Chapter 2

Extraction of plant material

Abstract

Selection of an appropriate extractant (when the chemical nature of the potentially active phytoconstituents is unknown) needs to be chosen with diligence. Different phytochemical groups are extracted by different extractants and specific compounds are frequently extracted by specific extractants. Ten different extracts were made from ten chosen extractants from each of *Pteleopsis myrtifolia* leaves, fruit and *Quisqualis littorea* leaves. Thin layer chromatograms indicated that the different extracts separated different compounds. There were however, also similarities elucidated in the chemical composition of non-polar and polar extracts (using extractants of widely varying polarities and belonging to different selectivity groups). This can only be explained by the presence of saponins that would solubilise very non-polar compounds into polar extractants. For all leaf and fruit material, the average largest amount of acetone soluble material (mg from one gram dry material) was isolated with the extractants tetrahydrofuran, followed by ethanol and acetone. From these results, one may decide that tetrahydrofuran is the extractant of choice, but after considering other properties than the mere quantity extracted, like toxicity, miscibility and volatility of the extractant, acetone was decided to be the most suitable extractant. Compared to tetrahydrofuran, acetone was not visible toxic to the test organisms below 30%, and it was miscible with polar and non-polar solvents. Tetrahydrofuran being less polar, was not as miscible with polar solvents as acetone.

2.1 Introduction

2.1.1 Selection of appropriate extraction solvents

Phytoconstituents can vary based on several factors, such as climate, habitat, soil nutrients, time of harvest, stress and physiological age of plants (Farnsworth and Soejarto, 1991). Selection of an appropriate extractant (when the chemical nature of the potentially active phytoconstituents is unknown) can be a daunting task. Furthermore, the presence of antagonistic substances could result in failing to detect individually active compounds – and result in obtaining essentially negative results. Many plants are known to accumulate large quantities of toxic inorganic constituents, i.e., selenium, nitrates, copper, etc. The predominant action of any one of these in a plant extract (containing organic compounds with potential biological activity) could result in these activities being undetected. Farnsworth *et al*., (1963) isolated 'leuroserine', an alkaloid with a high degree of activity against the P-1534 leukemia in DBA/2 mice, from a crude fraction of *Catharanthus lanceus* alkaloids, which was devoid of activity. Likewise, six alkaloids from this plant (leurosine sulphate, lochnerine, vindoline, vindolinine dihydrochloride, catharathine hydrochloride and tetrahydroalstonine which are at least equal to tolbutamide in hypoglycaemic action when administered orally to rats) were derived from a crude extract that failed to elicit a hypoglycaemic response (Svoboda *et al*., 1964).

Different phytochemical groups are extracted by different extractants (Table 2.1) and specific compounds are frequently extracted by specific extractants (Table 2.2).

Table 2.1. Type of phytochemicals extracted by different solvents (Houghton and Raman, 1998).

Polarity	Solvent	Chemical class extracted			
Low	*n*-hexane	waxes	fats	fixed oils	volatile oils
	chloroform	alkaloids	aglycones		volatile oils
Medium	dichloromethane	alkaloids	aglycones		volatile oils
	di-ethyl ether	alkaloids	aglycones		
	ethyl acetate	alkaloids	aglycones	glycosides	
	acetone	alkaloids	aglycones	glycosides	
	ethanol			glycosides	
	methanol	sugars	amino acids	glycosides	
High	water	sugars	amino acids	glycosides	
	aqueous acid	sugars	amino acids		bases
	aqueous alkali	sugars	amino acids		acids

Bands of an extract separated on thin layer chromatograms and viewed under ultra violet (UV) light, may give a specific fluorescence colour and thereby indicate the type of compounds present. All compounds are, however not visible under UV light. Table 3.3 lists examples of colours of phytoconstituents detected under UV light according to Wagner and Bladt (1996).

Table 2.2. Specific compound-groups usually extracted by specific extractants (Cowan, 1999).

Water	Ethanol	Methanol	Chloroform	Dichloromethanol	Ether	Acetone
Anthocyanins (Kaul *et al*., 1985)	Tannins (Silva *et al*., 1997))	Anthocyanins	Terpenoids (Ayafor *et al*., 1994)	Terpenoids (Mendoza *et al*., 1997)	Alkaloids	Flavonols (Afolayan and Meyer, 1997)
Starches	Polyphenols (Nakahara *et al*., 1993)	Terpenoids (Taylor *et al*., 1996)	Flavonoids (Perrett *et al*., 1995)		Terpenoids	
Tannins (Scalbert, 1991)	**Polyacetylenes** (Brandao *et al*., 1997)	Saponins			**Coumarins**	
Saponins (De Pasquale *et al*., 1995)	Flavonol (Hufford *et al*., 1993))	Tannins (Taylor *et al*., 1996)			**Fatty acids**	
Terpenoids	Terpenoids (Habtemariam *et al*., 1993)	**Xanthoxyllines**				
Polypeptides	**Sterols** (De Pasquale *et al*., 1995)	**Totarol** (Kubo *et al.,* 1995)				
Lectins	Alkaloids (Ivanovska *et al.,* 1996)	**Quassinoids** (Kitagawa *et al*., 1996)				
	Propolis	**Lactones** (Rao *et al*., 1993)				
		Flavones (Sato *et al*., 1996))				
		Phenones (Peres *et al*., 1997)				
		Polyphenols (Vijaya *et al*., 1995)				

Compounds in **bold** are commonly obtained in only one solvent.

