17.18	17.17
15	30.91	26.29	Int Glc		
16	25.81	22.95	1’’’	104.94	104.96
17	41.65	37.46	2’’’	75.51	75.55
18	51.40	42.30	3’’’	76.23	76.48
19	37.28	47.00	4’’’	78.80	78.44
20	31.69	31.25	5’’’	77.15	77.25
21	33.59	34.66	6’’’	61.28	61.37
22	25.99	31.71	Rha		
23	64.59	65.11	1’’’’	102.75	102.81
24	12.68	13.27	2’’’’	72.75	72.60
25	18.63	17.81	3’’’’	72.56	72.18
26	19.53	18.40	4’’’’	73.93	74.00
27	19.81	25.30	5’’’’	70.35	70.47
28	76.98	68.71	6’’’’	18.63	18.53
29	23.54	23.79			
30	35.00	33.31			
MeO		53.91			

Term. Glc: terminal glucose; Int. Glc: internal glucose

[a)] : interchangeable signals

Compound C



Verbascoside, or acteoside.

3,4-dihydroxy-β-phenethyl-*O*-α-rhamnopyranosyl-(1→3)-4-*O*-caffeoyl-β-glucopyranoside.

$C_{29}H_{36}O_{15}$

MW 624

Yellow amorphous powder

$[\alpha]_D^{20} =$ -84.9 (MeOH, c.0.1)

Mp 145-149

UV $\lambda_{\max}^{MeOH} nm$: 330, 290, 246

APCI-MS *m/z* (rel. Int.): 642.0 $[M+H_2O]^+$ (100), 624.9 (8), 479.0 (20), 470.9 (10), 356.9 (15), 325.0 (85), 195.1 (32), 163.2 (55)

ESI-MS *m/z* (rel. Int.): 641.9 $[M+H_2O]^+$ (18), 624.8 (15), 478.7 (55), 324.9 (100), 210.8 (27), 163.0 (48).

^{1}H NMR (500 MHz, methanol-*d4*): see Table 9

^{13}C NMR (125 MHz, methanol-*d4*): see Table 9

Table 9: ^{1}H NMR and ^{13}C signals of compound **C** (500/125 MHz, in methanol-*d4*, TMS as internal standard); chemical shifts in ppm

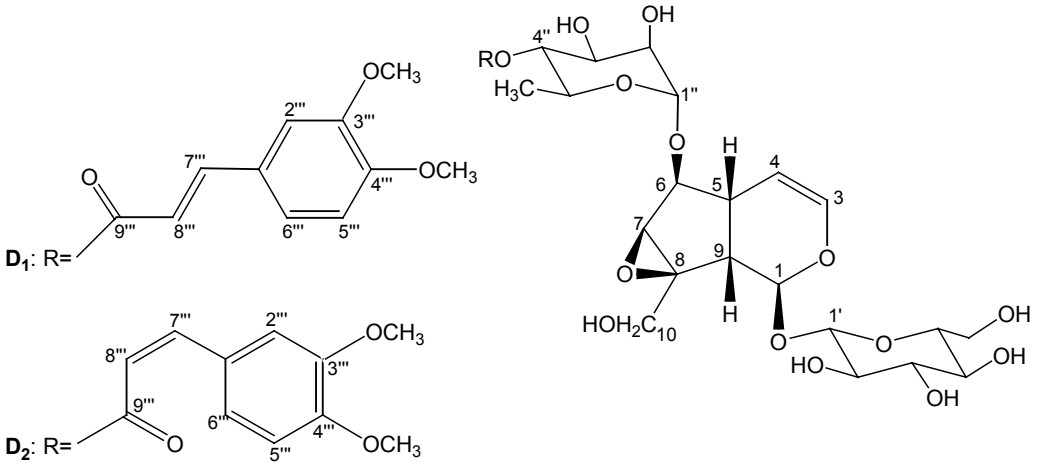
Signals	**^{1}H**	**^{13}C***
PhPr		
1	-	131.41
2	6.56 d, *J*=7.8 Hz	121.24
3	6.67 d, *J*=8.8 Hz	116.27
4	-	144.60
5	-	146.05
6	6.70 s	117.08
7	2.80 s	36.51
8	3.72 q, *J*=8 Hz	72.22
Glc		
1’	4.38 d, *J*= 10.8 Hz	104.42
2’	3.38 t, *J*=8.5 Hz	76.14
3’	3.81 t, *J*=9.0 Hz	81.63
4’	4.90 t, *J*=9.3 Hz	70.51
5’	3.54 m	75.94
6’	3.54-3.56 [a)]	62.30
	3.62 d, *J*=9.8 Hz	
Rha		
1’’	5.20 s	102.99
2’’	3.92 s	72.29
3’’	3.58 t, *J*=10.1 Hz	71.99
4’’	3.28 t, *J*=9.0 Hz	73.73
5’’	3.54-3.56 [a)]	70.38
6’’	1.08 d, *J*=5.9 Hz	18.44
CM		
1’’’	-	127.60
2’’’	7.06 s	115.18
3’’’	-	146.76
4’’’	-	149.72
5’’’	6.79 d, *J*=8.3 Hz	116.48
6’’’	6.95 d, *J*=8.3 Hz	123.21
7’’’	7.60 d, *J*=16.2 Hz	147.98
8’’’	6.27 d, *J*=15.6 Hz	114.64
9’’’	-	168.26

[a)] Signal unclear due to overlapping

Multiplicities: m: multiplet, s: singlet, d: doublet

Ph Pr: phenylpropane; Glc: glucose; Rha: rhamnose; CM: caffeoyl moiety

Compounds D_1/D_2



D_1: Buddlejoside A8

6-*O*-[4–*O*-(3,4-dimethoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] catalpol.

$C_{32}H_{42}O_{17}$
MW 698
White amorphous powder
$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 699.8 $[M+H]^+$ (122), 698.5 (100), 680.9 (5), 518.6 (8), 368.8 (9), 336.9 (42), 191.0 (10)
APCI-MS *m/z* (rel. Int.): 699.1 $[M+H]^+$ (8), 448.9 (10), 368.9 (30), 337.1 (100), 223 (100), 191.2 (100)
LC/^{1}H NMR (500 MHz): see Table 10

D_2:

6-*O*-[4–*O*-(3,4-dimethoxy-*Z*-cinnamoyl)-α-rhamnopyranosyl] catalpol

$C_{32}H_{42}O_{17}$
MW 698
White amorphous powder
$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 699.6 $[M+H]^+$ (6), 698.4 (20), 680.8 (35), 662.9 (20), 518.7 (20), 500.9 (23), 368.5 (30), 336.9 (100), 191.0 (65)
APCI-MS *m/z* (rel. Int.): 699.2 $[M+H]^+$ (8), 368.7 (25), 337.1 (80), 307.0 (37), 223 (92), 191.2 (100)
LC/^{1}H NMR (500 MHz): see Table 10

In-mixture analysis:

DC/I-MS (CH_4): *m/z* (rel. Int.) *m/z* 698.4 $[M]^-$ (54), 498.3 (28), 354.3 (30), 352.3 (65), 308.3 (90), 207.3 (100), 146.3 (62).
^{1}H NMR (500 MHz): see Table 10
^{13}C NMR (125 MHz) see Table 12

Table 10: ^{1}H NMR signals of compounds **D_1** and **D_2** in-mixture in methanol-*d4*; and LC/^{1}H-NMR data of **D_1** and **D_2** in methanol-D_2O (500 MHz, TMS as internal standard) chemical shifts in ppm

