Bioactivity of extracts and components of *Pteleopsis myrtifolia*

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"It can seem a formidable task, faced with a litre of fermentation broth - a dark, viscous sludge - knowing that in there is one group of molecules that has to be separated from all the rest. Those molecules possibly represent only about 0.0001%, or 1 ppm of the total biomass and are dispersed throughout the organism, possibly intimately bound up with other molecules. Like the proverbial needle in a haystack, you have to remove lot of hay to be left with just the needle, without knowing what the needle looks like or where in the haystack it is."

Richard J.P. Cannell

SUMMARY

Combretaceae contain several species with bioactive properties - especially the genera *Combretum* and *Terminalia*. *Pteleopsis myrtifolia* and *Quisqualis littorea* belong to this family and have not previously been thoroughly investigated for their bioactivity. Leaf and fruit extracts of *P. myrtifolia* and leaf extracts of *Q. littorea* were separated by three different thin layer chromatography eluent systems. For all leaf and fruit material, the largest amount of acetone soluble material was extracted with extractants of intermediate polarity. Antibacterial activity of 30 extracts was investigated using a microplate serial dilution method and bioautography. The four most important nosocomial pathogens that are used worldwide namely two Gram-positive: *Staphylococcus aureus* and *Enterococcus faecalis*, and two Gram-negative bacteria: *Pseudomonas aeruginosa* and *Escherichia coli* were used as test organisms. Areas of growth inhibition were best defined after an eluent system that separates compounds of intermediate polarity had been used. The Gram-positive bacteria were most sensitive to some extracts of *P. myrtifolia* leaves. Fruit extracts exhibited minimum inhibitory concentrations (MIC) values as low as 0.04 mg/ml, less than that of the allopathic antibiotics, ampicillin and chloramphenicol. *Q. littorea* leaf extracts had an average MIC value of 0.32 mg/ml for Gram-negative bacteria. The average antibacterial activity expressed as total activity for each bacterium was higher in the leaves than in the fruit of *P. myrtifolia*. After considering the amount of antibacterial compounds extracted, toxicity of extractants to test organisms and miscibility of several extractants, acetone was eventually chosen as the best extractant for future extractions.

Results obtained in this investigation showed clearly that *P. myrtifolia* leaves, fruit, and *Q. littorea* leaves contain several antibacterial compounds.

Five different extracts of *P. myrtifolia* leaves were tested for growth inhibitory effects on different

human cell lines (MCF-12, MCF-7, H157, $WHCO_3$, HeLa). The non-cancerous MCF-12A cell line's growth was not inhibited extensively, and the cancer cell lines - MCF-7, H157, $WHCO_3$ and HeLa, differed in their sensitivity to the plant extracts. This indicated that the plant extracts' effects were selective and not due to general toxicity. The effect of some extracts on certain cell lines, especially $WHCO_3$, was growth inhibitory but not lethal. This is the desired effect – to inhibit growth of cancer cells, but not to be toxic to cells in general. The presence of tannin in extracts either promoted or inhibited growth inhibition of different cell lines.

The same extractants that were used for cytotoxic tests were investigated for their antioxidant activity. All extracts gave positive scavenging capacity with the 1,2-diphenyl-2-picrylhydrazyl assay. The cold water, methanol and hot water extracts had vitamin C equivalents of 0.34, 0.20 and 0.147 mg/g respectively, all more than that of black tea.

The solvent-solvent separation of *P. myrtifolia* leaves was started with acetone as an initial extractant. Separation was undertaken with immiscible solvents of different polarities. All fractions had antibacterial activity against the Gram-positive bacteria. The chloroform fraction was antibacterial to all bacteria tested, and had the largest amount of antibacterial compounds.

Pure compounds were isolated from the chloroform fraction by column chromatography. One pure compound's structure was elucidated as a pentacyclic triterpenoid, taraxerol ($C_{30}H_{50}O$).

Taraxerol had MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli* respectively. It significantly inhibited growth of the human lung cancer cell line H157 and did not display free radical scavenger activity.

This is the first report of the antibacterial activity of several extracts from *P. myrtifolia* and *Q. littorea,* growth inhibition effects of several *P. myrtifolia* leaf extracts on the human cell lines

MCF-12, MCF-7, H157 and $WHCO_{3,}$ the isolation of taraxerol from *P. myrtifolia* leaves, taraxerol's antibacterial activity for above-mentioned test organisms, and growth inhibition effects on human cancer cell lines MCF-7, H157, $WHCO_3$ and HeLa.

OPSOMMING

Combretaceae word gekenmerk deur die teenwoordigheid van baie spesies met bioaktiewe eienskappe, veral van die genusse *Combretum* en *Terminalia*. *Pteleopsis myrtifolia* en *Quisqualis littorea,* wat tot hierdie familie hoort, is nog nie voorheen deeglik vir bioaktiwiteit ondersoek nie. Blaar- en vrugekstrakte van *P. myrtifolia,* en blaarekstrakte van *Q. littorea* is met drie verskillende elueermiddels en dunlaagchromatografie (DLC) ontwikkel. Van al die blaar- en vrugmateriaal is die grootste hoeveelheid asetoon-oplosbare materiaal deur ekstraheermiddels met intermediêre polariteit geëkstraheer. Antibakteriële aktiwiteit van 30 ekstrakte was ondersoek met serie verdunnings in multi-putjie plate en bioautografie. Die vier belangrikste nosokomiale patogene wat wêreldwyd gebruik word, naamlik twee Gram-positiewe: *Staphylococcus aureus* en *Enterococcus faecalis*, en twee Gram-negatiewe bakterië: *Pseudomonas aeruginosa* en *Escherichia coli* was as toetsorganismes gebruik. Areas van groei-inhibisie was die duidelikste ná ontwikkeling met ‘n elueermiddel van intermediëre polariteit. Die Gram-positiewe bakterië was die sensitiefste vir sommige ekstrakte van *P. myrtifolia* blare en vrugte met sommige minimum inhibitoriese konsentrasie (MIK) waardes so laag as 0.04 mg/ml - minder as die van die allopatiese antibiotika, ampisillien en chlooramfenikol. *Q. littorea* blaarekstrakte het ‘n gemiddelde MIK waarde van 0.32 mg/ml vir Gram-negatiewe bakterië gehad. Die gemiddelde aktiwiteit voorgestel as totale aktiwiteit, was vir elke bakterium hoër in die blare as in die vrugte van *P. myrtifolia*. Nadat hoeveelheid antibakteriële verbindings geëkstraheer, toksisiteit vir toetsorganismes en mengbaarheid van verskeie ekstrakte in ag geneem is, is daar eventueel besluit dat asetoon die beste ekstraheermiddel vir toekomstige ekstraksies is.

Resultate uit hierdie ondersoek verkry, toon duidelik dat *P. myrtifolia* blare, vrugte en *Q. littorea* blare verskeie antibakteriële verbindings bevat.

Vyf verskillende *P. myrtifolia* blaar ekstrakte is vir groei-inhiberende effekte teen menslike sellyne (MCF-12, MCF-7, H157, $WHCO_3$ en HeLa) getoets. Die nie-kankeragtige MCF-12A sellyn se groei is nie ekstensief geïnhibeer nie, en die kanker sellyne - MCF-7, H157, $WHCO_3$ en HeLa, het verskil in hul sensitiwiteit vir verskillende plant ekstrakte. Dit toon dat die plantekstrakte se effek selektief was en nie as gevolg van algemene toksisiteit nie. Die effek van sommige ekstrakte op spesifieke sellyne, veral $WHCO_3$, was groei inhiberend maar nie dodelik (toksies) nie. Hierdie is die gewensde effek – dat kankerselle se groei geïnhibeer sal word, maar nie sterf as gevolg van toksisiteit in die algemeen nie. Die teenwoordigheid van tanniene in ekstrakte het die sellyne se groei minder of soms meer geïnhibeer.

Dieselfde ekstrakte as wat vir sitotoksiese toetse gebruik was, is ondersoek vir hul antioksidant aktiwiteit. Alle ekstrakte het ‘n positiewe suiwerings aktiwiteit met 1,2-difeniel-2-pikrielhidrasiel getoon. Die koue water, metanol and warm waterekstrakte het vitamien C ekwivalente van of 0.34, 0.20 en 0.147 mg/g respetiewelik gehad, almal meer as dié van swart tee.

Die groepskeiding van *P. myrtifolia* blare is met asetoon as inisiële ekstraheermiddel begin. Skeiding is bewerkstellig met onmengbare vloeistowwe van verskillende polariteite. Alle fraksies was aktief teen Gram-positiewe bakterië. Die chloroformfraksie was antibakteriëel vir all die bakterië, en het die meeste antibakteriële verbindings gehad.

Suiwer verbindings is deur kolomkromatografie uit die chloroformfraksie geïsoleer. Een suiwer verbinding se struktuur is geïdentifiseer as ‘n pentasikliese triterpenoïed, taraxerol ($C_{30}H_{50}O$).

Taraxerol het MIK waardes van 0.04, 0.016, 0.63 en 0.31 mg/ml vir die bakterië *S. aureus*, *E. faecalis, P. aeruginosa* en *E. coli* respektiewelik gehad. Dit het die groei van die menslike longkanker sellyn H157, betekenisvol geïnhibeer en nie antioksidant aktiwiteit getoon nie.

Hierdie is die eerste verslag van antibakteriële aktiwiteit van verskeie ekstrakte van *P. myrtifolia* en *Q. littorea,* inhibisie van groei van die menslike sellyne MCF-12, MCF-7, H157 en $WHCO_3$ deur verskeie *P. myrtifolia* blaarekstrakte, die isolasie van taraxerol uit *P. myrtifolia* blare, taraxerol se antibakteriële aktiwiteit vir bogenoemde toetsorganismes, antioksidant aktiwiteit en groei-inhibeerende effekte op die bogenoemde menslike kanker sellyne.

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List of abbreviations

Abbreviation	Explanation
ATCC	American Tissue Culture Collection
BEA	benzene: ethanol: ammonia (36:5.4:4)
^{13}C-NMR	carbon-nuclear magnetic resonance
CEF	chloroform: ethyl acetate: formic acid (5:4:1)
COSY	correlated spectroscopy
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	dimethylsulfoxide
DPPH	1,2-diphenyl-2-picrylhydrazyl
E. coli	*Escherichia coli* (ATTC 27853)
EMW	ethyl acetate: methanol: water (40:5:4.4)
GI_{50}	growth inhibition by 50%
^{1}H	proton
HMQC	heteronuclear multiple quantum correlation
^{1}H-NMR	proton-nuclear magnetic resonance
INT	p-iodonitrotetrazolium violet
LC	lethal concentration
MIC	minimum inhibitory concentration
MS	mass spectroscopy
MWP	multiwell plate
MWP-96	96-multiwell plate
E. faecalis	*Enterococcus faecalis* (ATTC 29212)
NCCLS	National Committee for Clinical Laboratory Standards
NMR	nuclear magnetic resonance (spectroscopy)

P. aeruginosa	*Pseudomonas aeruginosa* (ATTC 25922)
rpm	revolutions per minute
S. aureus	*Staphylococcus aureus* (ATTC 29213)
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultra violet

Chapter 1

Introduction, hypotheses, aims and objectives

1.1 Introduction

1.1.1 Serious underestimation of plant-derived medicines

The importance of plant-derived medicines is seriously underestimated in modern medicine. Only approximately 15% of the Angiosperms (flowering plants) have been chemically investigated for their medical potential (Farnsworth, 1966; Farnsworth and Soejarto, 1991). Of the 300 plant species tested by Noristan Pty, Ltd. (Pretoria, RSA), 31% displayed high activity (activity being: analgesic, anti-inflammatory, anti-hypertensive, antimicrobial, antifungal, anti-ulcer, antagonism of acetylsalicylic acid induced gastric damage, narcotic analgesic, anti-convulsent, anti-depressant, anti-arrhythmic, diuretic and general toxicological and central nervous system effects), 48% were moderately active and 21% had no activity (Fourie *et al.*, 1992).

Plant secondary compounds are frequently associated with plant taxons. From data provided by Cunningham (1990), Eloff (1998) calculated that while the Combretaceae is a relatively small family the scale of use in KwaZulu-Natal is large, relative to most other plant families. This suggests that the Combretaceae plant family has the potential to offer novel or alternative phytomedicines. The intent of this research was therefore to investigate the assumed bioactivity potential of relatively unknown members in the Combretaceae namely; *Pteleopsis myrtifolia* and *Quisqualis littorea.*

1.1.2 Tendency towards unrefined or “natural’, assuming non-toxic

The majority of people from rural communities, also including some shack dwellers on the outskirts of cities, depend entirely on herbal medicines for their health. Due to increased

awareness of toxicities in refined products amongst modern city dwellers, individuals are progressively focusing their attention towards herbal medicine in an effort to find an alternative approach to living healthier. This is indicated by the steady increase in the number of retail outlets who solely merchandise herbal medicine and conventional pharmacies who stock herbal medicines. The United Nations Development Programme predicts (UNDP, 2002), that despite the severe impact of HIV/ AIDS, the African continent will still experience a slight growth in its human population (0,4% instead of 1,4%). The demand on natural resources will therefore not decline, but maintain the current pressure of intrusion into natural habitat areas of phytomedicines. A greater demand for land in terms of pastoral agriculture and commercial development will therefore reduce the available areas for conservation and subsequently impact negatively on areas for medicinal plants. A dynamic and clear cut plan for cultivating and conserving medicinal plants, as well as more knowledge about the Plant Kingdom's medicinal properties (many of which are undiscovered yet), urgently need to be implemented to ensure their future beneficial use.