Table 2.3. Examples of colours of phytoconstituents detected under 254 and 365 nm UV light.

Type of compound	UV-254 nm	UV-365 nm
Alkaloids	Pronounced quenching of some alkaloids of indoles, quinolines, isoquinolines, and purines. Weak quenching e.g. atropine alkaloids.	Blue, blue-green or violet fluorescence. Yellow fluorescence e.g. colchicine, berberine.
Flavonoids	All flavonoids cause fluorescent quenching. Caffeic acid, its derivatives and isoflavones show quenching.	Dark yellow, green or blue fluorescence depending on structure type. Caffeic acid, its derivatives and isoflavones fluoresce blue.
Triterpenes and essential oils	Compounds containing at least two conjugated double bonds quench fluorescence and appear as dark zones against the light green fluorescent background of the TLC plate.	No characteristic fluorescence of terpenoids and propylhenols is noticed.
Saponins	Not detectable.	Not detectable.

To determine the best extractants for the plants used in this study, a range of different extractants, presenting a polarity range from non-polar to polar was used to extract compounds from leaves and fruit of *Pteleopsis myrtifolia* and leaves of *Quisqualis littorea.*

2.2 Material and Methods

2.2.1 Plant material

Leaves and fruit of *P. myrtifolia* (the same tree that has voucher specimens number 24/2000 in Lowveld NBI herbarium) and leaves of *Q. littorea* (that correspond to voucher specimens number 13/1995 in Lowveld NBI herbarium) were collected in the National Botanical Garden of Nelspruit during March 2000 and 2001. These trees were the same ones that Eloff (1999) used in an earlier investigation. The seeds were originally collected in Punda Milia in Kruger National Park and planted in this garden. All old, insect damaged or fungus-infected leaves, and fruit were removed. Intact material was dried in the shade at room temperature to a constant mass.

The leaves and fruit from *P. myrtifolia* as well as leaves of *Q. littorea* were ground separately to a fine powder in a Jankel and Künkel Model A10 mill. The ground power was stored in tightly closed containers and protected from light.

2.2.2 Extracts

A 500 mg quantity of leaves and fruit were separately extracted 3 times (10 min for one extraction), with 5 ml of each of the 10 extractants. The extractants were chosen on the ground of their polarity, relatively low boiling points as well as their ability to evaporate, and represented a polarity range from non-polar to polar. Each extractant is listed with polarity and selectivity group according to Snyder and Kirkland (1979) in brackets behind each solvent's name. The polarity is the first number in brackets and the selectivity group the second number in brackets. The extractants were: *n*-hexane (0.10, 0), *di*-isopropyl ether (2.4,I), *di*-ethyl ether (2.8, I), methylene dichloride (3.1, V), tetrahydrofuran (4.0, III), ethyl acetate (4.4, VIa), acetone (5.1, VIa), ethanol (4.3, II), methanol (5.3, II) and water (10.2, VIII). Extractants used were of reagent grade (Merck). For *P. myrtifolia* fruit and *Q. littorea* leaf material, a solution of 50% water and 50% acetone was used instead of water (after water alone was found not to extract acetone soluble material). The material was extracted in polyethylene centrifuge tubes while shaking vigorously in a Vortex model K-500-4 test tube mixer for 5 min. The tubes were then balanced and centrifuged at 3500-x g for 5 minutes; the extract was decanted in pre-weighed amber coloured glass containers. This was done three times, and each extract was decanted into the same container. The combined extracts were dried in a stream of air at room temperature. Extractants that had difficulty drying (water and methanol) were dried by vacuum distillation in a Büchi rotary evaporator and placed in a desiccator overnight. Yield was determined and the dried extracts were redissolved in acetone to a final concentration of 10 mg/ml and stored in tightly sealed dark glass containers at 5 °C.

It was noticed that some extracts did not completely redissolve in acetone, even in the same extractant as used originally. This was in agreement with an observation made by Eloff (2004). To circumvent this problem, a small aliquot of a specific extract was taken, dried and values obtained used to calculate the original concentration of the extract.