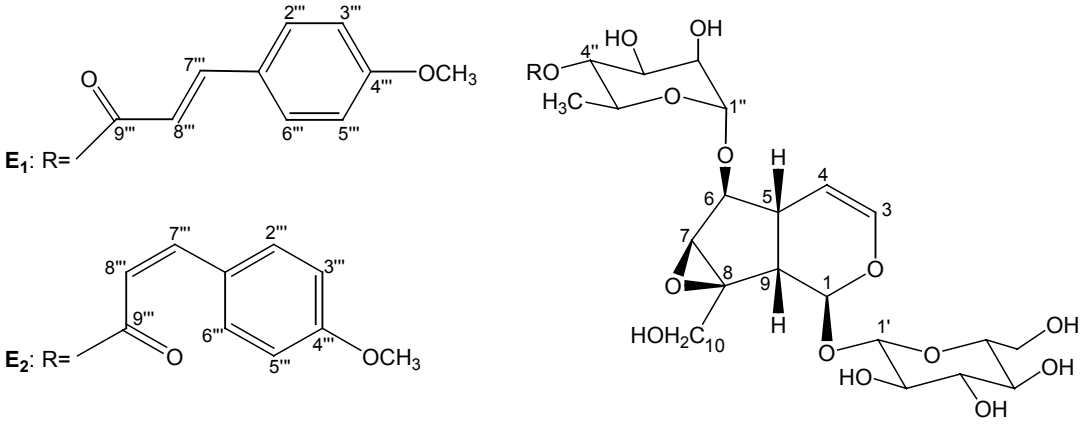
In-mixture ^{1}H-NMR analyses (methanol-*d4*)			**LC/^{1}H-NMR analyses**		
^{1}H	**D_1**	**D_2**	**^{1}H**	**D_1**	**D_2**
1	5.09 u	idem	1	4.80- 5.00 [a)]	4.85- 5.00 [a)]
3	6.38 t, *J*=5.6 Hz	idem	3	6.35 d, *J*=6.0 Hz	6.34 d, *J*=5.0 Hz
4	5.06 u	idem	4	4.80- 5.00 [a)]	4.85- 5.00 [a)]
5	2.42 m	idem	5	2.37 m	2.31 m
6	4.03 d, *J*=8.3 Hz	4.01 d, *J*=8.3 Hz	6	4.01 d, *J*=8.3 Hz	3.95 d, *J*=7.7 Hz
7	3.67 u	idem	7	3.10- 4.00 [a)]	3.10- 4.00 [a)]
9	2.56 m	idem	9	2.56 t, *J*=7.7 Hz	2.55 t, *J*=8.5 Hz
10	3.83 u	idem	10	3.94 d, *J*=12.1 Hz	3.92 m
	4.15 dd, *J*=13.2;3.4Hz	idem		4.14 d, *J*=13.2 Hz	4.13 d, *J*=13.2 Hz
Glc					
1'	4.78 u	idem	1'	[b)]	4.77 d, *J*=8.2 Hz
2'	3.28 u	idem	2'	3.10- 4.00 [a)]	3.10- 4.00 [a)]
3'	3.41 t *J*= 9 Hz	idem	3'	3.10- 4.00 [a)]	3.10- 4.00 [a)]
4'	3.27 u	idem	4'	3.10- 4.00 [a)]	3.10- 4.00 [a)]
5'	3.33 u	idem	5'	3.10- 4.00 [a)]	3.10- 4.00 [a)]
6'	3.63 u	idem	6'	3.10- 4.00 [a)]	3.10- 4.00 [a)]
	3.92 u	idem		3.10- 4.00 [a)]	3.10- 4.00 [a)]
Rha					
1''	5.01 u	5.00 m	1''	4.80- 5.00 [a)]	4.85- 5.00 [a)]
2''	3.94 u	3.94 m	2''	3.10- 4.00 [a)]	3.10- 4.00 [a)]
3''	3.95 u	3.88 m	3''	3.10- 4.00 [a)]	3.10- 4.00 [a)]
4''	5.08 u	5.04 m	4''	4.80- 5.00 [a)]	4.85- 5.00 [a)]
5''	3.90 u	3.81 m	5''	3.10- 4.00 [a)]	3.10- 4.00 [a)]
6''	1.18 d, *J*=6.4 Hz	1.16 d, *J*=6.4 Hz	6''	1.12 d, *J*=6.1 Hz	1.07 d, *J*=6.1 Hz
CM					
2'''	7.23 u	7.72 br s	2'''	7.19- 7.20 [a)]	7.36 s
3'''	-	-	3'''	-	-
5'''	6.98 d ,*J*=8.3 Hz	6.92 d, *J*=8.3 Hz	5'''	6.99 d ,*J*=8.8 Hz	6.93 d, *J*=8.8 Hz
6'''	7.19 d, *J*=8.3 Hz	7.25 m	6'''	7.19- 7.20 [a)]	7.12 d, *J*=8.8 Hz
7'''	7.67 d, *J*=16 Hz	6.94 d, *J*=12.7 Hz	7'''	7.64 d, *J*=15.9 Hz	7.02 d, *J*=12.1 Hz
8'''	6.46 d, *J*=16 hz	5.90 d, *J*=12.7 Hz	8'''	6.40 d, *J*=15.9 Hz	5.88 d, *J*=12.6 Hz
MeO 3	3.86	3.84	MeO 3	3.82	3.78
MeO 4	3.86	3.85	MeO 4	3.82	3.83

[a)] : signal pattern unclear due to overlapping; [b)] overlapped with suppressed solvent signal

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

CM: cinnamoyl moiety

Compounds E_1/E_2



E_1: Verbascoside A

6-*O*-[4–*O*-(4- methoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] catalpol.

$C_{31}H_{40}O_{16}$

MW 668

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 669.7 $[M+H]^+$ (25), 668.6 (100), 651.0 (5), 488.9 (6), 338.9 (12), 307.0 (32), 161.1 (12).

APCI-MS *m/z* (rel. Int.): 669.9 $[M+H]^+$ (8), 368.9 (30), 337.1 (90), 223 (100), 191.2 (95).

LC/^{1}H NMR (500 MHz): see Table 11

E_2:

6-*O*-[4–*O*-(4- methoxy-*Z*-cinnamoyl)-α-rhamnopyranosyl] catalpol

$C_{31}H_{40}O_{16}$

MW 668

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 669.4 $[M+H]^+$ (25), 668.6 (50), 650.9 (18), 632.5 (16), 488.8 (22), 471.0 (20), 338.7 (32), 306.9 (100), 161.0 (50).

APCI-MS *m/z* (rel. Int.): 669.1 $[M+H]^+$ (8), 338.9 (100), 307.0 (100), 193.1 (50), 161.2 (62).

LC/^{1}H NMR (500 MHz): see Table 11

In-mixture analyses:

DC/I-MS (CH_4): *m/z* (rel. Int.): *m/z* 668.4 $[M]^-$ (32), 324.3 (28), 306.3 (12), 177.2 (100), 146.3 (48).

1H NMR (500 MHz): see Table 11

^{13}C NMR (125 MHz): see Table 12

Table 11: ^{1}H NMR signals of compounds **E_1** and **E_2** in-mixture in methanol-*d4*; and LC/^{1}H-NMR data of **E_1** and **E_2** in acetonitrile-D_2O (500 MHz, TMS as int. standard) chemical shifts in ppm