In addition to identifying phytomedicines which can offer solutions to modern day diseases like AIDS and certain cancers, increased knowledge about phytomedicines can:

- serve as alternative solutions where orthodox medicines have limitations, for example antibiotics (in cases of antibacterial-drug-resistance), and anticancer drugs from plants, like tubulin polymerization inhibitors (which is less toxic than current anti-cancer drugs, such as Actinomycin D) and;
- provide man with necessary knowledge to avoid or minimize unwanted side effects from toxicities resulting from use of herbal medicines.

1.1.3 Ethno-medicinal uses represent leads for discovery of modern drugs

Folk or ethno-medicinal uses represent leads that may guide pharmaceutical researchers to

discover modern therapeutic drugs. Scepticism may still exist amongst some researchers (especially individuals who strongly advocate the traditional Western approach to medicine), regarding indigenous folk traditions that have been handed down from generation to generation. However, many important modern compounds for example, atropine, digitoxin, *d*-tubocurarine, ephedrine, morphine, reserpine and many other are of natural origin. Indeed, some have been discovered through following up leads derived from ethnographic research into folk use and information on integration of the indigenous people with their ecosystem. For example, the discovery of thiarubrine-A, a potent antibiotic, from the genus *Aspillia* (Compositae), took place by careful observation of chimpanzees' dietary habit (Rodriguez *et. al*, 1985). Winter (1955) investigated antimicrobial properties of two groups of plants. One group was randomly selected and the other obtained from Herbal Remedy sources, which was previously documented to have properties that are useful for the treatment of infections. Of the former randomly selected plants only 29,5% exhibited antimicrobial activity, while 65% of the latter selected group was active.

1.1.4 Expansion of human civilizations pose a threat to plant biomes

The rapid expansion of human civilization and its health care needs, poses a threat to certain plant biomes (such as rain forests) and human cultures living in and around such biomes. Their disappearance will be accompanied by the extinction of medicinal genetic resources as well as folk knowledge about how to use them for existence and survival. In each of the nine provinces of South Africa, savannas, grassland, forests, fynbos and karoo (to mention a few) are gradually being replaced by agriculture and urban development. These will deplete plant biomes from their resources and in future, they will virtually become extinct. Time is running short to investigate these biomes before they become extinct.

1.1.5 Plants' extraordinary ability to synthesize secondary metabolites

Plants' defence mechanisms are sophisticated which allow them to survive even though they have a sedentary existence. They have to be able to defend themselves against all the hazards to be able to survive. They do this with an enormous variety of secondary metabolites that they synthesize. Several tens of thousands of secondary metabolites have already been isolated and their structures elucidated (Wink, 1999).

The main roles of secondary metabolites have been identified to be:

- defence against herbivores (insects, vertebrates),
- defence against fungi and bacteria,
- defence against viruses,
- defence against other plants competing for light, water and nutrients,
- signal compounds to attract pollinating and seed dispersing animals,
- signals for communication between plants and symbiotic microorganisms (N-fixing Rhizobia or mycorrhizal fungi) and;
- protection against UV-light or physical stress (Wink, 1999).

1.1.6 Bioactive properties derived from plants

Large numbers of surveys have been conducted in which plant extracts have been evaluated for various biological activities. Only a small sample of species are listed in Table 1.1:

Table 1.1. Plants with medicinal uses indicating biological activity, drug name, type of extract or plant part, plant species and reference.

Biological activity	Drug/ extract/ plant part	Plant name	Reference
Antineoplastic	Combretastatin A-4 and B-1 (Subjects of patents)	*Combretum caffrum* *Combretum kraussi*	Pettit *et al*. (1987) Pettit *et al*. (1995)
Antibacterial	Juice	*Vaccinium* spp. (cranberry)	Ofek *et al*. (1996)
Antifungal	Grapefruit peel	*Citrus paradisa*	Stange *et al*. (1993)
Antiviral - AIDS	Glycyrrhizin (flavonoid)	*Glycyrrhiza rhiza*	Watanbe *et al*. (1996)
Antimalarial (plasmodium)	Solvent extract	*Mahonia aquifolia*	Omulokoli *et al*. (1997)
Insecticide	Phenantrenes	*Combretum apiculatum*	Malan and Swinny (1993)
Molluscicide Schistisomiasis	Mollic acid	*Combretum molle*	Rogers (1989)
Hypoglycemic	Leurosine sulphate (alkaloid)	*Catharanthus roseus*	Svoboda *et al*. (1964)
Cardiotonic activity	Extract	*Carissa sp.*	Thorpe and Watson (1953)
Andro- or Estrogenic	Extract	*Butea superba*	Schoeller *et al*. (1940)
CNS	Morphine	*Papaver somniferum*	Schmitz (1985)
Antihelminthic	Dried nuts	*Quisqualis indica* *Combretum molle*	Ladion (1985) Rogers and Verotta (1997)

1.1.7 Compounds from plants that regulate or participate in disease resistance

Plants have developed sophisticated active defence mechanisms against infectious agents (Barz *et al*., 1990). The main aim of these reactions appears to be inhibition of microorganisms with antibiotic compounds, hydrolytic enzymes, inactivation of microbial exoenzymes with specific inhibitors and isolation of lesions. These defence mechanisms operate at different stages of infection (Kuć, 1990a). The external plant surfaces are often covered with biopolymers (fatty acid esters) that are difficult to penetrate. In addition, external surfaces can be rich in compounds (phenolic compounds, alkaloids and steroid glycoalkaloids) that will inhibit

the development of fungi and bacteria (Reuveni *et al*., 1987). Once pathogens have passed the external barriers, they may encounter plant cells that contain sequestered glycosides (Kuć, 1990b). The glycosides may be antimicrobial *per se* or may be hydrolysed to yield antimicrobial phenols; these in turn may be oxidised to highly reactive quinones and free radicals (Noveroske *et al*., 1964). Damage to a few cells may rapidly create an extremely hostile environment for a developing pathogen. This rapid, but restricted disruption of a few cells after infection can also result in the biosynthesis and accumulation of low molecular weight antimicrobial, liphophylic compounds, called phytoalexins.

Phytoalexins differ in structure, with some structural similarities within plant families (Carr and Klessig, 1989). Some are synthesized by the malonate pathway, others by the mevalonate or shikimate pathways, whereas still others require participation of two or all three of the pathways (Kuć, 1990b). Phytoalexins can induce constitutive or other secondary metabolite pathways and link to various metabolic pathways (Barz *et al*., 1990). Since phytoalexins are not translocated, their protective effect is limited to the area of the infection, and their synthesis and regulation are accordingly restricted. Phytoalexins are degraded by some pathogens and by the plant; thus, they are transient constituents and their accumulation is a reflection of both synthesis and degradation.

Often associated with phytoalexin accumulation is the deposition around sites of injury or infection of biopolymers, which both mechanically and chemically restrict further development of pathogens (Hammerschmidt and Kuć, 1982). These biopolymers include: lignin, a polymer of oxidized phenolic compounds; callose, a polymer of β-1,3-linked glucopyranose; hydroxy-proline-rich glycoproteins, and suberin. The macromolecules produced after infection or some forms of physiological stress include enzymes, which can hydrolyse the walls of some pathogens (Carr and Klessig, 1989), including chitinases, β-1,3-glucanases and proteases.

Unlike the phytoalexins and structural biopolymers, the amounts of these enzymes increase systemically in infected plants even in response to localized infection. They are often found intercellular where they would contact fungi and bacteria. These enzymes are part of a group of stress or infection-related proteins commonly referred to as pathogenesis-related (PR) proteins. The function of many of these proteins is unknown. Some may be defence compounds; others may regulate the response to infection (Tuzun *et al.*, 1989).

Another group of systemically produced biopolymer defence compounds comprises the peroxidases and phenoloxidases (Hammerschmidt *et al.,* 1982). Both can oxidize phenols to generate protective barriers to infection, including lignin. Phenolic oxidation products can also cross-link to carbohydrates and proteins in the cell walls of plants and fungi to restrict further microbial development (Stermer and Hammerschmidt, 1987). Peroxidases also generate hydrogen peroxide, which is strongly antimicrobial. Associated with peroxidative reactions after infection is the transient localized accumulation of hydroxyl radicals and superoxide anion, both of which are highly reactive and toxic to cells. Both plant and microbial compounds regulate the expression of genes that encode products that contribute to disease resistance. The speed and degree of gene expression and the activity of the gene products (and not the presence or absence of genes for resistance mechanisms) determine disease resistance in plants (Kuć, 1990b).

The future will probably see the restriction of pesticide use and a greater reliance on resistant plants generated using immunization and other biological control technologies, genetic engineering and classical plant breeding. However, as with past and current technology, we may create unique problems. The survival of our planet may significantly depend upon anticipating these problems and meeting the challenge of their solution.

1.1.8 Antibacterial resistance – research to find alternative or natural antibiotics remains a matter of urgency

We are largely dependant on the pharmaceutical industry to continue to provide us with new antimicrobial agents to which bacteria have not yet developed resistance. Antibiotic resistance, which resulted from the frequent and unwise use of antibiotics, is a problem in hospital environments and can lead to the spread of resistant strains to communities. Resistance is determined by the bacterial genome, which may change rapidly (Berkowitz, 1995). A 'new' antibiotic may have a limited time in which bacteria have developed little or no resistance to it; thus the search for new antibiotics remains a continuous urgent priority.

The most common bacterial pathogens causing nosocomial infections are *Escherichia coli* (commonest pathogen in adult services), *Staphylococcus aureus* (commonest pathogen in paediatric and newborn services), *Enterococcus faecalis* (antibiotic resistant, some also against Vancomycin) and *Pseudomonas aeruginosa* (Sacho and Schoub, 1977). Despite the availability of a wide range of antibiotics (e. g. penicillin, cephalosporins, tetracycline, amino-glucosides, monobactams, carbapenems, macrolides, streptogranins and dihydrofolate reductase inhibitors), the percentage of people who die in hospitals is steadily increasing, because of resistant bacterial infections. Table 1.2 lists resistance mechanisms of pathogens to antimicrobial agents.

Table 1.2. Major resistance mechanisms of pathogens to antimicrobial agents (Jacoby and Archer, 1991).

Type of antimicrobial class	Specific resistance mechanism of pathogen
Quinolones	Altered DNA gyrase
Rifampicin	Altered RNA polymerase
Sulfonamides	New drug-insensitive dihydropteroate reductase
Tetracycline	Ribosomal protection
Trimetoprim	New drug-insensitive dihydrofolate reductase
Vancomycin	Altered cell wall stem peptide
Main action of antimicrobial and antibiotic that addresses it	**Specific resistance mechanism of pathogen**
Detoxifying enzyme	
Aminoglucosides (amikacin, gentamycin, kanamycin, netilmycin, tobramycin)	Acetyltransferase, nucleotidyltransferase, Phospho transferase
B-Lactam antibiotics (carbapenems, cephalosporins, monobactams, penicillin)	B-Lactamase
Chloramphenicol	Acetyltransferase
Decreased uptake	
Diminished permeability	
B-lactam antibiotics, chloramphenicol, quinolones, tetracycline, trimethoprim	Alterations in outer membrane proteins
Active efflux	
Erythromycin	New membrane transport system
Tetracycline	New membrane transport system

1.1.8.1 Useful antimicrobial phytochemicals

Useful antimicrobial phytochemicals can be divided into several categories, as shown in Table 1.3.

Table 1.3. Major classes of antimicrobial compounds from plants (Cowan *et al*., 1999).

Class	Subclass	Example(s)	Mechanism	References
Phenolics	Simple phenols	Catechol Epicatechin	Substrate deprivation Membrane disruption	Peres *et al*. (1997) Toda *et al*. (1992)
	Phenolic acids	Cinnamic acid		Fernandez *et al*. (1996)
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, Inactivate enzymes	King and Tempesta (1994)
	Flavonoids	Chrysin	Bind to adhesins	Perrett *et al*. (1995)
	Flavones	Abyssinone	Complex with cell wall Inactivate enzymes Inhibit HIV reverse transcriptase	 Brinkworth *et al.* (1992) Ono *et al*. (1989)
	Flavonols	Totarol	?	Kubo *et al*. (1993)
	Tannins	Ellagitannin	Bind to proteins Bind to adhesins Enzyme inhibition Substrate deprivation, complex with cell wall, Membrane disruption, metal ion complexation	Stern *et al*. (1996) Scalbert (1991) Haslam (1996)
	Coumarins	Warfarin	Interaction with eucaryotic DNA (antiviral activity)	Bose (1958)
Terpenoids, essential oils		Capsaicin	Membrane disruption	Cichewicz and Thorpe (1995)
Alkaloids		Berberine Piperine	Intercalate into cell wall/ or DNA	Rahman and Choudhary (1995)
Lectins and polypeptides		Mannose-specific agglutinin Fabatin Polyacetylenes	Block viral fusion or adsoption 8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	Zhang and Lewis (1997) Estevez-Braun *et al*. (1994)

In recent studies, several antibacterial compounds were isolated in the Combretaceae plant family, some for the first time from this family. The flavanols: kaemferol, rhamnocitrin, rhamnazin and quercitin 5,3'-dimethylether and flavones apigenin, genkwanin and 5 hydroxy-7,4'-dimethoxyflavone were isolated from *Combretum erythrophyllum* (Martini, 2002), and the stilbene, 2',3,4-trihydroxyl,3,5,4'-trimethoxybibenzyl (combretastatin B5) from *C. woodii.* This is the first report of antimicrobial activity of combretastatin B5 (Famakin, 2002). Two flavanones: alpinentin, pinocembrin and one chalcone: flavokwavain were isolated from *C. apiculatum* subsp. *apiculatum* (Serage, 2003). Terminoic acid isolated from *Terminalia sericea*, was compared to commercial gentamycin cream for use as a topical antibacterial remedy on mice's skin by Kruger (2004).