2.2.3 Thin layer chromatography (TLC)

The extracts were separated (in duplicate) by thin layer chromatography (TLC) (10 µl of a 10 mg/ml final concentration) on Merck Silica gel F_{254} plates with each of the following eluent systems (selected to separate high, intermediate and low polarity): BEA (benzene: ethanol: ammonia, (36:5.4:4)), CEF (chloroform: ethyl acetate: formic acid, (5:4:1)) and EMW (ethyl acetate: methanol: water, (40:5:4.4)). Separated compounds were examined under visible and ultraviolet light with a Camac UV lampTL-600. Bands of quenching fluorescence (254 nm) or fluorescing (236 nm) were marked with a soft pencil.

The majority of plant ingredients react with vanillin-sulphuric acid and anisaldehyde-sulphuric acid with coloured zones (Wagner and Bladt, 2001), therefore these two spray reagents were used. They were i) 5% anisaldehyde in 5% H_2SO_4 in ethanol and ii) 0.34% vanillin in 3.5% H_2SO_4 in methanol (Stahl, 1969). Heat (at 100°C for 2 - 5 minutes) was used after spraying, until the development of the colour bands was complete.

2.3 Results and Discussion

2.3.1 Extraction of plant material

The mass of plant material extracted, using different extractants, are given in Table 1.4. The highest percentage of (acetone soluble) material extracted for *Pteleopsis* (from 1 g dry material (found by doubling the amount from 500 g)) was as follow: *Pteleopsis* leaves - the extractants: tetrahydrofuran, ethanol and acetone; *Pteleopsis* fruit - the extractants: tetrahydrofuran, ethyl

acetate and equally *di*-ethyl ether, methylene dichloride and ethanol and *Quisqualis* leaves - the extractants tetrahydrofuran, ethanol and ethyl acetate.

Table 2.4. Milligram of plant material extracted per gram dry mass (using different extractants); listed from left to right as mg and percentage acetone soluble material extracted for *P. myrtifolia* leaves, *Q. littorea* leaves and *P. myrtifolia* fruit.

Extractant	*Pteleopsis myrtifolia* and *Quisqualis littorea* leaves						*Pteleopsis myrtifolia* fruit			All Ave mg
	Mg extracted/ g		acetone soluble /g				mg extracted/ g	Acetone soluble		
			Pm		Ql					
	Pm	Ql	mg	%	mg	%		mg	%	
n-hexane	22	16	16	1.6	10	1.0	10	10	1.0	12.0
di-isopropyl ether	18	30	12	1.2	14	1.4	14	10	1.0	12.0
di-ethyl ether	34	35	22	2.2	16	1.6	22	12	1.2	16.7
methylene chloride	64	152	20	2.0	20	2.0	114	12	1.2	17.3
tetrahydrofuran	88	56	70	7.0	48	4.8	52	20	2.0	46.0
ethyl acetate	46	30	28	2.8	24	2.4	24	14	1.4	22.0
acetone	52	40	38	3.8	22	2.2	30	10	1.0	23.3
ethanol	84	50	44	4.4	32	3.2	30	12	1.2	29.3
methanol	188	130	14	1.4	22	2.2	116	10	1.0	15.3
water	402	na	2	0.2	na	na	na	na	na	2.0
(water: acetone) 1 : 1	na	260	na	na	10	1.0	184	1.08	0.1	5.5
Average	99.8	79.9	26.6	50.9	21.8	79.4	59.6	11.1	40.4	18.3

Pm = *Pteleopsis myrtifolia*, Ql = *Quisqualis littorea,* na = not applicable, Ave = average.

Q. littorea's fruit were not analysed since it was not available. Figure 2.1 is a graphical presentation of the data in Table 2.4.