In-mixture ^{1}H-NMR analyses (methanol-*d4*)			**LC/^{1}H-NMR analyses**		
^{1}H	**E_1**	**E_2**	**^{1}H**	**E_1**	**E_2**
1	5.14 u	5.10 u	1	4.90- 5.10 [a)]	4.80- 4.95 [a)]
3	6.38 dd, *J*= 7.3, 1.5 Hz	idem	3	6.38 d, *J*= 6.0 Hz	6.31 d, *J*= 6.0 Hz
4	5.06 u	4.98 u	4	4.90- 5.10 [a)]	4.80- 4.95 [a)]
5	2.42 m	idem	5	2.39 m	2.26, m
6	4.03 d, *J*= 7.8 Hz	4.02 d, *J*= 8.3 Hz	6	4.03 d, *J*= 8.2 Hz	3.93 d, *J*= 7.7 Hz
7	3.66 u	idem	7	3.20- 4.00 [a)]	3.20-4.00 [a)]
9	2.58 m	2.55 m	9	2.59 t, *J*= 9.3 Hz	2.52 t, *J*= 8.8 Hz
10	3.81 u	idem	10	3.81 d, *J*= 12.1 Hz	3.20- 4.00 [a)]
	4.16 dd, *J*= 13.2, 3.9 Hz	idem		4.15 d, *J*= 13.2 Hz	4.10 d, *J*= 13.2 Hz
Glc					
1'	4.78 dd, *J*= 8.3, 2.0 Hz	idem	1'	4.77 d, *J*= 8.2 Hz	4.70 d, *J*= 7.7 Hz
2'	3.27 u	idem	2'	3.20- 4.00 [a)]	3.20- 4.00 [a)]
3'	3.41 t *J*= 9 Hz	idem	3'	3.20- 4.00 [a)]	3.20- 4.00 [a)]
4'	3.26 u	idem	4'	3.20- 4.00 [a)]	3.20- 4.00 [a)]
5'	3.33 u	idem	5'	3.20- 4.00 [a)]	3.20- 4.00 [a)]
6'	3.64 u	idem	6'	3.20- 4.00 [a)]	3.20- 4.00 [a)]
	3.94 u	idem		3.20- 4.00 [a)]	3.20- 4.00 [a)]
Rha					
1''	5.01 u	idem	1''	4.90- 5.10 [a)]	4.80- 4.95 [a)]
2''	3.92 u	3.94 u	2''	3.20- 4.00 [a)]	3.20- 4.00 [a)]
3''	3.95 u	3.86 u	3''	3.20- 4.00 [a)]	3.20- 4.00 [a)]
4''	5.09 u	5.02 u	4''	4.90- 5.10 [a)]	4.80- 4.95 [a)]
5''	3.89 u	3.78 u	5''	3.20- 4.00 [a)]	3.20- 4.00 [a)]
6''	1.18 d, *J*= 5.9 Hz	1.15 d, *J*= 6.3 Hz	6''	1.15 d, *J*= 6.0 Hz	1.05 d, *J*= 6.0 Hz
CM					
2'''	7.57 d, *J*= 8.8 Hz	7.72 d, *J*= 8.8 Hz	2'''	7.61 d, *J*= 8.2 Hz	7.50 d, *J*= 8.8 Hz
3'''	6.99 d, *J*= 8.8 Hz	6.90 d, *J*= 8.8 Hz	3'''	7.01 d, *J*= 8.8 Hz	6.89 d, *J*= 8.8 Hz
5'''	6.99 d, *J*= 8.8 Hz	6.90 d *J*= 8.8 Hz	5'''	7.01 d, *J*= 8.8 Hz	6.89 d, *J*= 8.8 Hz
6'''	7.57 d, *J*= 8.8 Hz	7.72 d, *J*= 8.8 Hz	6'''	7.61 d, *J*= 8.2 Hz	7.50 d, *J*= 8.8 Hz
7'''	7.69 d, *J*= 17 Hz	6.98 d, *J*= 12.5 Hz	7'''	7.72 d, *J*= 15.9 Hz	7.04 d, *J*= 12.6 Hz
8'''	6.44 d, *J*= 17 Hz	5.88 d, *J*= 12.5 Hz	8'''	6.42 d, *J*= 15.9 Hz	5.84 d, *J*= 12.6 Hz
MeO 4	3.83 s	3.81 s	MeO 4	3.82 s	3.74 s

[a)] : signal pattern unclear due to overlapping

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

CM: cinnamoyl moiety

Table 12: ^{13}C NMR signals of compounds **D_1/D_2** and **E_1/E_2** in-mixture (125 MHz, in methanol-*d4*); chemical shifts in ppm

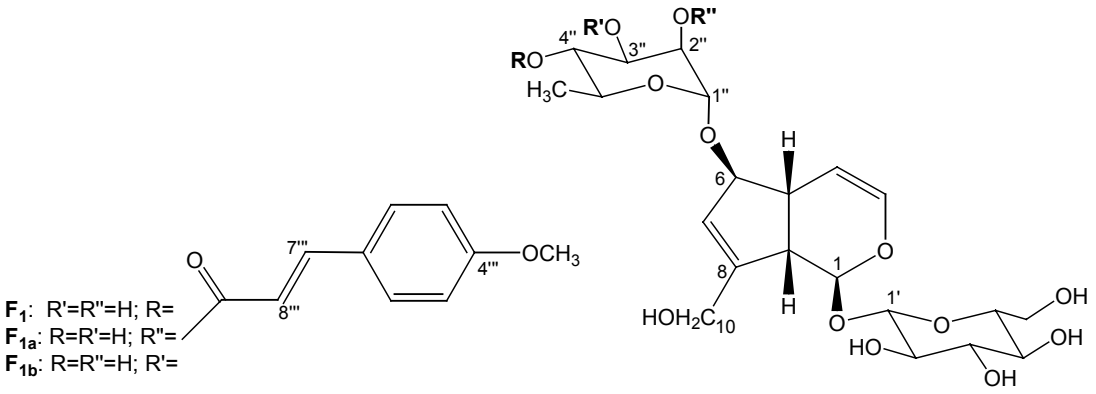
^{13}C	**D_1**	**D_2**	**E_1**	**E_2**
1	95.19	idem	95.16	idem
3	142.30	idem	142.30	idem
4	103.44	idem	103.44	103.41
5	37.27	37.30	37.30	37.25
6	84.02	84.14	84.12	83.89
7	59.47	59.40	59.35	59.46
8	66.57	idem	66.58	idem
9	43.28	43.31	43.24	43.28
10	61.43	idem	61.43	idem
Glc				
1’	99.72	idem	99.69	idem
2’	74.81	idem	74.80	idem
3’	77.68	idem	77.65	idem
4’	71.75	idem	71.74	idem
5’	78.58	idem	78.59	idem
6’	62.91	idem	62.90	idem
Rha				
1’’	100.44	100.34	100.44	100.27
2’’	72.44	idem	72.38	72.43
3’’	70.30	70.21	70.26	70.17
4’’	75.37	75.11	75.33	75.06
5’’	68.27	68.14	68.27	68.11
6’’	17.86	idem	17.87	idem
CM				
1’’’	128.81	129.20	128.32	128.76
2’’’	111.65	115.02	131.05	133.40
3’’’	150.77	149.67	115.44	114.34
4’’’	152.88	151.75	163.20	162.05
5’’’	112.68	111.94	115.44	114.34
6’’’	124.03	126.25	131.05	133.40
7’’’	146.75	145.43	146.53	145.14
8’’’	116.43	117.64	116.05	117.62
9’’’	168.68	167.58	168.73	167.60
MeO(3’’’)	56.44[a]	56.36[b]	-	-
MeO(4’’’)	56.54[a]	56.51[b]	55.77	55.89

CM: cinnamoyl moiety

[a)] : interchangeable in D_1

[b)] : interchangeable in D_2

Compounds $F_1/F_{1a}/F_{1b}$



F_1: 6-*O*-[4–*O*- methoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] aucubin

$C_{31}H_{40}O_{15}$

MW 652

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 669.7 $[M+H_2O]^+$ (25), 652.5 (12), 634.9 (28), 472.9 (17), 324.8 (100), 307.0 (80), 161.1 (28)

F_{1a}: 6-*O*-[2–*O*-(4- methoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] aucubin.

$C_{31}H_{40}O_{15}$

MW 652

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 669.7 $[M+H_2O]^+$ (25), 652.5 (4), 634.8 (9), 338.7 (16), 324.8 (24), 306.9 (100), 288.9 (30), 161.0 (40).

F_{1b}: 6-*O*-[3–*O*-(4- methoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] aucubinl

$C_{31}H_{40}O_{15}$

MW 652

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 669.7 $[M+H_2O]^+$ (19), 652.5 (7), 634.3 (5), 472.6 (5), 324.8 (100), 306.8 (65), 161.0 (70).