1.1.9 Plant compounds' role in the treatment of cancers

Some confusion exists in the use of the terms 'cytotoxicity', 'antineoplastic' and 'antitumour'. The National Cancer Institute (NCI) has defined these terms: cytotoxicity refers to *in vitro* toxicity of tumour cells, while antineoplastic and antitumour should refer to *in vivo* activity in experimental systems (Ghisalberti, 1993). Between 1955 and 1982, NCI screened 35 000 plant species representing 1551 genera compromising 114 000 extracts for *in vitro* cytotoxicity and *in vivo* activity against various animal tumour systems (Hamburger *et al*., 1991). Estimates indicate that there are approximately 250 000 terrestrial species of higher plants. Since plants have four or five different plant parts, a comprehensive screening program would require a million or more samples per assay. Approaches that are more pragmatic are usually followed (for example: leads from ethno-medicines or chemotaxonomy). Existing *in vivo* test systems, for example, xenografts on immune deficient mice, are far too slow, complex, expensive and probably immoral to be used as a mass screen. Using cells derived from human cancers in an *in vitro* setting, on the other hand, is quite compatible with the desired goal (Lednicer and Narayanan, 1993). Plant constituents able to kill cancer cells, and hence described as being

"cytotoxic" exhibit a very large range of structural types. The presence of tannins or other polyphenolic materials should be taken into account when enzyme-based bioassays are being used, because false positive results are often observed (Cordell *et al*., 1993).

Conventional anti-cancer drugs are designed to arrest and kill rapidly dividing cancer cells. They are however, non-selective, chemotherapy and radiation will kill both normal and tumour cells therefore, drugs with selective pharmacodynamics are sought after. A number of secondary metabolites and their derivatives of plant origin, as well as natural products of marine and microbial origin are currently in preclinical and clinical trials as potential anticancer agents. One such is 'combretastatin' from the Combretaceae plant family. It was isolated from the bark of the South African *Combretum caffrum* by Dr Gordon Gragg (an ex-South African organic chemist) in the laboratories of Prof Pettit at Arizona State University (Pettit *et al*., 1982). Since the yield was very low – 26,4 mg was isolated from 77 kg dry stem bark – several forms were synthesized and tested. Experiments examining the effect of combretastatin A4 and combretastatin A4 phosphate on murine tumours demonstrated that combretastatin A4 phosphate caused selective extensive vascular shutdown of tumours (more detail in 1.1.9.2). The vascular shutdown was followed by large-scale cell death and necrosis within 24 h after administration (Chaplin *et al.*, 1999).

In Tanzania 47 plants were evaluated for cytotoxic activity by testing their methanolic extracts on three human cancer cell lines. Of the nine plants traditionally used to treat cancer, only two exhibited a cytotoxic effect. Of the 38 plants that are used to treat non-cancer diseases, 14 exhibited a cytotoxic effect. *Pteleopsis myrtifolia* was one of the plants not traditionally used to treat cancers that had cytotoxic effects: at 100 μg/ml *in vitro* 75-100% inhibition of growth was obtained for the HT29 (colon adenocarcinoma) and A431 (skin carcinoma), and 25-50% for HeLa (cervical carcinoma) cells (Kamuhabwa *et al.*, 2000).

Cancer itself creates oxidative stress and impairs antioxidant status in the organism as a whole. Chemotherapy can overwhelm the antioxidant defence systems in the cell, which will lead to an increase in lipid peroxidation, which in turn leads to a decrease in cellular proliferation and therefore to a decrease in the effectiveness of chemotherapeutic agents. Patients with an impaired antioxidant status may become relative resistant to chemotherapy. There is also evidence that antioxidants improve the antitumour response to antineoplastic agents (Drisko *et al.*, 2003).

1.1.9.1 Incidence of cancer

Cancer is the second leading cause of death amongst Americans. One out of every four deaths in the U.S. is due to cancer. Figures for the year 1990 showed that the rate of growth in cancer cases (2,1% per year) was superseding that of the overall population increase (1,7%/year) (Kinghorn *et al.*, 1999). In the United States in 1999, over 1500 people were expected to die of cancer each day. A United States Cancer Report was released (by the Centres for Disease Control and Prevention (CDC), National Cancer Institute (NCI) and North American Association of Central Cancer Registries (NAACCR)) in November 2003 (to date the most current available) with cancer incidence data up to the year 2000. In 1994, U.S. death rates (for all cancer sites combined) decreased up to 1998 and stabilized from 1998 through 2000. Increases in breast cancer amongst woman and prostate cancer amongst men are masked by statistics of a decrease in all cancer sites combined.

1.1.9.2 Combretastatin

When a study on cancer cell growth inhibitors of the African willow tree (*Combretum caffrum*) was carried out, several active phenanthrenes, stilbenes and bibenzyls were isolated. Two potent cell growth and tubulin polymerisation inhibitors, the bibenzyls combretastatin A-1 (Lin *et al*., 1989) and combretastatin A-4 (Pettit *et al*., 1989) were of particular importance. Combre-

tastatin was found to prevent astrocyte maturation (Baden *et al*., 1981) and to inhibit tubulin polymerisation (Boyd, 1993). Table 1.4 show results of an evaluation of the combretastins A-1 to A-6 in the US NCI screens (Boyd, 1993).

Table 1.4. Antitumour evaluation of combretastatins in the NCI *in vitro* panel of 60 human tumour cell lines.

Combretastatins	Mean panel GI_{50} (x 10^{-8}M)
A-1	1.62
A-2	3.16
A-4	0.32
A-5	165.00
A-6	>10000

Combretastatin A-4 has been studied intensively because of its potent cytotoxicity. The drug seems to induce apoptosis of cells, suggesting that it may activate at least one specific intracellular signalling pathway. *In vivo* studies support the suggestion that combretastatin A-4 causes a rapid vascular collapse by increasing tumour vessel permeability (Dark *et al*., 1997).

Although combretastatin A-4 exhibits potent biological activity, it has poor pharmacokinetic properties due to its high lipophilicity and low water solubility (Ohsumi *et al*., 1998). Synthesis of more water-soluble analogues in order to enhance and promote more desirable qualities such as chemical stability, bioavailability and decrease of side effects, are required. Several derivatives have been prepared and were evaluated as pro-drugs, but it was proven to be insoluble in water. The analogues with dipotassium and disodium phosphate (Figure 1.1) had good water solubility.

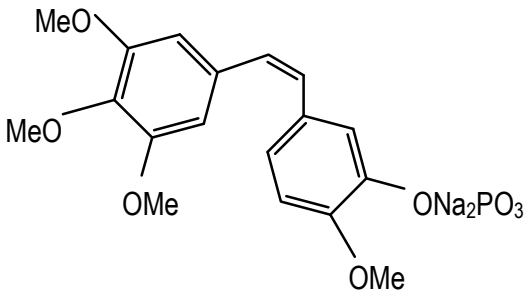


Figure 1.1. Structure of the combretastatin A-4 disodium phosphate analogue.

The combretastatin A-4 disodium phosphate analogue, known as CA4P, is undergoing phase I clinical trials (Dowlati *et al*., 2002) and has potential for cancer treatment in combination with other conventional antitumour drugs (Chaplin *et al*, 1999). CA4P is itself inactive but there is rapid phosphate hydrolysis *in vivo* to produce combretastatin A-4 (Chaplin *et al*, 1996). Histological studies carried out by the Pettit group (Chaplin *et al*., 1999), demonstrated that 90% of vessels were non-functional 6 h post-treatment with 100 mg/kg ip. Further, it showed that normal vessels were unaffected. This selectivity seems to come from a process of *in vivo* phosphate hydrolysis by endogenous non-specific phosphates, with greater rates in tumour vascular systems than in the normal vascular system (Griggs *et al*., 2001).

Since there was considerable interest in the possibility of separating the cytotoxic activity of CA4P from its ability to effect vascular shutdown, analogues have been prepared that do indeed display this type of selectivity (Hadimani *et al*., 2003). Future work in this area will be of interest to those studying the various retinopathies and other vascular diseases (Cirla and Mann, 2003).

1.1.10 Antioxidative properties of plants

Since reactive oxygen radicals play an important part in carcinogenesis, antioxidants present in consumable fruits, vegetables, neutraceuticals and beverages have received considerable attention as cancer chemopreventative agents (Muktar *et al.*, 1994). The balance between an

individual's intake of antioxidants and exposure to free radicals may literally be the balance between life and death (Holford, 1997). Several compounds from plants play cancer preventative roles. The antioxidant activity of several plant constituents, beyond the vitamins, in the form of crude extracts and isolated compounds, has been put into consideration (Gazzani *et al*., 1998). Many phenolic compounds, including flavonoids, have attracted considerable attention because the antioxidant activity thereof has been reported to be more powerful than vitamins, C, E and β-carotene (Vinson *et al*., 1998). Consumption of the flavonoids and their potential significance as antagonists of oxidative stress has been an interesting subject of many investigations. One of the best approaches for discovering new antioxidants, is the screening of plant extracts (Souri *et al*., 2004). Recently it has been found that proanthocyanidins from grape seeds inhibited the activation of mitogen-activated protein kinases (MAPK) and nuclear factor κB (NFκB) pathways in human prostate carcinoma cells, thus preventing cancer (Vayalil *et al*., 2004).

In more recent years, a variety of substances normally included in the diet have come under more critical investigation for the neutraceutical value thereof. The occurrence or lack of certain diseases in specific demographically defined areas of the world led to comparative analysis of the population's diets. Food items known for their antioxidant value have been investigated. Recently studies on tea (the most popularly consumed beverage aside from water and associated with decreased risk of various proliferative diseases such as cancer and arteriosclerosis in humans), provided evidence that green tea catechins, in addition to their antioxidative properties, also effect the molecular mechanisms involved in angiogenesis, extra cellular matrix degradation, regulation of cell death and multidrug resistance (Demeule *et al.*, 2002).

1.1.11 Phytochemistry of the Combretaceae plant family

Medicinal uses from the Combretaceae plant family by traditional healers in Africa have almost exclusively been of species from the genus *Combretum* and to a lesser extent, *Terminalia.* These species have been used for the treatment of a wide range of disorders, but only about 25% percent of the African species of *Combretum* have been subjected to scientific study. With the exception of a few species of *Terminalia, Annogeissus* and *Guiera*, very little have been reported on the phytochemistry of the remaining genera (Rogers and Verotta, 1997). In a preliminary investigation of the antibacterial activity of 27 members of the South African Combretaceae, Eloff (1999) found that other genera in the Combretaceae (like *Pteleopsis* and *Quisqualis*) displayed antibacterial activity similar to that of the *Combretum* genus.

Combretum genera secrete triterpenoid mixtures onto the surface of their leaves and fruit through epidermal trichomes. The anatomy of the trachoma's and the chemical composition of the triterpenoids are both species specific and are of taxonomic importance (Lawton *et al*., 1991). Treatment of leaves from South American and Indian species yielded mixtures of acidic triterpenoids similar to those found in South African species (Rogers, C. B., unpublished data). These results as suggest that their distribution must have been established when continents from Gondwanaland had separated by a significant amount approximately 120 million years ago. The genus *Pteleopsis* occurs in the sub-tribe Pteleopsidinae and comprises 10 spp. Moreover, the genus *Quisqualis* occurs in the sub-tribe Combretiae and comprises 16 spp.

For more information on metabolites isolated in Combretaceae so far, see 3.1.3 of Chapter 3.

1.1.12 Distribution of *Pteleopsis myrtifolia*

Pteleopsis *myrtifolia* (common names - Myrtle Bush willow, 'basterraasblaar' or 'stinkboswilg', Mnepa, Mgoji) of the family Combretaceae occurs in Botswana, Zimbabwe, Angola, Zambia,

Mozambique, Malawi, Tanzania, Kenya and South Africa. In South Africa, the occurrence thereof is north of the Soutpansberg in the vicinities of Messina and Sibasa, in the Punda Milia area in the Kruger National Park and in the North Eastern part of KwaZulu-Natal, from Ndumu and Kosi Bay reaching as far south as the Hluhluwe vicinity (Figure 1.2).

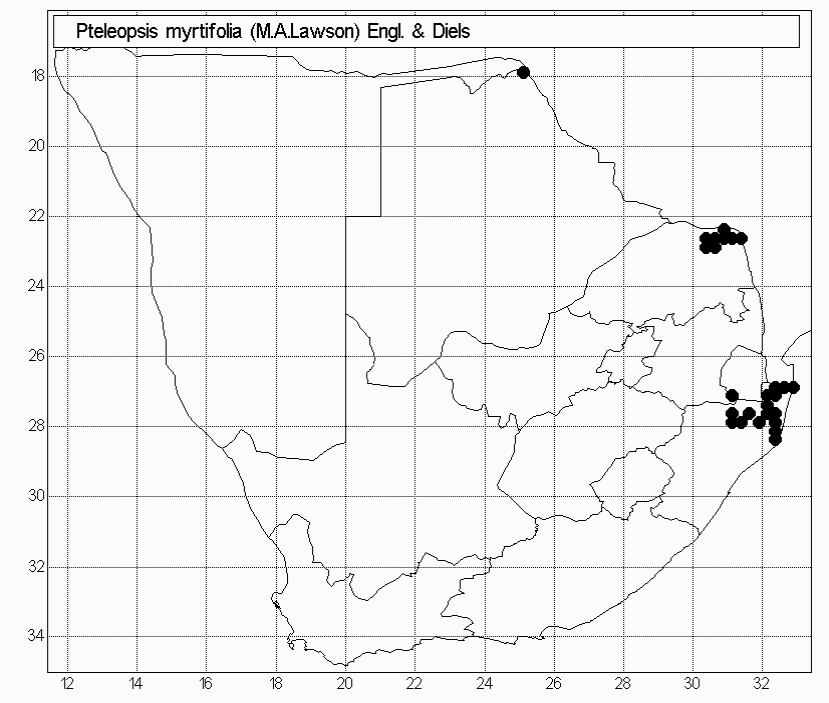


Figure 1.2. Distribution map of *Pteleopsis myrtifolia* (Precis data from SANBI, 2005).