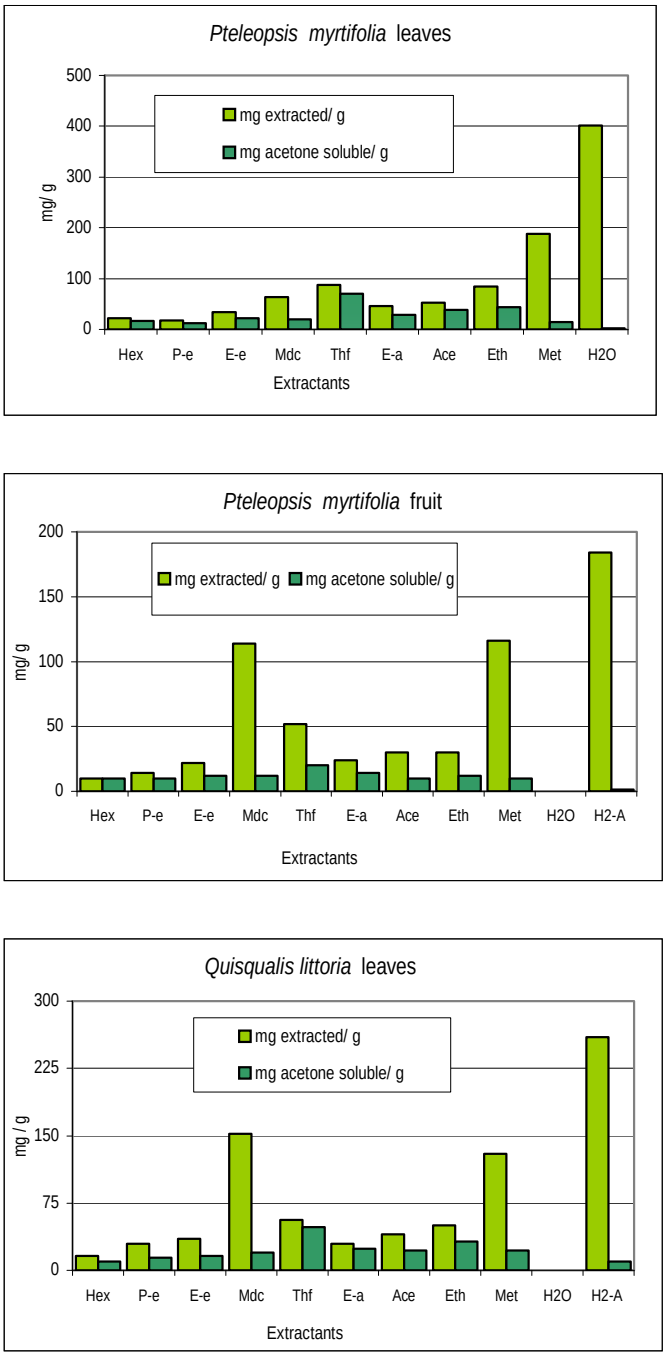


Figure 2.1. Milligram plant material extracted (■) (per gram dry mass) as well as mg acetone soluble plant material extracted (■) for *Pteleopsis myrtifolia* leaves (top), *Pteleopsis myrtifolia* fruit (middle) and *Quisqualis littorea* leaves (bottom) and different extractants. Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Eth = ethanol, Met = methanol, H_2O =

water and H_2-A = (1: 1) (water : acetone)).

Table 2.4 and Figure 2.1 show that the total mg extracted for *P. myrtifolia* leaf material increased as the polarity of the solvents increased. Only the acetone soluble part of each extractant was used for antibacterial assays. Redissolving in acetone ensured that:

- particles (which is not in solution and do not contribute towards antibacterial activity) remained behind,
- all extracts are (redissolved) in a solvent that has a relative low toxicity to the test organisms (Eloff, 1998),
- all extracts are (redissolved) in a solvent that is miscible with polar and non-polar solvents (Eloff, 1998) and
- all extracts are (redissolved) in a solvent that is volatile and drying time is not very long (Eloff, 1998).

The largest amounts (in mg) of acetone soluble material extracted for each plant material type, were:

P. myrtifolia leaves - tetrahydrofuran, ethanol and acetone

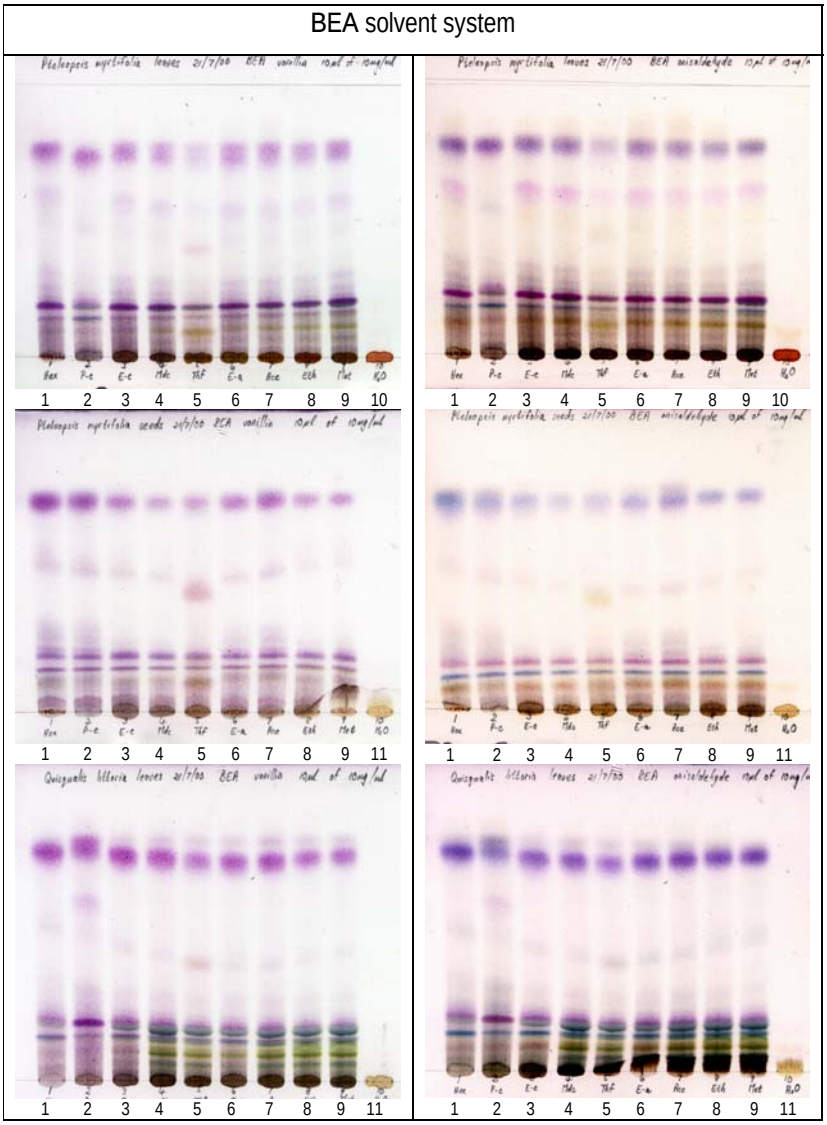
P. myrtifolia fruit - tetrahydrofuran, ethyl acetate and equally *di*-ethyl ether, methylene dichloride and ethanol