In –mixture analyses:

1H NMR (500 MHz): see Table 13

^{13}C NMR (125 MHz): see Table 12

Table 13: ^{1}H NMR signals of compounds **F_1**, **F_{1a}** and **F_{1b}** in-mixture (methanol-*d4*, 500 MHz, TMS as internal standard) chemical shifts in ppm

^{1}H	**F_1**	**F_{1a}**	**F_{1b}**
1	4.94 d, *J*= 7.3 Hz	idem	idem
3	6.34 dd, *J*= 5.9; 2.0 Hz	idem	idem
4	5.14 dd, *J*= 5.9; 3.9 Hz	idem	5.17 dd, *J*= 5.9 ; 3.9 Hz
5	2.82 m	idem	idem
6	4.48 m	idem	idem
7	5.88 s	5.89 s	5.89 s
9	2.92 br dd (u)	idem	idem
10	4.18 br d, *J*= 16.1 Hz	idem	idem
	4.38 br d, *J*= 15.6 Hz	idem	idem
Glc			
1’	4.69 dd, *J*= 7.8; 2.0 Hz	idem	idem
2’	3.23 dd, *J*= 5.9; 9.3 Hz	idem	idem
3’	3.38 t, *J*= 8.5 Hz	idem	idem
4’	3.30 u	idem	idem
5’	3.27 u	idem	idem
6’	3.64 dd, *J*= 12.0; 5.0 Hz	idem	idem
	3.86 u	idem	idem
Rha			
1’’	4.87 d, *J*= 1.8 Hz	4.91 d, *J*= 1.6 Hz	4.85 d, *J*= 1.6 Hz
2’’	3.83 u	5.06 u	4.0 m
3’’	3.88 u	3.88 u	5.06 u
4’’	5.08 u	3.49 t, *J*= 9.8 Hz	3.68 u
5’’	3.91 u	3.76 u	3.84 u
6’’	1.16 d, *J*= 6.4 Hz	1.31 d, *J*= 6.4 Hz	1.32 d, *J*= 6.4 Hz
CM			
2’’’	7.57 d, *J*= 8.8 Hz	7.21 d, *J*= 1.9 Hz	7.25 d, *J*= 1.9 Hz
3’’’	6.96 d, *J*= 8.8 Hz	idem	idem
5’’’	6.96 d, *J*= 8.8 Hz	idem	idem
6’’’	7.57 d, *J*= 8.8 Hz	7.17 d, *J*= 8.3 Hz	7.18 d, *J*= 8.3 Hz
7’’’	7.68 d, *J*= 16.1 Hz	7.68 d, *J*= 16.1 Hz	7.72 d, *J*= 16.1 Hz
8’’’	6.43 d, *J*= 16.1 Hz	6.48 d, *J*= 16.1 Hz	6.46 d, *J*= 16.1 Hz
MeO	3.82 s	idem	idem

CM: cinnamoyl moiety

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

Idem: identical signals between the three forms

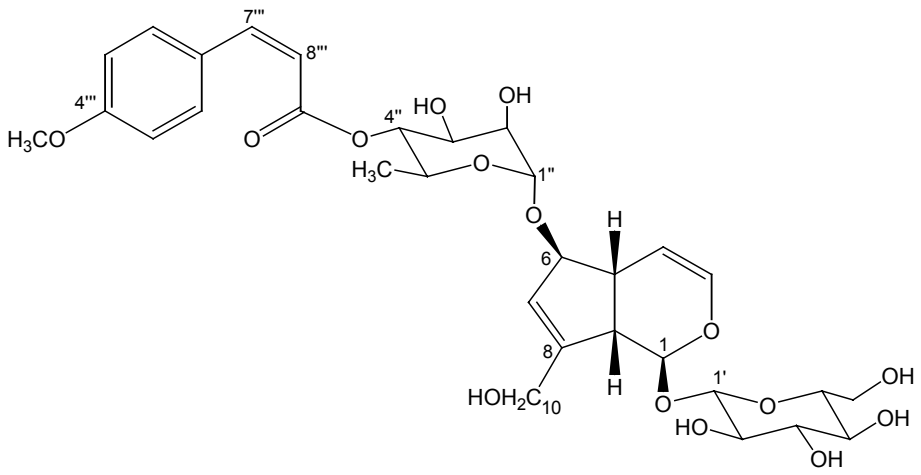
Table 14: ^{13}C NMR signals of compounds **F_1/F_{1a}/F_{1b}** in-mixture (125 MHz, in methanol-*d4*); chemical shifts in ppm

^1H	**F_1**	**F_{1a}**	**F_{1b}**
1	97.99	98.42	98.06
3	142.0	idem	idem
4	105.50	105.45	105.57
5	44.29	idem	idem
6	89.33	89.41	89.07
7	127.18	idem	idem
8	149.66	149.80	149.60
9	48.21	idem	idem
10	61.46	idem	idem
Glc			
1’	100.02	idem	idem
2’	74.95	idem	idem
3’	77.93	idem	idem
4’	71.59	idem	idem
5’	78.29	idem	idem
6’	62.69	idem	idem
Rha			
1’’	101.26	98.42	101.11
2’’	72.71	74.50	70.45
3’’	70.41	70.45	75.50
4’’	75.54	74.35	71.45
5’’	68.32	70.30	70.45
6’’	17.93	18.10	18.05
CM			
1’’’	128.82	128.45	128.39
2’’’	131.03	idem	idem
3’’’	115.46	idem	Idem
4’’’	163.24	idem	idem
5’’’	115.46	idem	idem
6’’’	131.03	idem	Idem
7’’’	146.48	146.70	146.29
8’’’	116.14	115.94	116.40
9’’’	168.75	168.25	167.75
MeO	55.90	idem	idem

CM: cinnamoyl moiety

Idem: identical signals between the three forms

Compound F_2



6-*O*-[3–*O*-(4-methoxy-*Z*-cinnamoyl)-α-rhamnopyranosyl] aucubin

$C_{31}H_{40}O_{15}$
MW 652
White amorphous powder

$[\alpha]_D^{20} =$ -95.5 (MeOH, c.0.1)

UV $\lambda_{\max}^{MeOH} nm$: 228, 312

HR-ESI-MS: 675.22427 $[M + Na]^+$
D/CI-MS *m/z* (rel. Int.) : 669.9 $[M+NH_3]^+$ (4), 652.2 (4), 636.3 (5), 510.3 (62), 476.5 (17), 196.02 (100).

^{1}H NMR (500 MHz, methanol-*d4*): see Table 15
^{13}C NMR (125 MHz, methanol-*d4*): see Table 16

Table 15: ^{1}H NMR signals of the pure compounds **F_2** and **G_2** (methanol-*d4*, 500 MHz, TMS as int. standard) chemical shifts in ppm.

^{1}H	**F_2**	**G_2**
1	4.92 d, *J*= 7.3 Hz	4.92 d, *J*= 7.3 Hz
3	6.35 dd, *J*= 5.9; 1.9 Hz	6.34 dd, *J*= 5.9; 1.96 Hz
4	5.09 d, *J*= 5.9 Hz	5.10 d, *J*= 5.9 Hz
5	2.80 m	2.80 m
6	4.46 m	4.46 m
7	5.88 d	5.88 s
9	2.89 t, *J*= 7.5 Hz	2.92 t, *J*= 7.3 Hz
10	4.18 br d, *J*= 15.6 Hz	4.18 br d, *J*= 15.6 Hz
	4.37 br d, *J*= 15.6 Hz	4.38 br d, *J*= 15.6 Hz
Glc		
1’	4.68 d, *J*= 6.8 Hz	4.69 d, *J*= 7.8 Hz
2’	3.21 t, *J*= 8.0 Hz	3.22 t, *J*= 8.3 Hz
3’	3.37 dd, *J*= 12.2; 5.4 Hz	3.38 t, *J*= 8.3 Hz
4’	3.28 u	3.28 u
5’	3.27 u	3.26 u
6’	3.64 dd, *J*= 12.2; 5.4 Hz	3.65 dd, *J*= 12.0; 5.0 Hz
	3.86 d, *J*= 12.7 Hz	3.85 u
Rha		
1’’	4.85 u	4.86 m
2’’	3.81 u	3.81 u
3’’	3.80 u	3.82 u
4’’	5.01 t, *J*= 9.8 Hz	5.03 t, *J*= 9.3 Hz
5’’	3.82 u	3.83 u
6’’	1.16 d, *J*= 6.4 Hz	1.17 d, *J*= 6.4 Hz
CM		
2’’’	7.72 d, *J*= 8.8 Hz	7.73 s
3’’’	6.90 dd, *J*= 8.8; 1.9 Hz	-
5’’’	6.90 dd, *J*= 8.8; 1.9 Hz	6.94 d, *J*= 8.3 Hz
6’’’	7.72 d, *J*= 8.8 Hz	7.23 dd, *J*= 8.3; 1.9 Hz
7’’’	6.96 d, *J*= 12.7 Hz	6.95 d, *J*= 13.2 Hz
8’’’	5.88 d, *J*= 12.7 Hz	5.90 d, *J*= 13.2 Hz