This tree occurs in Brachystegia, Mopane and Baikieae woodland, also in *Acacia* or Combretaceae savannah, evergreen forest and riverine forest, from near sea level up to 1500m. It thrives in sand and under such conditions can be the dominant species. In sand, its growth form can be shrub in dense thickets, reaching 2 – 4 m. In *Acacia* or Combretaceae savannah, its growth form is trees that reach 10 – 12 m (30 m in Tanzania) (Carr, 1988; Van Wyk 1974). The growth form, flowers and fruit of *P. myrtifolia* are shown in Figure 1.3.

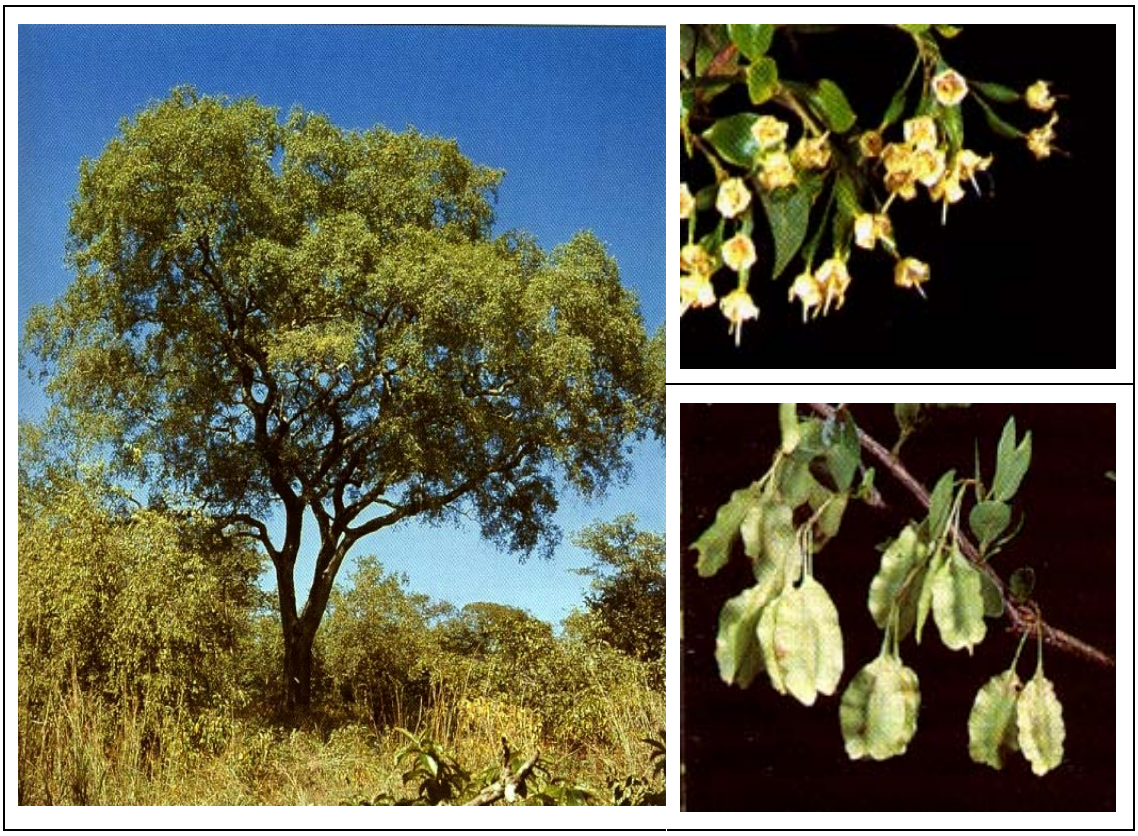


Figure 1.3. *Pteleopsis myrtifolia* tree (left), flowers (top right) and fruit (bottom right) (Van Wyk *et al*., 2000).

1.1.13 Distribution of *Quisqualis littorea*

The genus *Quisqualis* consists of 17 species of woody vines and climbing shrubs native to the Old World tropics. The name is from the Latin '*quis'*, who?, and '*qualis'*, what ? This name was given by the early botanist Rumphius as an expression of his surprise at the variability of the plant's growth and flower colour. *Q. littorea*'s growth form is a climber, it occurs mainly in forests in central Africa, with a few plants in the Laeveld National Botanical Garden (Jongkind, 1993). No distribution map could be found and the South African National Botanical Garden in Pretoria (SANBI) could not provide data for a map.

Blossoms and fruit of *Q. indica* are shown in Figure 1.4.

Figure 1.4. *Quisqualis indica* blossoms (left) and fruit (right) (A Short Story About *Quisqualis* Fruit, 2003).

1.1.14 Bioactivities of *Pteleopsis species*

Pteleopsis suberosa: The aqueous extract of bark has anti-ulcer activity against indomethacin-induced ulcers in rats (De Pasquale *et al.*, 1995). In Mali, it is used locally (10 ml/kg) for the treatment of gastric ulcers. The aqueous extract's mechanism of action appears to be similar to other known triterpenoids (derivatives of glycyrrhetinic acid, a triterpenoid saponin from liquorice roots), now used in treatment of gastric ulcers. The mechanism of action may be due to its coating property that has a protective effect on the gastric mucosa. Sodium carbenoxolone, a triterpenoid related compound, is effective as an anti-ulcer agent because it protects the mucosa from gastric effects by selectively inhibiting prostaglandin $PGF_{2\alpha}$ (Aguwa and Okunji, 1986). The methanolic extract of stem bark was effective (at MIC's of 31.25 – 250 μg/ml) against gastric ulcers associated with *Helicobacter pylori* infections in rat (Germano *et al.*, 1998).

Traditional use of this plant for the treatment of cough and other respiratory diseases was confirmed by the fact that a 1000 mg/ kg dose of a decoction, reduced citric acid induced cough in guinea pigs by 73.72 % (Occhiuto *et al.*, 1999).

Preliminary results of a previous investigation indicated that polar substances were responsible

for antimicrobial activity and that the tannins were also involved in antibacterial activity. The methanolic extracts had antimicrobial activity against some microorganisms that are responsible for skin infections (*Staphylococcus aureus*, *S. capitatis*, *S. epidermidis*, *S. saprophyticus*, *Bacillus subtilus*, *Pseudomonas aeruginosa*, *P. cepacia*, *Cochlospermum tinctorium* but not against *Escherichia coli*, *Proteus vulgaris* and *P. mirabilis*). The antibacterial activity established, possibly justify the traditional use of these plants in folk medicine for treatment of skin diseases (Bisignano *et al.*, 1996). Antifungal activity of shoot and stem-bark samples (MIC's 0.25 – 1 mg/ml) against *Candida albicans*, *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* can be contributed to the tannins and saponins, which occurs richly in these plants (Baba-Moussa *et al.*, 1999).

Pteleopsis hylodendron is highly valued in folk medicine in Cameroon. The aqueous concoction of the stem bark is used in the treatment of sexually transmitted diseases, female sterility, liver and kidney disorders as well as dropsy. The ethyl acetate extract was found to be active against the bacteria *Bacillus cereus*, *Corynebacterium diptheriae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Streptococcus pyogenes* (Ngounou *et. al*, 1999).

Pteleopsis myrtifolia: Decoctions of these trees' roots have been used by Zulus (Hutchings *et al.*, 1996) and by the traditional healers of Tanzania for venereal diseases (Kokwaro, 1976). Root decoctions and leaf sap have also been used for dysentery (Neuwinger, 2000). Methanolic extracts of roots (that contain many polar compounds) showed cytotoxic activity (100 μg/ml) *in vitro,* 75-100% inhibition of growth against HT29 (colon adenocarcinoma) and A431 (skin carcinoma) and 25-50% inhibition of growth against HeLa (cervical carcinoma) (Kamuhabwa *et al.*, 2000).

1.1.15 Bioactivities of *Quisqualis species*

Quisqualis littorea's synonyms are *Q. falcata, C. falcatum, C. mussaendiflorum, Q. mussaendiflora, C. sericogyne, C. pellegrinianum, Q. pellegriniana* and *Cacoucia littoria* (Jongkind, 1993).

Although Jongkind (1991) suggested that '*Quisqualis*' be incorporated into '*Combretum*', '*Quisqualis*' is still recognised as the official name in South Africa (Germishuizen and Meyer, 2003) and Central Africa (Lebrun and Stork, 1991).

Quisqualis indica is antiviral (at non-cytotoxic concentrations), minimally in the range of 30 – 80 μg plant material/ ml) against the double-stranded DNA murine cytomegalovirus (MCMV) and the single-strand RNA Sindbus virus (SV) (Yip *et al*., 1991). Fruit and roots were found to be antihelminthic (Monzon, 1995). Extracts (50% ethanol and ethanol) inhibited (> 30%) phosphodiesterase (Thein *et al*., 1995). Amoebicidal, antimalarial, antibacterial, and antispastic drugs appear active in phosphodiesterase inhibition tests (Weinryb *et al.* (1972). Further more, it was found to be antibacterial (Nyein and Zaw, 1995), and the acetone extracts of leaves displayed antifungal activity (inhibition of germ-tube elongation) (Ganesan, 1992). Leaf extracts in India were used as a vermifuge (Cirla and Mann, 2003).

Previous investigations of species from the Combretaceae at the University of Pretoria, have isolated and determined the structure and biological characteristics of compounds and extracts. Some extracts had such good activity that commercial applications in the protection of animal health are currently under way. There are a strong probability that similar activities and applications may be discovere*. myrtifolia* and *Q. littorea.*

P. myrtifolia and *Q. littorea* have previously only been included in preliminary investigations (Eloff, 1999; Kamuhabwa *et al*., 2000) and no report of a thorough investigation exists. The

absence of information on *P. myrtifolia* and *Q. littorea* motivated this study.

1.2 Hypothesis, aim and objectives of this study

1.2.1 Hypothesis: *Pteleopsis myrtifolia* and *Quisqualis littorea* are under-evaluated species of the Combretaceae and have antibacterial activity. *P. myrtifolia* has cytotoxic and antioxidant activity as well.

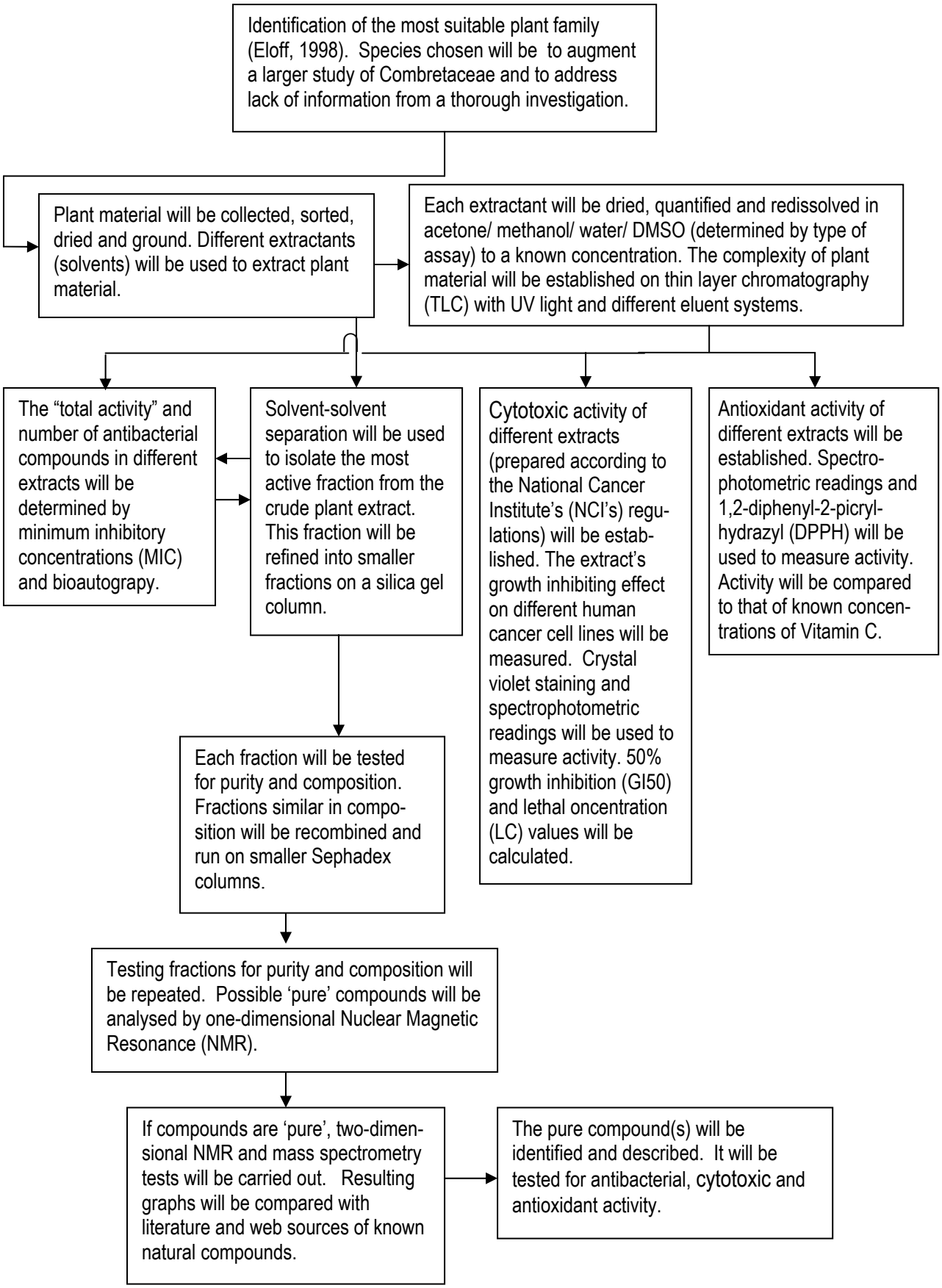
2.2 The aim of this study (as part of a comprehensive project to explore less known genera of the Combretaceae is):

- to investigate extracts of *P. myrtifolia* and *Q. littorea* for antibacterial activity, to investigate extracts of *P. myrtifolia* for cytotoxic and antioxidant activity, as well as to determine the chemical structure and activities (antibacterial, cytotoxic and antioxidant) of possible pure compound(s) isolated by bioassay- guided fractionation from *P. myrtifolia* leaves.