Q. littorea leaves - tetrahydrofuran, ethanol and ethyl acetate

On average, the largest amount of acetone soluble material (mg from one gram dry material) for all plant materials was isolated with tetrahydrofuran, followed by ethanol and acetone. For all leaf and fruit material, the largest amount of mg of acetone soluble material was extracted with extractants of intermediate polarity or slightly polar, like methylene dichloride, tetrahydrofuran, ethyl acetate, acetone and ethanol.

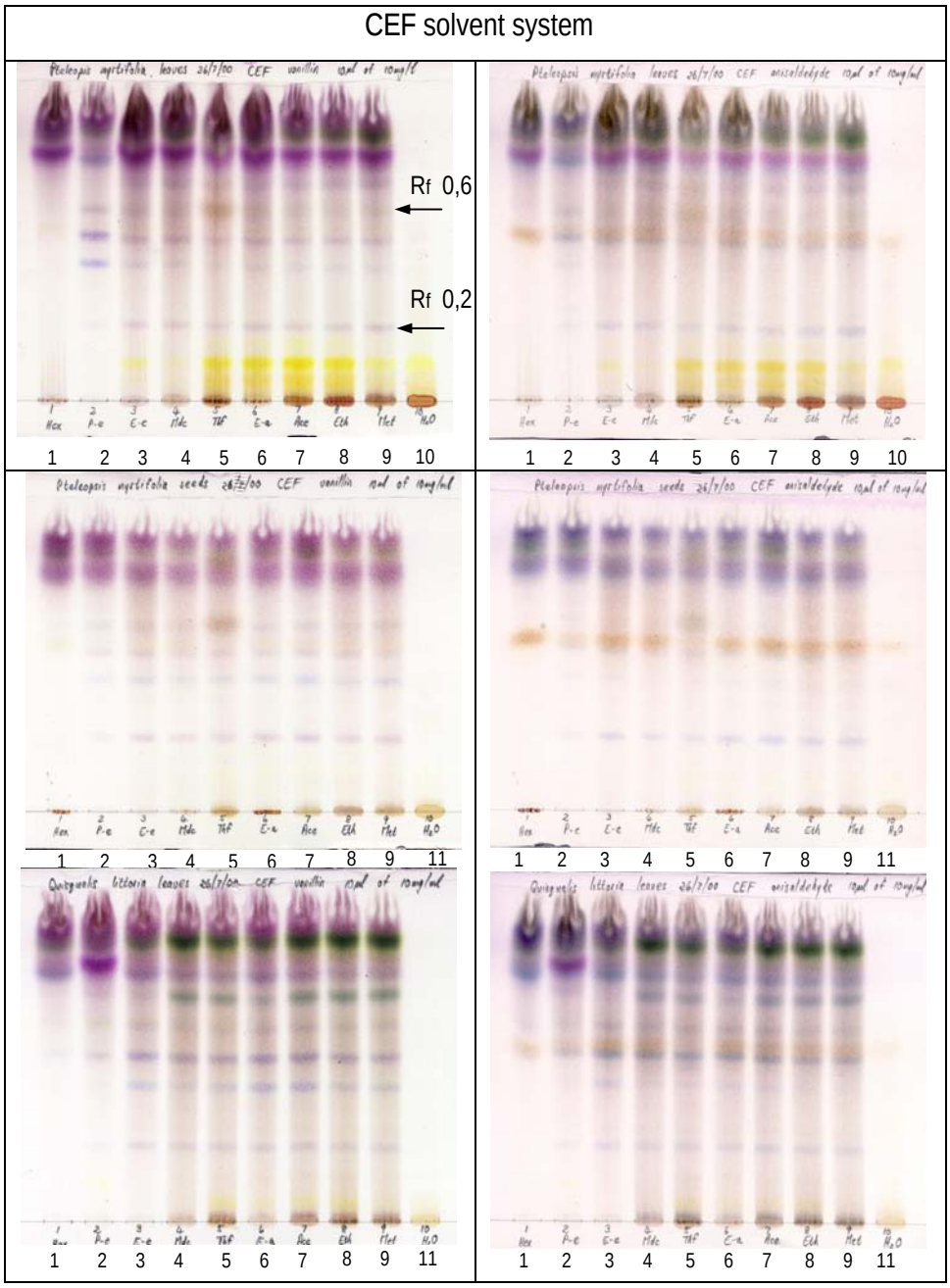
2.3.2 Chemical composition of extracts

Visualisation of the developed TLC plates enabled one to see how complex or simple the different extracts were and to what extent the solvent systems used, were effective in separating the different compounds (indicated by colour bands) of a specific extract (Figure 2.2). For all extracts and plant material types, more compounds were on average visible on TLC for the extracts of intermediate to polar polarity, namely methylene dichloride, tetrahydrofuran, ethyl



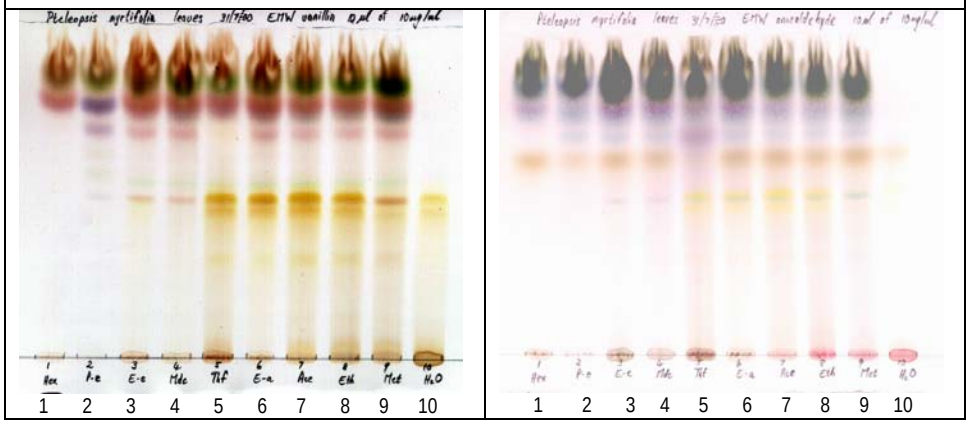
(a)