MeO 4'''	3.81 s	3.85 s
MeO 3'''	-	3.84 s

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals
CM: cinnamoyl moiety

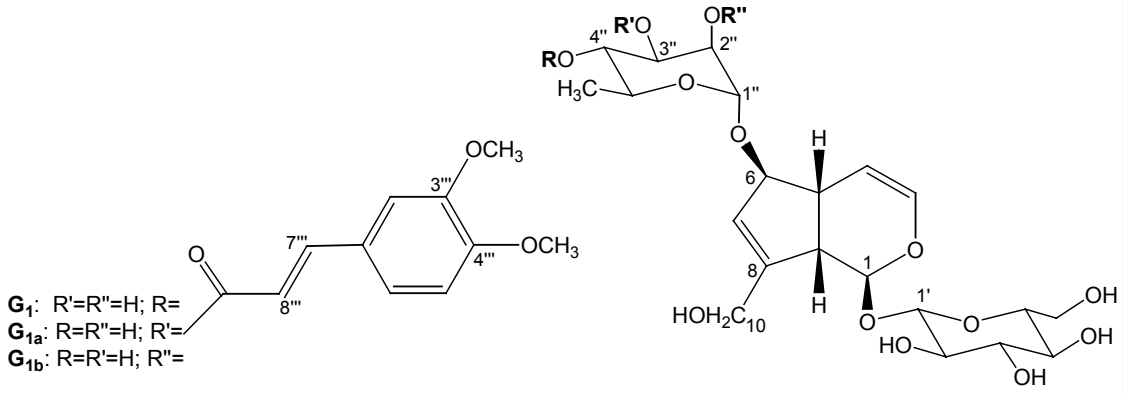
Table 16: ^{13}C NMR signals of the pure compounds **F_2** and **G_2** (125 MHz, in methanol-*d4*); chemical shifts in ppm

^{13}C	**F_2**	**G_2**
1	98.00	97.96
3	142.01	142.00
4	105.48	105.49
5	44.34	44.29
6	89.21	89.24
7	127.12	127.12
8	149.62	149.64
9	48.17	48.18
10	61.47	61.46
Glc		
1'	99.97	99.96
2'	74.94	74.94
3'	77.92	77.90
4'	71.58	71.57
5'	78.32	78.29
6'	62.68	62.67
Rha		
1''	101.19	101.20
2''	72.67	72.66
3''	70.31	70.32
4''	75.24	75.27
5''	68.18	68.19
6''	17.93	17.95
CM		
1'''	128.80	129.25
2'''	133.42	114.97
3'''	114.36	149.68
4'''	162.10	151.76
5'''	114.36	111.89
6'''	133.42	126.24
7'''	145.07	145.42
8'''	117.66	117.71

9'''	167.54	167.60
MeO	55.78	56.35
MeO (4''')	-	56.48

CM: cinnamoyl moiety

Compounds $G_1/G_{1a}/G_{1b}$



G_1: R'=R"=H; R=
G_{1a}: R=R"=H; R'=
G_{1b}: R=R'=H; R"=

G_1.: 6-*O*-[4–*O*-(3,4- dimethoxy-*E*-cinnamoyl)-α-rhamnopyranosyl] aucubin

$C_{32}H_{42}O_{16}$

MW 682

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 682.6 $[M+H]^+$ (5), 664.2 (22), 502.9 (20), 354.8 (54), 337.0 (100), 318.9 (5), 301.1 (10), 278.9 (20), 248.8 (10), 214.0 (18), 191.1 (39), 163.1 (14).

G_{1a}:6-*O*-[3–*O*-(3,4-dimethoxy-*E*-cinnamoyl)-α-rhamnopyranoside] aucubin.

$C_{32}H_{42}O_{16}$

MW 682

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 682.7 $[M+H]^+$ (10), 664.8 (22), 502.8 (20), 369.9 (5), 354.9 (60), 337.0 (100), 318.9 (8), 301.1 (10), 191.1 (55), 163.0 (5).

G_{1b}: 6-*O*-[2–*O*-(3,4-dimethoxy-*E*-cinnamoyl) -α-rhamnopyranoside] aucubinl

$C_{32}H_{42}O_{16}$

MW 682

White amorphous powder

$[\alpha]_D^{20}$, Mp, and UV $\lambda_{\max}^{MeOH} nm$ not determined (mixture)

ESI-MS *m/z* (rel. Int.): 682.6 $[M+H]^+$ (5), 664.7 (22), 502.9 (8), 368.6 (5), 354.8 (40), 337.0 (100), 318.9 (30), 191.0 (55), 163.0 (4).

In –mixture analyses:

1H NMR (500 MHz): see Table 13

^{13}C NMR (125 MHz): see Table 12

TMS as internal standard) chemical shifts in ppm

^{1}H	G_1	G_{1a}	G_{1b}
1	4.93 d, *J*= 7.3 Hz	idem	idem
3	6.34 dd, *J*= 6.4; 2.0 Hz	idem	idem
4	5.14 dd, *J*= 5.9; 3.9 Hz	idem	5.17 dd, *J*= 5.9 ; 3.9 Hz
5	2.82 m	idem	idem
6	4.48 m	idem	idem
7	5.88 s	5.89 s	5.89 s
9	2.92 t, *J*= 7.3 Hz	idem	idem
10	4.18 br d, *J*= 16.1 Hz	idem	idem
	4.38 br d, *J*= 15.6 Hz	idem	idem
Glc			
1’	4.70 d, *J*= 7.8 Hz	idem	idem
2’	3.22 dd, *J*= 8.5 Hz	idem	idem
3’	3.38 t, *J*= 8.8 Hz	idem	idem
4’	3.28 u	idem	idem
5’	3.27 u	idem	idem
6’	3.64 dd, *J*= 12.0; 5.0 Hz	idem	idem
	3.86 u	idem	idem
Rha			
1’’	4.88 d, *J*= 1.5 Hz	4.91 d, *J*= 1.6 Hz	4.85 d, *J*= 1.6 Hz
2’’	3.85 u	5.09 u	4.00 m
3’’	3.88 u	3.89 u	5.06 u
4’’	5.07 t, *J*= 9.8 Hz	3.49 t, *J*= 9.8 Hz	3.70 m
5’’	3.92 u	3.85 u	3.78 m
6’’	1.20 d, *J*= 6.4 Hz	1.32 d, *J*= 6.4 Hz [a)]	1.32 d, *J*= 6.4 Hz [a)]
CM			
2’’’	7.24 d, *J*= 1.9 Hz	7.21 d, *J*=1.9 Hz	7.25 d, *J*= 1.9 Hz
3’’’	-	-	-
5’’’	6.99 d, *J*= 8.3 Hz	idem	idem
6’’’	7.19 d, *J*= 8.3 Hz	7.17 d, *J*= 8.3 Hz	7.18 d, *J*= 8.3 Hz
7’’’	7.67 d, *J*= 16.1 Hz	7.68 d, *J*= 16.1 Hz	7.70 d, *J*= 16.1 Hz
8’’’	6.44 d, *J*= 16.1 Hz	6.48 d, *J*= 16.1 Hz	6.48 d, *J*= 16.1 Hz
MeO 4	3.86 s	idem	idem