2.3 The objectives of this study are:

- to prepare extracts of *P. myrtifolia* and *Q. littorea* over a wide polarity range in order to identify range in order to identify extracts with antibacterial activity, using *Staphylococcus aureus, Enterococcus faecalis* (Gram-positive); *Pseudomonas aeruginosa* and *Escherichia coli* (Gram-negative) recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1990).
- to investigate cytotoxic activity of different leaf extracts from *P. myrtifolia* on the following human cell lines: oesophagus (WHCO3), breast (MCF-7), lung (H157), cervix (HeLa) (transformed) and breast (MCF12) (non-transformed).
- to identify extracts from *P. myrtifolia* with oxidant scavenging activity.
- to isolate pure compounds from active extracts of *P. myrtifolia* and;
- to investigate antibacterial, cytotoxic and oxidant scavenging activity of pure compounds.

1.3 Schematic representation of the research methodology



1.4 Envisaged contributions of this study:

- Information about antibacterial activity of extracts from *P. myrtifolia* and *Q. littorea* will be determined.
- Cytotoxic activities of different *P. myrtifolia* leaf extracts will be established (Preliminary investigations indicated that *Q. littorea* material would not be sufficient for several assays).
- Antioxidant activities of different *P. myrtifolia* leaf extracts will be determined.
- Possible pure compound's structure from *P. myrtifolia* will be elucidated with NMR and MS. This may contribute to existing knowledge about Combretaceae's phytochemistry.
- Antibacterial, cytotoxic and antioxidant activities of possible pure compounds will be established.
- This knowledge may not only help in the discovery or development of new therapeutic agents, it will also contribute to the knowledge of where new sources of economic viable materials (such as tannins and gums, precursors for the synthesis of complex chemical substances) can be found.
- The results of this research will be submitted as articles (Chapters 2 and 3 combined, Chapter 5, Chapter 6, and Chapters 7 and 8 combined, with some modifications).

1.5 Statistical considerations:

Dr P. Becker from the Medical Research Council (MRC) in Pretoria was involved in the analyses and interpretation of the results. Growth inhibition, lethal concentration and 50% data (growth inhibition of 50% [GI_{50}]) were summarized and displayed graphically (taking into account extraction methods using descriptive statistics, mean and SD). Outcome data was analysed in appropriate analysis of variance for the functional designs of the different experiments. The main effects of the different experiments were cell lines, extraction methodology and concentration. Of great importance was the interpretation of interactions between the factors that were present. Testing was done at the 0.05 level of significance and the Strata Release

statistical software was employed.

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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.

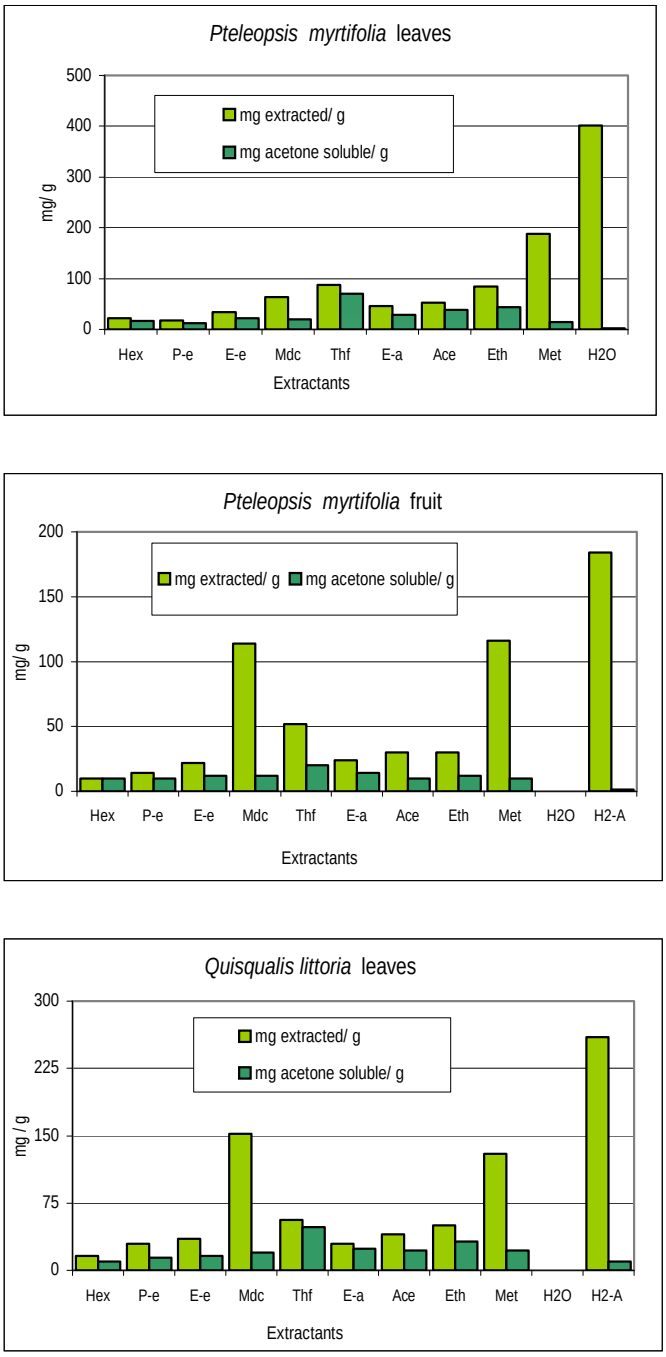


Figure 2.1. Milligram plant material extracted (■) (per gram dry mass) as well as mg acetone soluble plant material extracted (■) for *Pteleopsis myrtifolia* leaves (top), *Pteleopsis myrtifolia* fruit (middle) and *Quisqualis littorea* leaves (bottom) and different extractants. Hex = *n*-hexane, P-e = *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

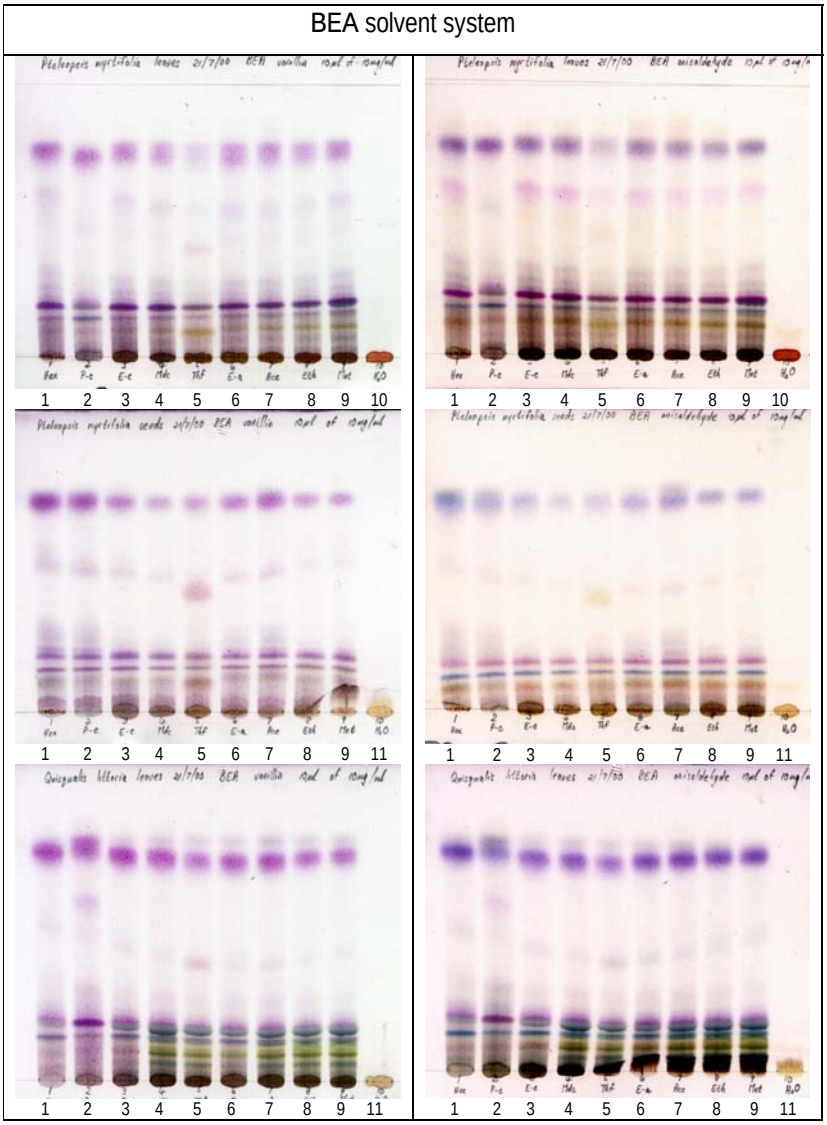
P. myrtifolia fruit - tetrahydrofuran, ethyl acetate and equally *di*-ethyl ether, methylene dichloride and ethanol

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.

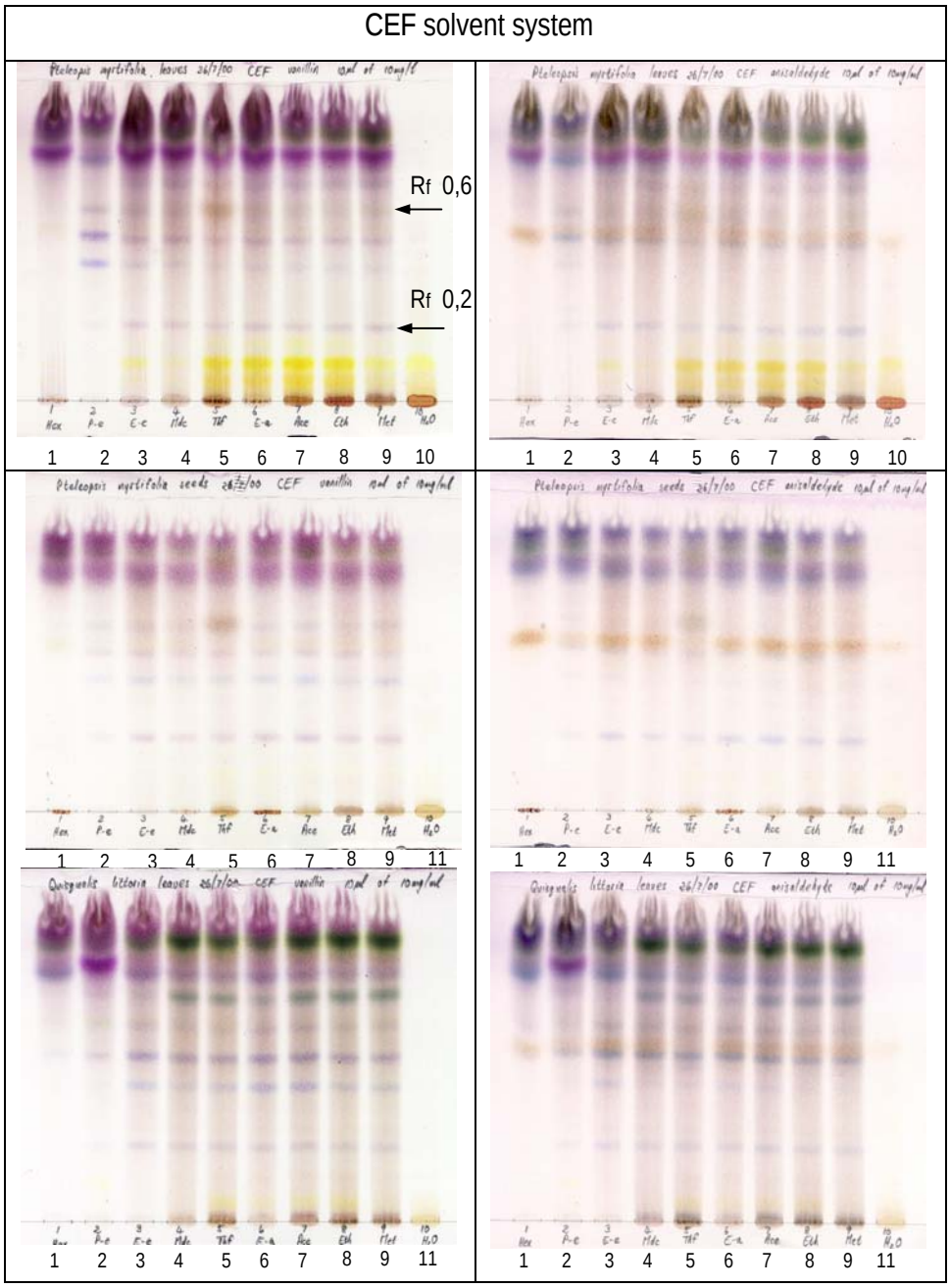
2.3.2 Chemical composition of extracts

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



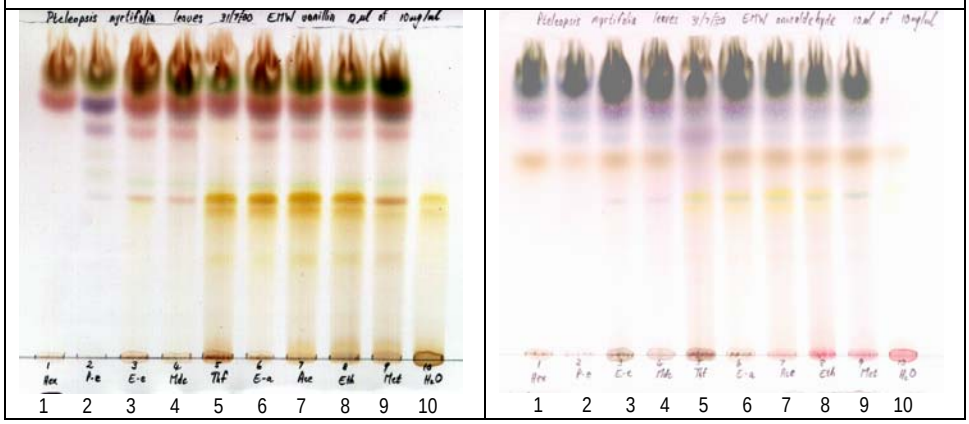
(a)