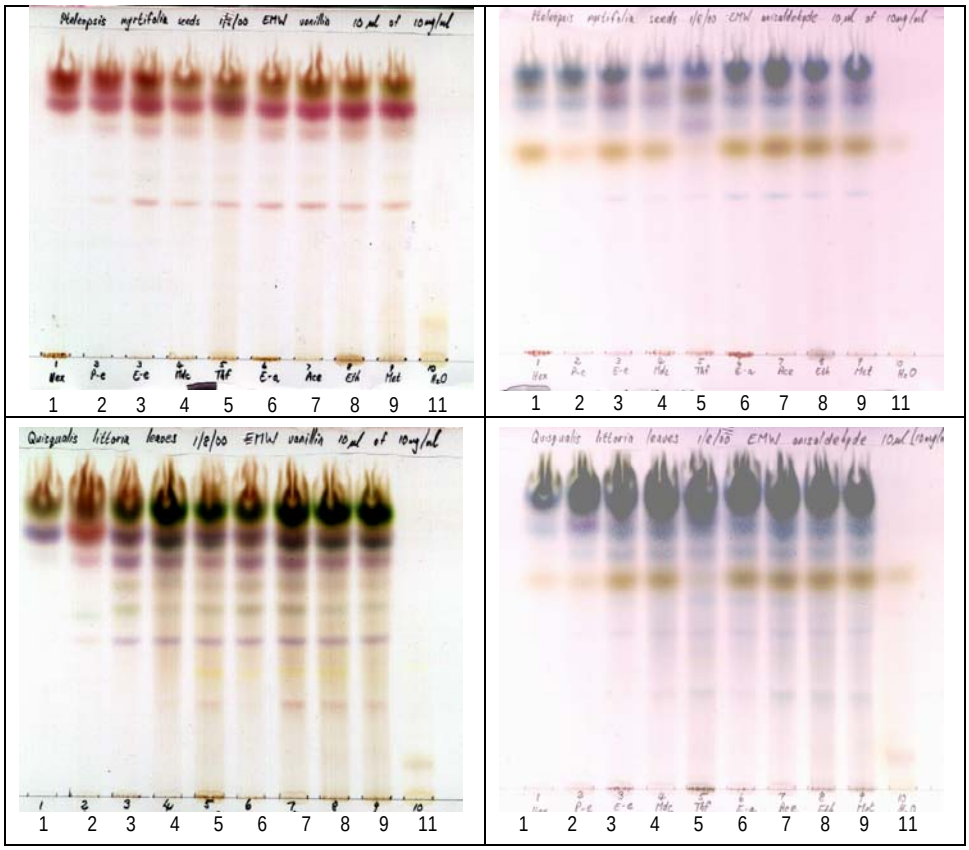
CEF solvent system



(b)

EMW solvent system





(c)

Figure 2.2. Chromatograms of *Pteleopsis myrtifolia* leaves (top) and fruit (middle) and *Quisqualis littorea* leaves (bottom), showing separation of compounds with the three eluent systems ((a) - BEA, (b) - CEF and (c) - EMW), for the different extractants. For each solvent system, the left chromatogram was sprayed with vanillin and right one with anisaldehyde. (1) = *n*-hexane, (2) = di-isopropyl ether, (3) = *di*-ethyl ether, (4) = methylene dichloride, (5) = tetrahydrofuran, (6) = ethyl acetate, (7) = acetone, (8) = ethanol, (9) = methanol, (10) = water and (11) = (water : acetone) (1 : 1).

acetate, acetone, ethanol, and methanol. Between 1 and 13 different compounds were separated on TLC for different extracts and eluent types.

There were major differences and some similarities between the different extracts. For example, in Figure 2.2, the top left chromatogram from the CEF eluent system, the colour and presence of bands of the hexane extract (first column), tetrahydrofuran extract (fifth column) and water extract (tenth column) differed for a Rf value of 0.58. At an Rf value of 0.24 the *di*-ethyl ether, methylene dichloride, tetrahydrofuran, ethyl acetate, acetone, ethanol and methanol

extracts all had a light purple band.

For the thin layer chromatograms developed with BEA, CEF and EMW eluent systems, sprayed with vanillin and anisaldehyde, on average more bands (representing different compounds) were made visible for the BEA eluent system with the anisaldehyde spray. The vanillin spray defined the compounds the clearest in the CEF and EMW eluent systems. It was observed that not all compounds seen under UV light became visible with anisaldehyde or vanillin sprays (an example is shown in Figure 7.2 of Chapter 7). For the plant material worked with, the best separation occurred with the CEF system. The vanillin spray was therefore routinely used.

2.4 Conclusions

Observations of the thin layer chromatograms indicated that the different extracts separated different compounds. There were however, also similarities elucidated in the chemical composition of non-polar and polar extracts (using extractants of widely varying polarities and belonging to different selectivity groups). This can only be explained by the presence of saponins that would solubilise very non-polar compounds into polar extractants.

For all leaf and fruit material, the average largest amount of acetone soluble material (mg from one gram dry material) was isolated with the extractants tetrahydrofuran, followed by ethanol and acetone. From these results, one may decide that tetrahydrofuran is the extractant of choice, but after considering other properties than the mere quantity extracted, like toxicity (see Chapter 3), miscibility and volatility of the extractant, acetone was decided to be the most suitable extractant. Compared to tetrahydrofuran, acetone was not visible toxic to the test organisms below 30%, and it was miscible with polar and non-polar solvents. Tetrahydrofuran being less polar, was not as miscible with polar solvents as acetone (Cowan, 1999; Eloff, 1998).

2.5 Literature references

Afolayan AJ, Meyer JJM (1997) Antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. Journal of Ethnopharmacology 57: 177-181

Ayafor JF, Tehuendem MHK, Nyasse B (1994) Novel active diterpenoids from *Afromomum aulacocarpos*. Journal of Natural Products 57: 917-923

Brandao MGL, Krettli AU, Soares LSR, Nery CGC, Marinuzzi HC (1997) Antimalarial activity of extracts and fractions from *Bidens pilosa* and other *Bidens* species (Asteraceae) correlated with the presence of acetylene and flavonoid compounds. Journal of Ethnopharmacology 57: 131-138

De Pasquale R, Germano MP, Keita A, Sanogo R, Lauk L (1995) Anti-ulcer activity of *Pteleop--sis suberosa*. Journal of Ethnopharmacology 47: 55–58

Eloff JN (1998) Which extractant should be used for the screening and isolation of antimicrobial components from plants ? Journal of Ethnopharmacology 60: 1-8

Eloff JN (1999) The antibacterial activity of 27 southern African members of the Combretaceae. South African Journal of Science 95: 148-152

Eloff JN (2004) Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. Phytomedicine 11: 370-371