MeO 3	3.86 s		idem

CM: cinnamoyl moiety

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

Idem: identical signals between the three forms

[a)] multiplicity and coupling constant taken from LC/^{1}H-NMR

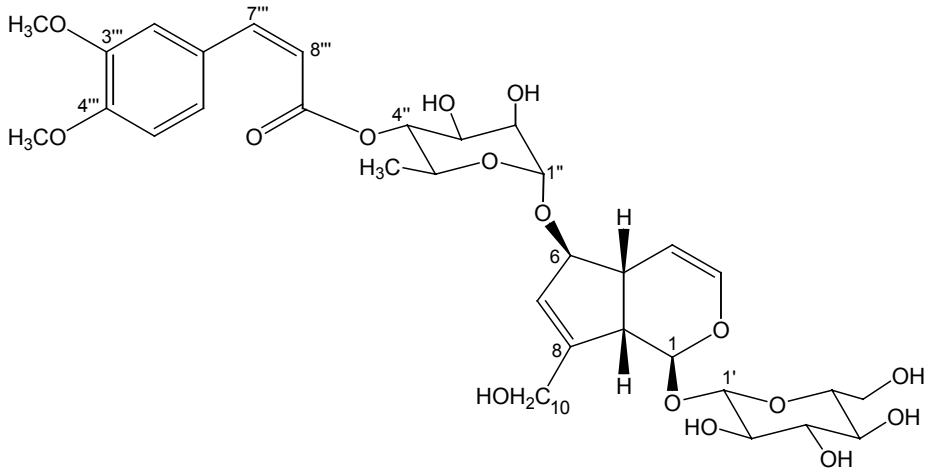
Table 18: ^{13}C NMR signals of compounds **G_1/G_{1a}/G_{1b}** in-mixture (125 MHz, in methanol-*d4*); chemical shifts in ppm

^1H	**G_1**	**G_{1a}**	**G_{1b}**
1	97.99	idem	98.05
3	142.01	idem	idem
4	105.47	105.45	105.50
5	44.30	idem	idem
6	89.32	89.40	89.06
7	127.15	idem	idem
8	149.62	idem	idem
9	48.49	idem	idem
10	61.44	idem	idem
Glc			
1’	99.98	idem	idem
2’	74.91	idem	idem
3’	77.75	idem	idem
4’	71.54	idem	idem
5’	78.26	idem	idem
6’	62.65	idem	idem
Rha			
1’’	101.25	98.20	101.09
2’’	72.67	74.47	70.45
3’’	70.39	70.45	75.50
4’’	75.51	74.47	71.45
5’’	68.30	70.30	70.45
6’’	17.94	18.10	18.10
CM			
1’’’	128.81	idem	128.90
2’’’	111.59	idem	idem
3’’’	150.77	idem	Idem
4’’’	152.88	idem	idem
5’’’	112.64	idem	idem
6’’’	124.03	124.23	123.95
7’’’	146.73	146.93	146.53
8’’’	116.44	116.23	116.69
9’’’	168.67	idem	168.62
MeO 3’’’	56.42	idem	idem
MeO 4’’’	56.51	idem	idem

CM: cinnamoyl moiety

Idem: identical signals between the three forms

Compound G_2



6-*O*-[3–*O*-(3,4-dimethoxy-*Z*-cinnamoyl)-α-rhamnopyranosyl] aucubin

$C_{32}H_{42}O_{16}$
MW 682
White amorphous powder

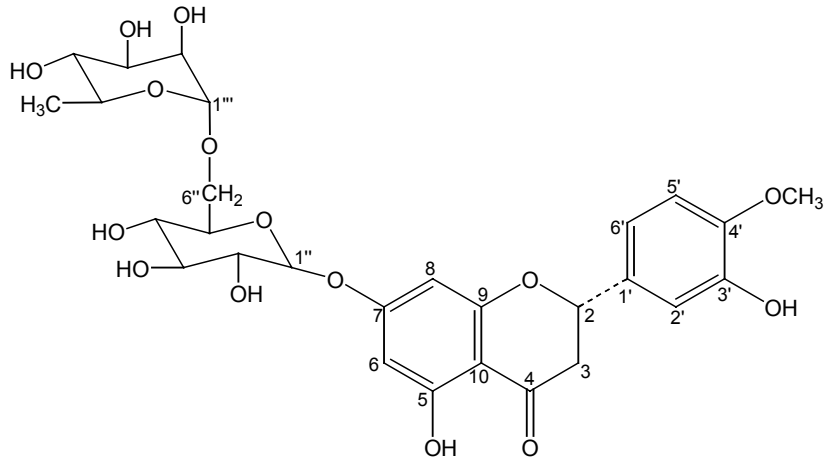
$[\alpha]_D^{20} =$ -95.5 (MeOH, c.0.1)

UV $\lambda_{\max}^{MeOH} nm$: 218, 330

HR-ESI-MS: *m/z* 705.23527 $[M + Na]^+$
APCI-MS *m/z* (rel. Int.): 699.9 $[M+H_2O]^+$ (6), 682.5 (3), 665.2 (3), 368.8 (30), 355.5 (20), 337.0 (95), 319.0 (20), 301.1 (10), 251.1 (25), 223.0 (85), 191.3 (100)

^{1}H NMR (500 MHz, methanol-*d4*): see Table 15
^{13}C NMR (125 MHz, methanol-*d4*): see Table 16

Compound H



Hesperidin:

7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl]-3', 5, 7-trihydroxy-4'-methoxyflavanone

$C_{28}H_{34}O_{15}$

MW 610

Yellow powder

$[\alpha]_D^{20} =$ -47.3 (pyr, c.0.1)

Mp 258-262 °C

UV $\lambda_{\max}^{MeOH} nm$: 286, sh 330

D/CI-MS *m/z* (rel. Int.) : 611.2 $[M+H]^+$ (15), 326.1 (22), 303.0 (100)

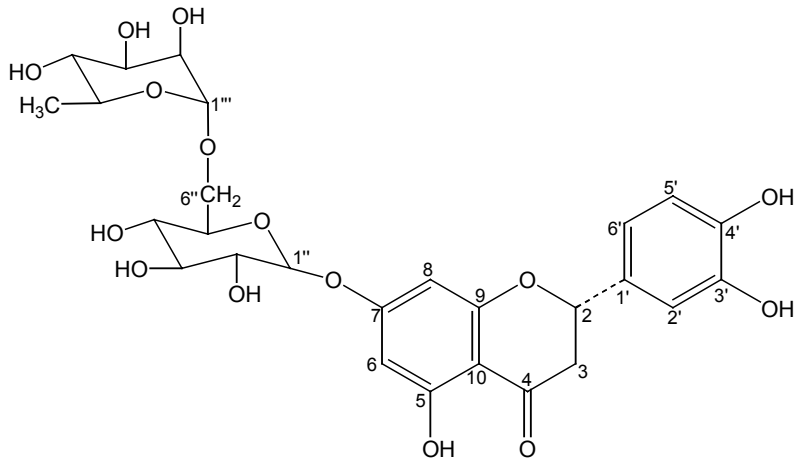
APCI-MS *m/z* (rel. Int.) : 612.0 $[M+H]^+$ (25), 610.9 (100), 465.0 (35), 449.1 (20), 303.2 (100)

ESI-MS *m/z* (rel. Int.): *m/z* 611.8 $[M+H]^+$ (20), 610.8 (100) 464.8 (10), 303.0 (10)

^{1}H NMR (500 MHz, methanol-*d4*): see Table 19

^{13}C NMR (125 MHz, methanol-*d4*): see Table 20

Compound I



Eriocitrin, or eriodictioside:

7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl]-3', 4', 5, 7 –tetrahydroxyflavanone

$C_{27}H_{32}O_{15}$

MW 596

Yellow powder

$[\alpha]_D^{20} =$ -62 (MeOH, c.0.1)

Mp 167-172 °C

UV $\lambda_{\max}^{MeOH} nm$: 288, sh 333

APCI-MS *m/z* (rel. Int.): 598.1 $[M+H]^+$ (25), 596.9 (87), 450.8 (48), 289.1 (100)

ESI-MS *m/z* (rel. Int.): 597.7 $[M+H]^+$ (22), 596.7 (100) 450.6 (18), 355.1 (12), 288.9 (15), 278.8 (78).