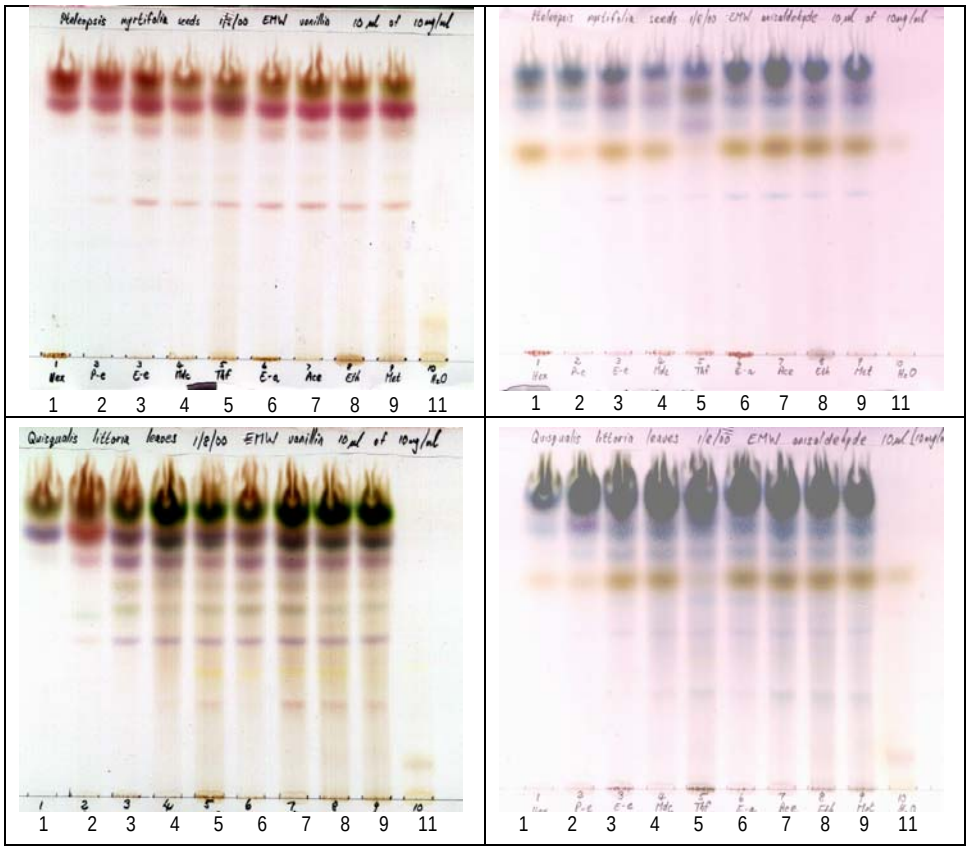
CEF solvent system



(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).

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Chapter 3

Antibacterial activity of extracts of *Pteleopsis myrtifolia* leaves and fruit and *Quisqualis littorea* leaves.

Abstract

Pteleopsis and *Quisqualis* are two of the less known genera of the Combretaceae plant family that have antibiotic activity. The aim of this research was to investigate several extracts of *Pteleopsis myrtifolia and Quisqualis littorea*, thereby facilitating the isolation of compounds from the complex blend of substances in the plant, some of which are antibacterial. Different extractants yielded between 1% and 40.2% of the dry mass and 0.1% to 7 % of the dry mass was acetone soluble. Thin layer chromatography (TLC) indicated that the best separation occurred with an eluent system that separated compounds of medium polarities, like CEF (chloroform: ethyl acetate: formic acid (5:4:1). The extracts of all plant materials (*P. myrtifolia* leaves and fruit and *Q. littorea* leaves) had antibacterial activity against Gram-positive bacteria, with MIC values between 0.039 and 0.63 mg/ml. With *P. myrtifolia* leaves, the lowest average MIC values were found (in increasing order) for *Enterococcus faecalis* (0.078 mg/ml), *Staphylococcus aureus* (0.195 mg/ml), *Pseudomonas aeruginosa* (0.75 mg/ml), *and Escherichia coli* (>0.97 mg/ml). The Gram-positive bacteria were most sensitive and compared with the MIC values of antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol respectively. With *P. myrtifolia* fruit, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.13 mg/ml), *S. aureus* (0.13 mg/ml), *P. aeruginosa* (2.4 mg/ml), and *E. coli* (>2.5 mg/ml). With *Q. littorea* leaves the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.09 mg/ml), *P. aeruginosa* (0.31 mg/ml), *E. coli* (0.33 mg/ml) and *S. aureus* (0.58 mg/ml). For *P. myrtifolia* leaves and fruit, the Gram-negative bacteria had higher MIC

values that varied between 5.0 and 1.25 mg/ml. With *Q. littorea* leaves the average MIC value for Gram-negative bacteria was 0.32 mg/ml compared to the average MIC values of 1.86 mg/ml and 2.44 mg/ml for *P. myrtifolia* leaves and fruit respectively. The average antibacterial activity for each bacterium was higher in the leaves than in the fruit. Results obtained in this study make it clear that *P. myrtifolia* leaves and fruit and *Q. littorea* leaves contain several antibacterial compounds. *Q. littorea* had high activity, expressed as total activity, 234.7 ml/g for the Gram-negative bacterium, *E. coli*.

3.1 Introduction

3.1.1 Antibiotic resistance

With the increasing resistance that microbes show against medicine and an increased awareness of toxicities in refined products amongst modern city dwellers, individuals are progressively focusing their attention towards herbal medicine in an effort to find an alternative approach to living healthier. Antibiotic resistance, which resulted from the frequent and unwise use of antibiotics, is a problem in especially hospital environments and can lead to the spread of resistant strains to communities. Resistance is determined by the bacterial genome, which may change rapidly (Berkowitz, 1995). A 'new' antibiotic may have a limited time in which no bacteria has resistance to it and the search for new antibiotics must carry on. The most common bacterial pathogens causing nosocomial infections are E. *coli* (commonest pathogen in adult services), *S. aureus* (commonest pathogen in paediatric and newborn services), E. *faecalis* (antibiotic resistant, some also against Vancomycin) and P. *aeruginosa* (Sacho & Schoub, 1977).

While antibiotic resistance "benefits" the microbes, it presents humans with two big problems: it makes it more difficult to purge infections from the body; and it heightens the risk of acquiring infections in a hospital. The Department of Healthcare Epidemiology and Infection Control at

the University of Pennsylvania Medical Centre reported that antibiotic-resistant "super bugs" cause an estimated 19 000 deaths in the US each year, compared to 372 deaths worldwide that have been attributed to severe acute respiratory syndrome (SARS) since its outbreak in November 2002. According to the centres for disease control and prevention (CDC) statistics:

- Nearly two million patients in the United states get an infection in the hospital each year;
- Of those patients, about 90 000 die each year as a result of their infection (compared to 13 300 patient deaths in the year 1992);
- More than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to kill them;
- Persons infected with drug resistant organisms are more likely to have longer hospital stays. In addition, they require treatment with second or third choice drugs that may be less effective, more toxic, and more expensive (http://www.niaid.nih.gov/factsheets/antimicro.htm, 2004).

3.1.2 Plant secondary compounds are frequently associated with plant taxons

Plant secondary components are frequently associated with plant taxons. From data provided by Cunningham (1990), Eloff (1998b) calculated that although the Combretaceae is a relatively small family, the scale of use in KwaZulu-Natal is large relative to most other plant families. The Combretaceae thus make up a major group of plants that has potential to offer novel or alternative phytomedicines.

In preliminary investigations of the antibacterial activity of 27 members of the South African Combretaceae family Eloff (1999) found that the MIC's of acetone leaf extracts of *P. myrtifolia* and *Q. littorea* were in the same order as that of other genera in the Combretaceae. In this investigation, one of the highest antibacterial activities found, less than 0.1 mg/ml, was for an acetone leaf extract of *Q. littorea* and *Staphylococcus aureus* (Gram-positive cocci).

In the Combretaceae plant family, traditional healers in Africa have confined themselves almost exclusively to the use of species from the genus *Combretum* and to a lesser extent, *Terminalia.* These species have been used for the treatment of a wide range of disorders, but only about 25% of the African species of *Combretum* have been subjected to scientific study. With the exception of a few species of *Terminalia, Annogeissus* and *Guiera*, very little have been reported on the phytochemistry of the remaining genera (Rogers & Verotta, 1997).

3.1.3 Metabolites isolated in Combretaceae

Metabolites isolated in Combretaceae so far include alkaloids, tannins, flavonoids, amino acids; substituted phenanthrenes, triterpenoid acids and their saponins mainly from the cycloartane and oleane types; unique stilbenes, their glucosides and macrocyclic lactones called combreta-statins. Many *Combretum* species exude gums similar to gum Arabic (Rogers & Verotta, 1997). Certain metabolites show cytotoxic, molluscicidal, anti-HIV, antimicrobial and anti-inflammatory activity and several triterpenoid mixtures strongly inhibit seed germination and seedling growth. In nearly all species Carr & Rogers (1987) studied, geographical and seasonal variation had little or no effect on the composition of extracts isolated.

3.1.4 Bioactive properties of *Pteleopsis*

Bioactive properties of *Pteleopsis* spp. are discussed in 1.14 of Chapter 1.

3.1.5 Bioactive properties of *Quisqualis*

Bioactive properties of *Quisqualis* spp. are discussed in 1.15 of Chapter 1.

As mentioned in Chapter 1, previous investigations of species of the Combretaceae at the University of Pretoria, have isolated and determined the structure and biological characteristics of compounds and some extracts had such good activity that commercial applications in the

protection of animal health are currently under way (Kruger, 2004). There is a strong probability that similar activities and applications may be discovered in an investigation of *P. myrtifolia* and Q. *littorea.*

The aim of this investigation was to identify extracts with antibacterial activity, from *P. myrtifolia* and *Q. littorea* and to quantify the antibacterial activity of active extracts.

3.2 Material and Methods

3.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

P. myrtifolia leaves from different environments and or years were compared to establish whether the antibacterial activity in the leaves differ significantly for different environments or different years from the same environment. Groups of leaves compared were from the Lowveld Botanical Garden March 2000, Lowveld Botanical Garden March 2001, Centurion March 2001, Tembe Reserve (KwaZulu-Natal) March 2001 and Durban March 2001.

3.2.2 Extracts

Extracts were prepared as described in 2.2 of Chapter 2.

3.2.3 Thin layer chromatography

Thin layer chromatography (TLC) were carried out as described in 2.3 of Chapter 2.

3.2.4 Determining antibacterial activity

3.2.4.1 Minimum inhibitory concentration

Four test organisms (recommended by the National Committee for Clinical Laboratory

Standards (NCCLS 1990)) were selected on the grounds that they are most often responsible for antibiotic resistance in hospitals (Sacho and Schoub, 1993). Two Gram-positive: *Staphylococcus aureus* American Type Culture Collection [ATCC 29213] and *Enterococcus faecalis* [ATCC 29212] and two Gram-negative: *Pseudomonas aeruginosa* [ATCC 25922] and *Escherichia coli* [ATCC 27853] were used to determine minimum inhibitory concentration (MIC) of the different plant extracts. Many researchers use agar diffusion to assay antibacterial activity. The technique works well with defined inhibitors (Hewitt and Vincent, 1989), but when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998a). A microplate serial dilution method (as described by Eloff (1998a)) was used. The development of a red formazan indicated bacterial growth (Lund & Lyon, 1975).

3.2.4.2 Total activity

Minimum inhibitory concentration (MIC) values do not give any indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity extracted (in mg) from 1 g of plant material (redissolved in acetone) by the MIC value in mg/ml (for the specific bacterium). The resultant value in ml/g indicate the degree to which the active compounds in one gram of plant material can be diluted and still inhibit the growth of the tested microorganism (Eloff, 2000). “Antibacterial activity” expressed as “total activity” provides a tool by which different plants can be compared, using the same measuring instruments.

If one compare MIC values of different plants without taking the amount extracted form the plant into account, one can easily make the wrong conclusions (Eloff, 2004). For example: extracts form plants A and B had MIC values of 0.2 mg/ml and 0.1 mg/ml respectively. Looking at MIC values only, one would have concluded that plant B is twice as active as plant A and would be a good source for bioprospecting. However, the calculated antibacterial activity per gram dry

mass for plant A and B, where 200 mg and 20 mg were extracted respectively from 1g, is 200 mg/0.2 mg/ml i.e. 1000 ml/g for plant A and 20 mg/0.1mg/ml i.e. 200 ml/g for plant B. Plant A has a much higher total activity than plant B, and thus much higher activity per gram dry mass. The situation is equivalent to the terms efficacy and potency used in pharmacology. The potency would be the activity in mg/ml of the extract and the efficacy would be the activity of the total plant material in ml/g.