Farnsworth NR, Loub WD, Blomster RN (1963) Studies on *Catharanthus lanceus* (*Vinca lancea)* I. Isolation of Leurosine, Perivine and Yohimbine. Journal of Pharmaceutical Science 52: 1114

Farnsworth NR, Soejarto DD (1991) Global Importance of Medicinal Plants. In Akerle O, Heywood V, Synge H (Eds.) Conservation of medicinal plants. Cambridge University Press, Cambridge, pp. 25-51. ISBN 0521392063

Habtemariam S, Gray AL, Waterman PG (1993) A new antibacterial sesquiterpene from *Premna oligitricha*. Journal of Natural Products 56: 140-143

Houghton PJ, Raman A (1998) Methods for extraction and sample clean-up. In: Laboratory Handbook for the Fractionation of Natural Extracts. Chapman and Hall, London. pp. 22-53. ISBN 0412749106

Hufford CD, Jia Y, Croom EM, Muhammed Jr I, Okunade AL, Clark AM, Rogers RD (1993)) Antimicrobial compounds from *Petalostemum purpurium*. Journal of Natural Products 56: 1878-1889

Ivanovska N, Philipov S, Istakova R, Gieorgieva P (1996) Antimicrobial and Immunological activity of ethanol extracts and fractions from *Isopyrum talictroides.* Journal of Ethnopharmacology 54: 143-151

Kaul TN, Middletown Jr E, Ogra PL (1985) Antiviral Effect of Flavanoids on Human Virusses. Journal of Medical Virology 15:71-79

Kitagawa I, Mahud T, Yokota K-I, Nakagawa S, Mayumi T, Kobayashi M, Shibuya H (1996) Indonesian medicinal plants. XVII. Characterization of quassinoids from the stems of *Quiassia indica*. Chemical and Pharmaceutical Bulletin 44: 2009-2014

Kubo I, Muroi H, Himejima M (1995) Antibacterial activity of totarool and its potentiation. Journal of Natural Products 55: 1436-1440

Mendoza L, Wilkens M, Urzua A (1997) Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids from some Chilean *Pseudognaphalium* (Asteraceae). Journal of Ethnopharmacology 58: 85-88

Nakahara K, Kawabata S, Ono H, Ogura K, Tanaka T, Ooshima T, Hamada S (1993) Inhibitory effect of oolong tea polyphenols on glucosyltransferases of mutans streptococci. Applied and Environment Microbiology 59: 968-973

Peres MTLP, Monache FD, Cruz AB, Pizzolatti MG, Yunes RA (1997) Chemical composition and antimicrobial activity of *Croton urucurana* Baillon (Euphorbiaceae). Journal of Ethnopharmacology 56: 233-226

Perrett S, Whitfield PJ, Sanderson L, Bartlett A (1995) The plant molluscicide *Millettia thonningii* (Leguminosae) as a topical antischistosomal agent. Journal of Ethnopharmacology 47: 49-54

Rao KV, Sreeramulu K, Gunasekar D, Ramesh D (1993) Two new sesquiterpene lactones from *Ceiba pentandra*. Journal of Natural Products 56: 2041-2045

Sato M, Fujiwara S, Tsuchiya H, Fujii T Iinuma M, Tosa H, Ohkawa Y (1996) Flavones with antibacterial activity against carcinogenic bacteria. Journal of Ethnopharmacology 54: 171-176

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30: 3875-3883

Silva O, Duarte A, Pimrntal M, Viegas S, Barroso A, Machado J, Pires I, Cabrita J, Gomes E (1997) Antimicrobial activity of *Terminalia macroptera* root. Journal of Ethnopharmacology 57: 203-207

Snyder LR, Kirkland JJ (1979) Introduction to modern Liquid Chromatography. John Wiley &

Sons Inc, New York, pp. 248–250. ISBN 0-471038229

Stahl E (1969) Thin layer Chromatography (A laboratory handbook), Springer Verlag, Berlin. p. 857. ISBN 0387047360

Svoboda GH, Gorman M, Root M (1964) Alkaloids in *Vinca rosea* (*Catharanthus roseus*) XXVIII. A preliminary report on hypoglycemic activity. Lloydia 27: 361-363

Taylor RS, Edel LF, Manandhar NP, Towers GHN (1996) Antimicrobial activities of southern Nepalese medicinal plants. Journal of Ethnopharmacology 50: 97-102

Vijaya A, Ananthan S, Nalini R (1995) Antibacterial effect of theaflavin, polyphenon 60 (*Camellia sinensis*) and *Euphorbia hirta* on *Shigella* spp. – a cell culture study. Journal of Ethnopharmacology 49: 115-118

Wagner H, Bladt S (2001) Plant drug analysis. A Thin Layer Chromatography Atlas. Second ed. Springer, Berlin, p. 351. ISBN3540586768