^{1}H NMR (500 MHz, pyridin-*d5*): see Table 19

^{13}C NMR (125 MHz, pyridin-*d5*): see Table 20

Table 19: ^{1}H NMR signals of the pure compounds **H** and **I** (pyridin-*d5*, 500 MHz, TMS as int. standard) chemical shifts in ppm

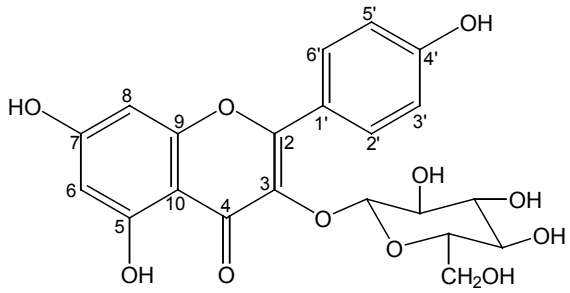
^{1}H	**H**	**I**
2	5.50 dd, *J*= 12.2; 2.9 Hz	5.44 m
3	2.77 dd, *J*= 17.1; 2.9 Hz	2.74 m
	3.28 m	3.26 u
5	-	-
6	6.12 d, *J*= 2.0 Hz	6.11 t, *J*= 2.4 Hz
7	-	-
8	6.14 d, *J*= 2.4 Hz	6.14 m
2’	6.93 u	6.76 m
3’	-	-
4’	-	-
5’	6.95 d, *J*= 8.3 Hz	6.70 m
6’	6.90 t, *J*= 8.3 Hz	6.89 s
Glc		
1’’	4.97 d, *J*= 7.3 Hz	4.98 t, *J*= 7.3 Hz
2’’	3.27 u	3.27 u
3’’	3.22 m	3.22 m
4’’	3.12 t, *J*= 9.3 Hz	3.13 dd, *J*= 9 ; 5 Hz
5’’	3.53 m	3.53 m
6’’	3.42 u	3.42 u
	3.80 m	3.80 d, *J*= 10.3 Hz
Rha		
1’’’	4.52 s	4.52 s
2’’’	3.64 m	3.64 m
3’’’	3.43 u	3.42 u
4’’’	3.14 t, *J*= 9.3 Hz	3.15 d, *J*= 10.2 Hz
5’’’	3.40 u	3.40 u
6’’’	1.08 d, *J*= 6.3 Hz	1.09 d, *J*= 6.3 Hz

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

Table 20: ^{13}C NMR signals of the pure compounds **H** and **I** (125 MHz, in methanol-*d4*); chemical shifts in ppm

^{13}C	**H**	**I**
2	78.50	79.31
3	42.21	43.00
4	198.40	198.21
5	162.64	163.20
6	95.58	96.60
7	165.28	166.18
8	96.52	98.12
9	163.50	164.22
10	103.40	103.40
1'	131.06	130.85
2'	114.23	116.10
3'	146.51	145.81
4'	148.11	146.23
5'	112.23	118.60
6'	118.05	115.01
MeO	55.85	-
Glc		
1''	99.61	100.20
2''	76.42	77.02
3''	73.13	73.50
4''	69.73	70.51
5''	75.58	76.22
6''	66.18	66.80
Rha		
1'''	100.75	101.40
2'''	70.40	71.20
3'''	70.84	71.50
4'''	72.21	72.63
5'''	68.45	69.24
6'''	17.96	18.21

Compound J



Astragalin, or kaempferol 3-glucoside:

3-*O*-β-glucopyranosyl-4', 5, 7-trihydroxyflavone

$C_{21}H_{20}O_{11}$

MW 448

Yellow powder

$[\alpha]_D^{20} =$ +17 (MeOH, c.0.1)

Mp 178°C

UV $\lambda_{\max}^{MeOH} nm$: 267, 350

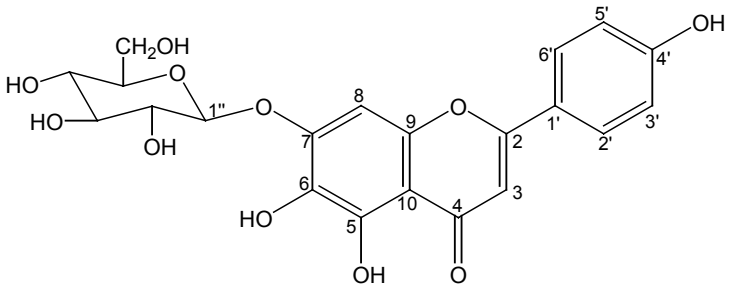
D/CI-MS *m/z* (rel. Int.) : 476.1 $[M+NH_4]^+$ (15), 449.3 (58), 286.9 (66), 178.1 (100)

APCI-MS *m/z* (rel. Int.) : 447.4 $[M-H]^-$ (100), 285.5 (35)

^{1}H NMR (500 MHz, methanol-*d4*): see Table 21

^{13}C NMR (125 MHz, methanol-*d4*): see Table 22

Compound K



Plantaginin, scutellarein 7-glucoside:

7-*O*-β-glucopyranosyl-4', 5, 6, 7-tetrahydroxyflavone

$C_{21}H_{20}O_{11}$

MW 448

Yellow powder

$[\alpha]_D^{20} =$ -73.5 (MeOH, c.0.1)

Mp 213-215 °C

UV $\lambda_{\max}^{MeOH} nm$: 285, 334

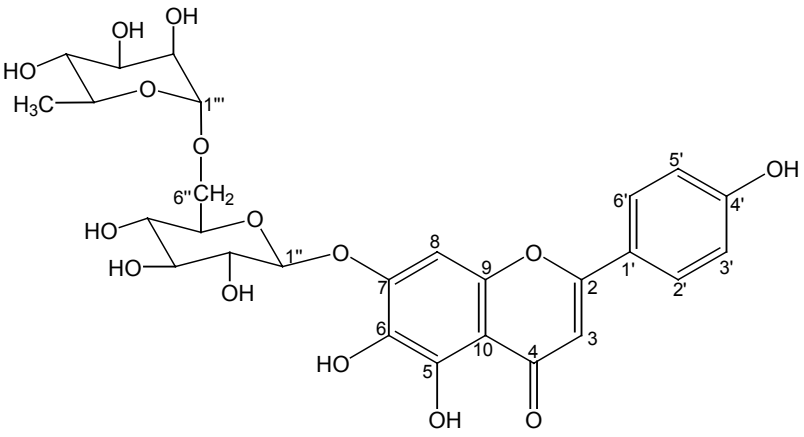
D/CI-MS *m/z* (rel. Int.) : 449.0 $[M+H]^+$ (12), 301.0 (100), 287.0 (72), 180.0 (70)

APCI-MS *m/z* (rel. Int.) : 447.3 $[M-H]^-$ (100), 285.4 (10)

^{1}H NMR (500 MHz, methanol-*d4*): see Table 21

^{13}C NMR (125 MHz, methanol-*d4*): see Table 22

Compound L



Scutellarein 7-rutinoside:

7-*O*-[α-rhamnopyranosyl-(1→6)-β-glucopyranosyl] -4', 5, 6, 7-tetrahydroxyflavone

$C_{27}H_{30}O_{15}$

MW 594

Yellow powder

$[\alpha]_D^{20}$ = -109 (EtOH, c.0.1)

Mp 185-188 °C

UV $\lambda_{\max}^{MeOH} nm$: 285, 333

APCI-MS *m/z* (rel. Int.) : 593.4 $[M-H]^-$ (100), 285.5 (26)

^{1}H NMR (500 MHz, methanol-*d4*): see Table 21

^{13}C NMR (125 MHz, methanol-*d4*): see Table 22

Table 21: ^{1}H NMR signals of the pure compounds **J**, **K** and **L** (methanol-*d4*, 500 MHz, TMS as int. standard) chemical shifts in ppm.