3.2.4.3 Bioautography

For bioautography on the thin layer chromatography (TLC) all the extracts (10 µl of a 10 mg/ml final concentration) were applied to Merck Silica gel F_{254} plates) and developed with non-polar BEA (benzene: ethanol: ammonia, (90:9:1)), intermediate polar CEF (chloroform: ethyl acetate: formic acid, (5:4:1)) and polar EMW (ethyl acetate: methanol: water, (40:5:4.4)) eluent systems. After TLC separation, chromatograms were dried overnight in a stream of air to remove the last traces of the TLC solvents. The plates were examined under ultraviolet (UV) light and bands of quenching fluorescence (254 nm) or fluorescing (236 nm) were marked with a soft pencil. Subsequently the plates were sprayed with a concentrated solution of actively growing cells of the relevant test organism, - a 24 h old culture in Hinton-Mueller (HM) broth, centrifuged at 3500 r p m for 15 min, the supernatant discarded and the sediment bacteria were resuspended in fresh HM broth. A fine spray was used to spray the bacterial suspension onto the TLC plates. The spraying of the bacteria was done in an extraction cabinet where the front glass panel could slide down. Gloves and a facemask were worn to prevent bacterial infection. The TLC plates were then dried until they appeared translucent and incubated overnight at 37°C and 100% relative humidity. The following day, the TLC plates were sprayed with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet (INT) solutions and reincubated at 37° C until the development of inhibition zones were complete. Clear zones developed where inhibition was

present and bacterial growth was indicated by the formation of a red formazan (Lund & Lyon, 1975).

3.2.5 Stability of extracts over time

To determine which plant extracts retained activity upon storage, MIC values of freshly made extracts were compared to extracts from material redissolved in acetone and stored at 4° C for 8 months. Total activity of the extracts was compared.

3.2.6 Test for toxicity of tetrahydrofuran

A test for toxic effects of tetrahydrofuran was carried out by drying 15 ml (the amount used for the different extracts) of tetrahydrofuran. CP-grade and AR-quality was used for the experiment. The AR-quality tetrahydrofuran did not dry completely, but formed oil, which would not dry. Three times 5 ml CP-grade of tetrahydrofuran were measured and dried in the same way as the extracts was, quantified and made to a final concentration of 10 mg/ml.

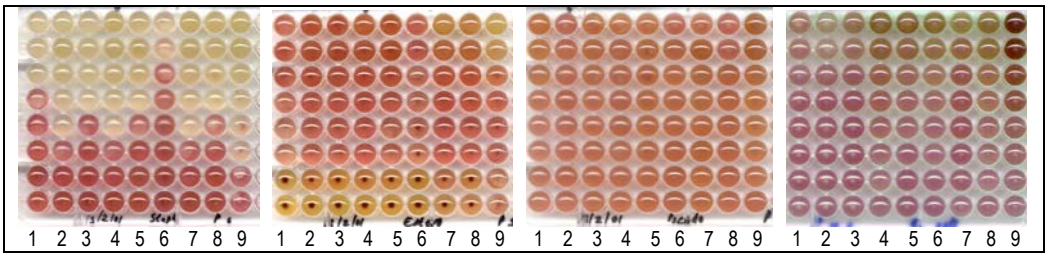
3.3 Results and Discussion

3.3.1 Antibacterial activity

3.3.1.1 Minimum inhibitory concentration

The MIC values for the different extractants and bacteria could be distinguished from the multiwell plates (Figure 3.1, and some controls in Figure 3.2) where bacterial growth was minimally inhibited, values gi

ven in Table 3.1.



Staphylococcus aureus | *Enterococcus faecalis* | *Pseudomonas aeruginosa* | *Escherichia coli*

Figure 3.1. 96-Multiwell plates (MWPs-96) showing MIC values of *Pteleopsis myrtifolia* fruit for *Staphylococcus aureus* (left), *Enterococcus faecalis* (second from left), *Pseudomonas aeruginosa* (second from right), *Escherichia coli* (right), after 40ul of 0.2 mg/ml p-iodonitrotetrazolium violet solution were added to each well. Lanes from left to right were (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. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml.

Gentamycin's antibiotic activity (as a positive control) was less effective than a similar quantity of plant extract against all the bacteria tested. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml (Figure 3.2).

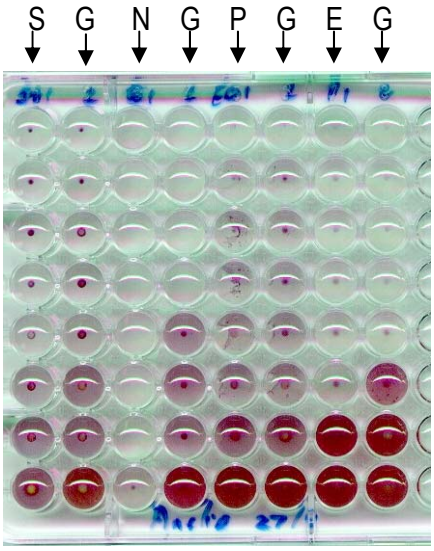


Figure 3.2. Multiwell plate showing minimum inhibitory concentrations of the antibiotic Gentamycin (G), which served as a positive control, (every even column), and a plant extract (every uneven column). The bacteria *Staphylococcus aureus* (S), *Enterococcus faecalis* (N),

Pseudomonas aeruginosa (P) and *Escherichia coli* (E) were used. The amount of colour formation (red formazan) is an indication of the MIC, 2 hours after 40ul of 0.2 mg/ml p-iodonitrotetrazolium violet solution was added to each well. All solutions tested were 10 mg/ml, the first row effectively 2.5 mg/ml.

The extracts of all plant materials (*P. myrtifolia* leaves and fruit and *Q. littorea* leaves) had antibacterial activity for Gram-positive bacteria, with MIC values between 0.039 and 0.63 mg/ml. With *P. myrtifolia* leaves, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.078 mg/ml), S. *aureus* (0.195 mg/ml), *P. aeruginosa* (0.75 mg/ml), *and E. coli* (>0.97 mg/ml). The Gram-positive bacteria were most sensitive and compared with the MIC values of antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol respectively (Martini, 1998). With *P. myrtifolia* fruit, the lowest average MIC values were found (in increasing order) for *E. faecalis* (0.13 mg/ml), *S. aureus* (0.13 mg/ml), *P. aeruginosa* (2.4 mg/ml), *and E. coli* (>2.5 mg/ml). With *Q. littorea* leaves the lowest average MIC values were found (in increasing order) for *E. faecalis* ((0.13 mg/ml), *P. aeruginosa* ((0.13 mg/ml), *E. coli* ((2.4 mg/ml) and *S. aureus* (>2.5 mg/ml). For *P. myrtifolia* leaves and fruit, the Gram- negative bacteria had higher MIC values that varied between 5 and 1.25 mg/ml. For *Q. littorea* leaves the average MIC value for Gram-negative bacteria was 0.32 mg/ml compared to the average MIC values of 1.86 and 2.44 mg/ml for *P. myrtifolia* leaves and fruit respectively.

A second set of MIC values were calculated which did not differ significantly from the first (results not shown).

3.3.1.2 Total (antibacterial) activity.

To determine which extracts were the most promising as sources of antibacterial compounds, not only the MIC of the extract but also the quantity present in the plant is important. Because the MIC value is inversely related to the quantity of antibacterial compounds present, an

arbitrary measure of the quantity of antibacterial compounds present was calculated by dividing the quantity extracted in milligrams from 1000 mg of leaves by the MIC value in mg/ml (Eloff 1999). The unit of this arbitrary measure is ml/g and Eloff (2004) called it "total activity". This value indicates the volume to which the biological active compounds present in one gram of dried plant material can be diluted and still kill bacteria. Total activity for each type of plant material and all extractants with the four different bacterial strains are listed in Table 3.1 and graphically represented in Figure 3.3.

With *P. myrtifolia* leaves, the highest single and average total activity was found (in decreasing order) for *E. faecalis* (1794.9 and 489.7 ml/g respectively), *S. aureus* (448.7 and 163.4 ml/g respectively), *P. aeruginosa* (223.6 and 74.2 ml/g respectively), and *E. coli* (70.4 and >31.0 ml/g respectively). For the fruit of *P. myrtifolia*, the highest average total activity was found (in

Table 3.1. Milligram plant extracted per gram dry plant material, milligram acetone soluble plant extract per extractant, MIC values and total activity for the different bacterial strains and plant materials.

Pteleopsis myrtifolia leaves												
X-ant	mg x/g	mg a sol/ g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	22	16	0.08	0.16	1.25	>4	1.37	205.1	102.6	12.8	>4	81.1
P-e	18	12	0.31	0.16	1.25	1.25	0.74	38.3	76.9	9.6	9.6	33.6
E-e	34	22	0.16	0.08	0.63	2.5	0.84	141.0	282.1	35.2	8.8	116.8
Mdc	64	20	0.16	0.08	0.31	0.63	0.29	128.2	256.4	63.9	32.0	120.1
Thf	88	70	0.16	0.04	0.31	1.25	0.44	448.7	1794.9	223.6	56.0	630.8
E-a	46	28	0.31	0.08	0.31	1.25	0.49	89.5	359.0	89.5	22.4	140.1
Ace	52	38	0.16	0.08	0.31	0.63	0.29	243.6	487.2	121.4	60.8	228.3
Eth	84	44	0.16	0.04	0.31	0.63	0.28	282.1	1128.2	140.6	70.4	405.3
Met	188	14	0.31	0.04	0.31	0.31	0.24	44.7	359.0	44.7	44.7	123.3
H_2O	402	2	0.16	0.04	2.5	1.25	0.99	12.8	51.3	0.8	1.6	16.6
Tot	998	266	1.95	0.78	7.50	>9.67	5.98	1634.1	4897.4	742.1	>310.3	1896.0
Ave	99.8	26.6	0.2	0.08	0.8	>0.97	0.60	163.4	489.7	74.2	>31.0	189.6
								Ave g+	326.6	Ave g-	52.6	

Pteleopsis myrtifolia fruit												
X-ant	mg x/g	mg a sol/g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	10	10	0.04	0.08	2.5	>2.5	1.28	256.4	128.2	4.0	>4.0	98.2
P-e	14	10	0.08	0.16	2.5	>2.5	1.31	128.2	64.1	4.0	>4.0	50.1
E-e	22	12	0.08	0.16	2.5	1.25	1.00	153.8	76.9	4.8	9.6	61.3
Mdc	114	12	0.08	0.16	2.5	>3.0	1.43	153.8	76.9	4.8	>4.0	59.9
Thf	52	20	0.08	0.04	2.5	>5.0	1.90	256.4	512.8	8.0	>4.0	195.3
E-a	24	14	0.04	0.08	1.25	>2.5	0.97	359.0	179.5	11.2	>5.6	138.8
Ace	30	10	0.16	0.08	2.5	>2.5	1.31	64.1	128.2	4.0	>4.0	50.1
Eth	30	12	0.08	0.16	2.5	>3.0	1.43	153.8	76.9	4.8	>4.0	59.9
Met	116	10	0.63	0.08	2.5	>2.5	1.43	16.0	128.2	4.0	>4.0	38.1
H_2O	-	-	-	-	-	-	-	-	-	-	-	-
H_2-A	184	1.08	0.08	0.313	2.5	0.313	0.80	13.8	3.5	0.4	3.5	5.3
Tot	596	111.1	1.33	1.29	23.75	>25.06	12.86	1555.5	1375.2	50.0	>46.7	756.8
Ave	59.6	11.18	0.13	0.13	2.38	>2.51	1.29	155.6	137.5	5.0	>4.7	75.7
								Ave g+	146.6	Ave g-	4.9	

Quisqualis littorea leaves												
X-ant	mg x/g	mg a sol/g	MIC in mg/ ml					Total activity in ml				
			S aur	E fae	P aer	E col	Ave	S aur	E fae	P aer	E col	Ave
Hex	16	10	0.31	0.04	0.31	0.63	0.323	32.0	255.8	32.0	16.0	84.0
P-e	30	14	0.63	0.04	0.31	1.25	0.557	22.4	358.1	44.8	11.2	109.1
E-e	35	16	0.63	0.08	0.31	0.31	0.332	25.6	204.9	51.2	51.2	83.2
Mdc	152	20	0.16	0.16	0.16	0.04	0.127	128.0	128.0	128.0	511.5	223.9
Thf	56	48	0.31	0.08	0.16	0.08	0.16	153.6	614.6	307.1	614.6	422.5
E-a	30	24	0.31	0.16	0.16	0.16	0.195	76.8	153.6	153.6	153.8	134.5
Ace	40	22	0.31	0.08	0.16	0.08	0.16	70.4	281.7	140.8	281.7	193.7
Eth	50	32	0.31	0.16	0.16	0.08	0.176	102.4	204.7	204.7	409.7	230.4
Met	130	22	0.31	0.08	0.16	0.08	0.16	70.4	281.7	140.8	281.7	193.7
H_2O	-	-	-	-	-	-	-	-	-	-	-	-
H_2A	260	10	2.50	0.08	1.25	0.63	1.113	4.0	128.0	8.0	16.0	39.0
Tot	799	208	5.78	0.94	3.13	3.32	3.29	685.6	2610.9	1210.9	2347.5	1713.8
Ave	79.9	20.8	0.58	0.09	0.31	0.33	0.33	68.6	261.1	121.1	234.7	171.4
Ave all	79.8	19.5	0.30	0.10	1.15	1.27	0.76	32.0	255.8	32.0	16.0	145.6
								Ave g+	164.9	Ave g-	177.9	

Ave = Average, a sol = acetone soluble, S = *Staphylococcus aureus*, N = *Enterococcus faecalis,* P = *Pseudomonas aeruginosa,* E = *Escherichia coli,* X-ant = extractant, mg x/g = milligram extractant per gram, mg a sol/g = milligram acetone soluble extractant per gram. Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf =

tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H_2O = water and H_2-A = water: acetone, Ave g+ = average for Gram-positive, Ave g- = average for Gram-negative.

decreasing order) for *S. aureus* (155.6 ml/g), *E. faecalis* (137.5 ml/g), *P. aeruginosa* (5.0 ml/g), and *E. coli* (>4.7 ml/g). For both *P. myrtifolia* leaves and fruit, the Gram-positive bacteria were more sensitive and compared well with the values of known antibiotics, 0.08 and 0.16 mg/ml for ampicillin and chloramphenicol. The average antibacterial activity for each bacterium was higher in the leaves than in the fruit.