^{1}H	**J**	**K**	**L**
2	-	-	-
3	-	6.59 br s	6.62 br s
5	-	-	-
6	6.18 d, *J*=,1.5 Hz	-	-
7	-	-	-
8	6.38 d, *J*= 1.5 Hz	6.98 s	6.92 s
2'	8.05 d, *J*= 8.8 Hz	7.84 d, *J*= 6.8 Hz	7.86 d, *J*= 6.8 Hz
3'	6.88 d, *J*= 8.8 Hz	6.91 d, *J*= 7.3 Hz	6.98 d, *J*= 7.3 Hz
4'	-	-	-
5'	6.88 d, *J*= 8.8 Hz	6.91 d, *J*= 7.3 Hz	6.98 d, *J*= 7.3 Hz
6'	8.05 d, *J*= 8.8 Hz	7.84 d, *J*= 6.8 Hz	7.86 d, *J*= 6.8 Hz
Glc			
1''	5.22 d, *J*= 7.3 Hz	5.09 d, *J*= 5.9 Hz	5.05 d, *J*= 5.9 Hz
2''	3.42 u	3.59 u	3.58 m
3''	3.44 u	3.56 m	3.70[a)]
4''	3.31 m	3.43 t, *J*= 8.3 Hz	3.46 m
5''	3.21 m	3.59 u	3.54 m
6''	3.53 dd, *J*= 11.6; 5.4 Hz	3.74 m	3.70 [a)]
	3.69 dd, *J*= 11.6; 2.4 Hz	3.98 d, *J*= 11.7 Hz	4.06 d, *J*= 9.3 Hz
Rha			
1'''			4.72 s
2'''			3.87 s
3'''			3.68 u
4'''			3.32 m
5'''			3.65 u
6'''			1.16 d, *J*= 6.3 Hz

multiplicities: m: multiplet, s: singlet, d: doublet; u: multiplicity unclear due to overlapped signals

[a)] interchangeable signals

Table 22: ^{13}C NMR signals of the pure compounds **J**, **K** and **L** (methanol-*d4*, 125 MHz) chemical shifts in ppm.

^{1}H	**J**	**K**	**L**
2	158.98	166.77	167.02
3	135.47	103.49	103.62
	179.40	184.39	184.44
5	163.01	147.92	148.11
6	100.27	131.86	132.02
7	167.11	152.75	152.68
8	95.03	95.82	95.62
9	158.59	151.75	151.38
10	105.43	107.47	107.58
1’	122.82	123.29	123.35
2’	132.26	129.56	129.61
3’	116.09	117.00	117.18
4’	161.59	162.78	162.77
5’	116.09	117.00	117.18
6’	132.26	129.56	123.35
Glc			
1’’	104.29	102.66	102.54
2’’	78.08	74.73	74.79
3’’	75.74	77.52	77.18[a)]
4’’	71.37	71.42	71.16
5’’	78.39	78.58	77.37[a)]
6’’	62.66	62.55	67.28
Rha			
1’’’			102.03
2’’’			72.15
3’’’			72.44
4’’’			74.09
5’’’			69.77
6’’’			17.85

[a)] interchangeable signals

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7 Abstract

Abstract

In certain African countries, medicinal plants represent the unique source of drugs to 90% of the population. The knowledge of traditional healers represents a basis for the pharmacological and phytochemical investigation of these natural medicines.

This work first focused on the validation of use of two plants frequently employed in traditional medicine, *Dioscorea sylvatica* (Dioscoreaceae) and *Urginea altissima* (Liliaceae), which produce mild inflammation and itching when rubbed on the skin. These cutaneous reactions were shown to be due, at least in part, to the presence of sharp needles of calcium oxalate, implying the risk of granulomatous lesions following a long term use. Histamine was not detected, but other compounds could be involved in the inflammatory process.

The second part of this work consisted of the detection, isolation and characterisation of new natural compounds of potential therapeutic interest from African plants.
Seventy extracts obtained from 28 higher plants of Zimbabwe were submitted to a chemical and biological screening. The methanol extracts of the whole plants of *Jamesbrittenia fodina* and *J. elegantissima* (Scrophulariaceae) were selected for their various activities.
An activity-guided fractionation of *J. fodina* led to the isolation of the saponins A and B, responsible for the antifungal, antibacterial and molluscicidal properties. Both saponins were equally active as inhibitors of acetylcholinesterase, a property that has, to our knowledge, never been described for this class of compounds.

A LC/UV/MS analysis of the extract allowed the identification of verbascoside as the product with radical scavenging activity, and indicated the presence of a series of potentially interesting cinnamic acid derivatives.
Two types of instability problems occurred in the course of their isolation, as some compounds could not be separated despite very good chromatographic conditions.
LC/^{1}H-NMR analyses combined with in-mixture NMR analyses enabled the attribution of the cause of the instability in one case to a *cis*/*trans* light-induced isomerisation, and in the other case to a transacylation of the cinnamoyl moiety on a sugar residue. These problems of instability have not been the object of previous studies.

12 cinnamic iridoid esters could be characterised, 8 of these being new natural compounds. Several similar substances have displayed significant anti-inflammatory properties in different *in vivo* models, suggesting a therapeutic interest for these new derivatives.

Two flavanones were isolated from the same extract. This class of compound has not been previously reported from species of the Scrophulariaceae family.

A comparative LC/UV/MS study of the polar extracts of the two species *J. elegantissima* and *J. fodina* was performed in order to detect possible common compounds. Saponins A and B and verbascoside were thus identified in *J. elegantissima*. Moreover, three supplementary flavonoids were isolated from *J. elegantissima*..

8 Résumé

Les plantes médicinales représentent la seule source de médicaments pour près de 90 % de la population de certains pays d'Afrique. Le savoir-faire des guérisseurs traditionnels, d'une valeur inestimable, représente un point de départ pour l'investigation pharmacologique et phytochimique de ces médicaments naturels.
Dans le cadre de ce travail, nous nous sommes dans un premier temps intéressés à valider l'utilisation en médecine traditionnelle de deux plantes, *Dioscorea sylvatica* (Dioscoreaceae) et *Urginea altissima* (Liliaceae), qui produisent, lorsqu'elles sont frottées sur la peau, une inflammation et des démangeaisons.
Ces réactions cutanées ont pu être expliquées, au moins en partie, par la présence d'aiguilles acérées d'oxalate de calcium dans les organes souterrains. Ces microtraumatismes répétés de l'épiderme risquent de provoquer, lors d'une utilisation prolongée, des lésions granulomateuses. L'histamine n'a pas été détectée, mais d'autres substances pourraient être impliquées dans le processus inflammatoire.

La seconde partie de ce travail a consisté en la détection, l'isolement et la caractérisation de nouveaux composés naturels présentant un intérêt thérapeutique potentiel. 70 extraits provenant de 28 plantes supérieures du Zimbabwe ont été soumis à un criblage chimique et biologique. Les extraits méthanoliques des parties aériennes de *Jamesbrittenia fodina* et *J. elegantissima* (Scrophulariaceae) ont été sélectionnés sur la base de leurs nombreuses activités.
Le fractionnement guidé par l'activité de *J. fodina* a permis l'isolement des saponines A et B, responsables des activités antifongique, antibactérienne et molluscicide de l'extrait. De plus, les deux saponines ont montré une activité équivalente en tant qu'inhibiteurs de l'acétylcholinestérase, propriété encore non décrite pour cette classe de composés.
Une analyse LC/UV/MS de l'extrait a permis d'attribuer l'activité antiradicalaire au verbascoside, un dérivé du phenylpropane; cette analyse a de plus montré la présence d'une série de dérivés de l'acide cinnamique, dont l'isolement a été entrepris.
Deux problèmes d'instabilité sont apparus, empêchant l'isolement des composés par des méthodes chromatographiques de pointe, en dépit de très bonnes conditions de séparations.

Des analyses LC/^{1}H-NMR combinées à des analyses RMN classiques des mélanges ont permis d'attribuer ces instabilités d'une part à une isomérisation *cis*/*trans* induite par la lumière, et d'autre part à une transacylation du groupe cinnamoyl sur une unité de sucre. Ceci a permis l'identification de 12 esters cinnamiques d'iridoïdes, dont 8 nouveaux produits naturels. Ces dérivés présentent un intérêt thérapeutique, car des composés similaires ont montré des propriétés anti-inflammatoires significatives dans différents modèles *in vivo*.

Deux flavanones ont aussi été isolées de l'extrait. Cette classe de composés n'a jamais été rapportée chez un membre des Scrophulariaceae.

Une analyse LC/UV/MS comparative des extraits polaires des deux espèces, *J. fodina* et *J. elegantissima*, a été effectuée pour détecter la présence éventuelle de composés communs. Les saponines A et B et le verbascoside ont été identifiés dans l'extrait de *J. elegantissima*. Trois flavonoïdes ont de plus été isolés de ce dernier par CPC et HPLC semi-préparative.