For *Q. littorea* leaves the highest average total activity were found (in decreasing order) for *E. faecalis* (261.1 ml/g), *E. coli* (234.7 ml/g), *P. aeruginosa* (121.1 ml/g) and *S. aureus* (68.6 ml/g) (Table 3.2 and figure 3.3). Total activity of *Q. littorea* leaves against Gram-negative bacteria is high and it might be worthwhile to cultivate plant material and attempt to isolate pure compounds from a fraction with activity against Gram-negative bacteria.

In a study where Kotzé (2000) investigated antibacterial activity of *Combretum microphyllum* leaves, the lowest MIC values were obtained with extractants ethanol, *di*-isopropyl ether and acetone. Famakin (2002) obtained the lowest average MIC values with extractants ethyl acetate, methylene dichloride, and acetone for *Combretum woodii* leaves. In this study, the lowest average MIC values for *P. myrtifolia* leaves were obtained with extractants methanol, ethanol, and equally acetone and methylene dichloride. Results of the average MIC and total activity values obtained by different researchers while investigating different genera of Combretaceae are listed in Table 3.2.

Table 3.2. Average MIC and total activity values obtained for each bacterium and extracts of each plant type.

	Average MIC value for each bacterium (mg/ml)				Average total activity for each bacterium (ml/g)			
	S	E	P	E	S	E	P	E
C. microphyllum leaves	0.46	0.29	0.30	0.31	234.6	359.0	296.4	332.0
C. woodii leaves	>0.59	>0.35	>0.80	>0.29	>235.1	>898.2	>246.9	>1155.0
P. myrtifolia leaves	0.2	0.08	0.8	>0.97	163.4	489.7	74.2	>31.0
P. myrtifolia fruit	0.13	0.13	2.38	>2.51	155.6	137.5	5.0	>4.7
Q. littorea leaves	0.58	0.09	0.31	0.33	68.6	261.1	121.1	234.7

S = *Staphylococcus aureus*, N = *Enterococcus faecalis*, P = *Pseudomonas aeruginosa* and E = *Escherichia coli, C. microphyllum = Combretum microphyllum, C. woodii = Combretum woodii, P. myrtifolia = Pteleopsis myrtifolia, Q. littorea = Quisqualis littorea.*

From Table 3.2, it is clear that different members of Combretaceae have different activities against the different test organisms.

In an investigation where Eloff (1999) investigated antibacterial activity (of acetone leaf extracts) of 27 members of Combretaceae, *P. myrtifolia* and *Q. littorea* included, the MIC values obtained were higher and total activity values lower than the MIC and total activity values obtained in this study (Table 3.3). A possible explanation for variation in values could have been due to the difference in plant material or bacterial cultures from different years.

Table 3.3. MIC and total activity values obtained for extracts of *P. myrtifolia* and *Q. littorea*

Plant and author	MIC (mg/ml)				Total activity (ml/g)	
	S	E	P	E	Ave Gram +	Ave Gram -
P myrtifolia leaves (Eloff)	0.4	0.4	0.8	1.6	65.8	88.6
P myrtifolia leaves (Rabie)	0.2	0.08	0.8	>0.97	326.5	>52.6
Q littorea leaves (Eloff)	<0.7	0.8	1.6	0.8	14.8	19.0
Q littorea leaves (Rabie)	0.58	0.09	0.31	0.33	164.8	177.9

Ave Gram + = average Gram-positive, Ave Gram - = average Gram-negative, S = *Staphylococcus aureus*, N = *Enterococcus faecalis,* P = *Pseudomonas aeruginosa,* E = *Eschericia*

coli.

Graphs drawn of the values in Table 3.1 give a 2-dimentional representation of the total activity for all plant material (Figure 3.3).

Results with *Pteleopsis* plant material indicated that on average the extractants tetrahydrofuran, ethanol and acetone gave the highest total activity for leaves and the extractants tetrahydrofuran, ethyl acetate and *n*-hexane gave the highest total activity for fruit. For *Q. littorea* leaves on average the extractants tetrahydrofuran, ethanol and methylene dichloride gave the highest total activity for leaves (Table 3.2). For all plant material types water or (1: 1) (water: acetone) gave the lowest total activity. Water probably did not extract acetone soluble material because it could not pass the lipids in the membranes. A mixture of water and acetone however, could break the plant membranes and extract some soluble cell contents.

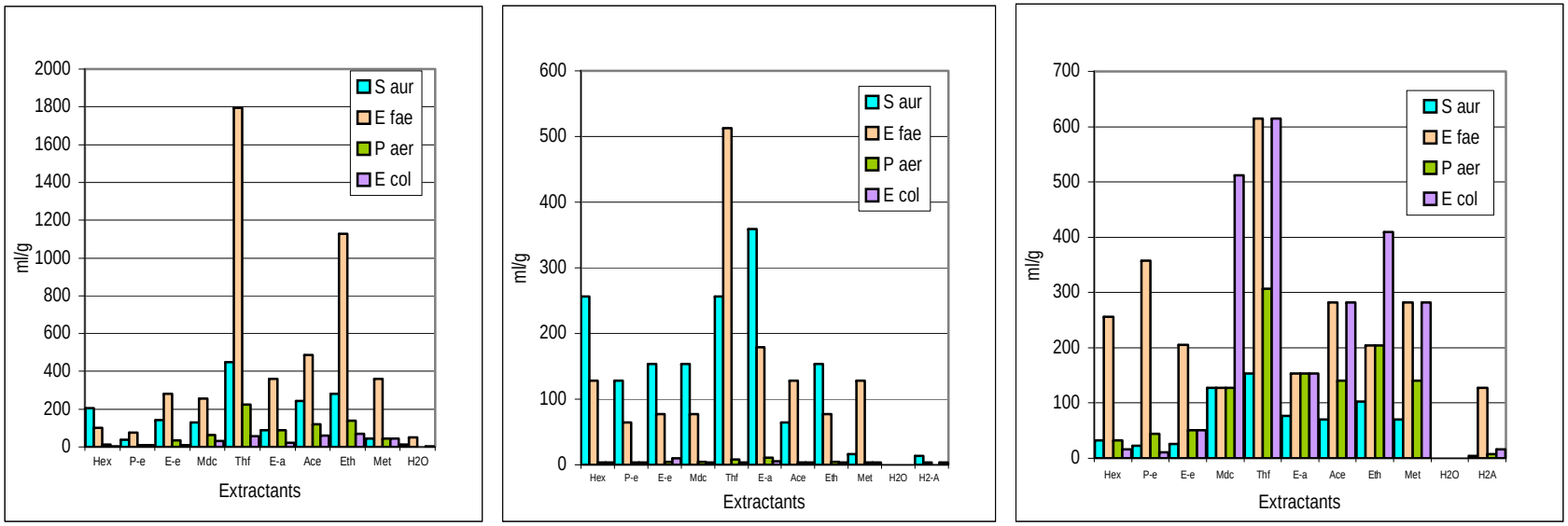
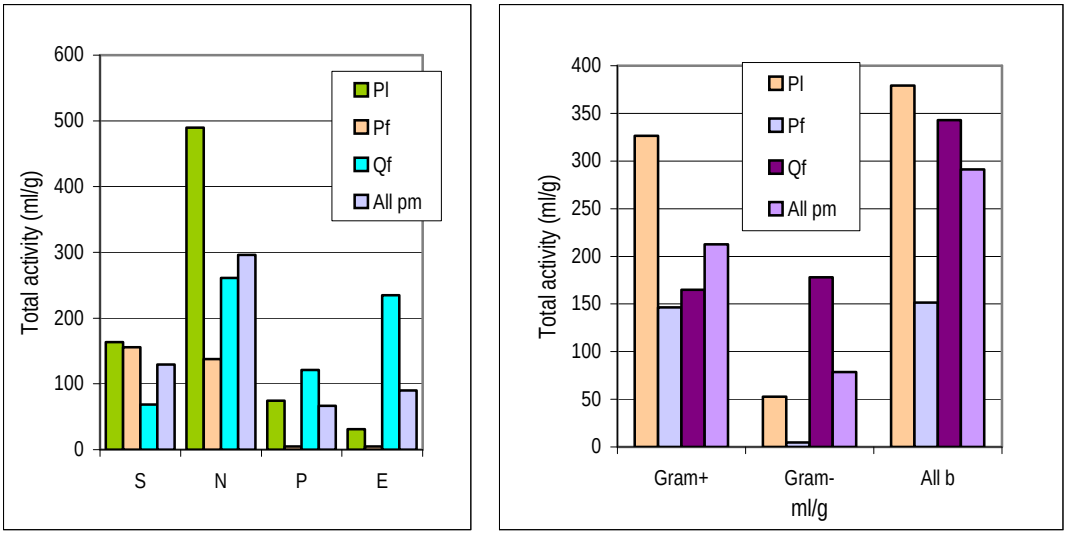


Figure 3.3. Total activity for *Pteleopsis myrtifolia* leaves (left), fruit (middle) and *Quisqualis littorea* leaves (right) for the bacteria *Staphylococcus aureus* (*S aur*), *Enterococcus faecalis* (*E fae*), *Pseudomonas aeruginosa* (*P aer*) and *Escherichia coli* (*E col*) in ml/g, with extractants *n*-hexane (Hex), *di*-isopropyl ether (P-e), *di*-ethyl ether (E-e), methylene dichloride (Mdc), tetrahydrofuran (Thf), ethyl acetate (E-a), acetone (Ace), ethanol (Eth), methanol (Met) and water (H_2O) or water: acetone (1:1) (H_2-A).

The average antibacterial activity (expressed as total activity) for each bacterium was higher in the leaves than in the fruit (although TLC separated more compounds in the fruit). The lowest average antibacterial activity was found for *P. aeruginosa* (Figure 3.4(a)).



(a) (b)

Figure 3.4. Average of total activity for each bacterium type and type of plant material ((a) left), and average total activity for Gram-positive (Gram+), Gram-negative (Gram–) and all bacteria (All b) for each plant material type as well as for all plant material (All pm) ((b) right). S = *Staphylococcus aureus*, N = *Enterococcus faecalis,* P = *Pseudomonas aeruginosa,* E = *Escherichia coli,* Pl = *Pteleopsis myrtifolia* leaves, Pf = *Pteleopsis myrtifolia* fruit, Ql = *Quisqualis littorea* leaves.

On average *E. faecalis* was more sensitive to leaf material and *S. aureus* was more sensitive to fruit material of *P. myrtifolia.* The Gram-negative bacteria *P. auruginosa* and *E. coli* were more sensitive to *Q. littorea* leaf material (Figure 3.4(a)). *P. myrtifolia* leaves and fruit support the observation of Vlietinck *et al*. (1995) - that plant extracts are frequently more active against Gram-positive bacteria. This was not true for *Q. littorea* leaves. The average total activity of *Q. littorea* leaves against Gram-negative bacteria was higher than for Gram-positive bacteria. The average total activity for Gram-positive bacteria was highest for P. *myrtifolia* leaf material and

the average total activity for Gram-negative bacteria was highest for *Q. littorea* leaf material (Figure 3.4 (b)).

The aim in an investigation of this type, is extracting the largest quantity of antibacterial activity, therefore not only the MIC, but also the (acetone soluble) quantity extracted should be considered. A very complex extract will not yield a lot of pure compound, therefore the main objective is to find a highly active extract with a low concentration of other compounds - found by looking for extracts with a low MIC and a large quantity of extract that redissolves in acetone. According to Table 3.2, the best extractants for this purpose are tetrahydrofuran, ethyl acetate, acetone and ethanol (631, 140, 228, 405 ml/g) for *P. myrtifolia* leaves, *n*-hexane, *di*-ethyl ether, tetrahydrofuran and ethyl acetate (98, 61, 195, 139 ml/g) for *P. myrtifolia* fruit and methylene dichloride tetrahydrofuran, ethanol and jointly acetone and methanol (224, 423, 230, 194 ml/g) for *Q. littorea* leaves.

To determine if the selectivity group of the extractants (Snyder and Kirkland, 1979) has a major effect, the results were grouped according to selectivity group (Table 3.4). A solvent's selectivity is the result of the interaction of three forces x_d, x_e and x_n, shown in more detail in Figure 4.1 of Chapter 4. When these latter values for each solvent are plotted in a triangular diagram (in Figure 4.1 of Chapter 4), it is found that various solvents are grouped into clusters of similar selectivity. All the solvents in the same cluster belong to the same 'selectivity group'. For example, group I will include all the H-acceptors such as amines and ethers, group II includes donor acceptors such as the alcohols, and group VIII consists of pure donor solvents such as chloroform. A graph was drawn with the solvents arranged according to selectivity group (Figure 3.5).

Table 3.4. The extractants listed within their selectivity groups of Snyder and Kirkland (1979), solvent strength, average amounts of plant material extracted per gram dry weight (third column) and acetone soluble (fourth column).

Extractant	Sel grp	Solvent strength	Ave all pm x/g	Ave all pm AS
Hex	-	0	16.0	12.0
P-e	I	2.4	20.7	12.0
E-e	I	2.8	30.3	16.7
Eth	II	4.3	54.7	29.3
Met	II	5.1	144.7	15.3
Thf	III	4.0	65.3	46.0
Mdc	V	3.1	110.0	17.3
E-a	VIa	4.4	33.3	22.0
Ace	VIa	5.1	40.7	23.3
H_2O	VIII	10.2	134.0	0.7
50H_A	-		148.0	3.7

Sel grp = selectivity group, Ave = average, pm = plant material, x/g = extracted per gram, AS = acetone soluble. Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H_2O = water and H_2-A = water: acetone.

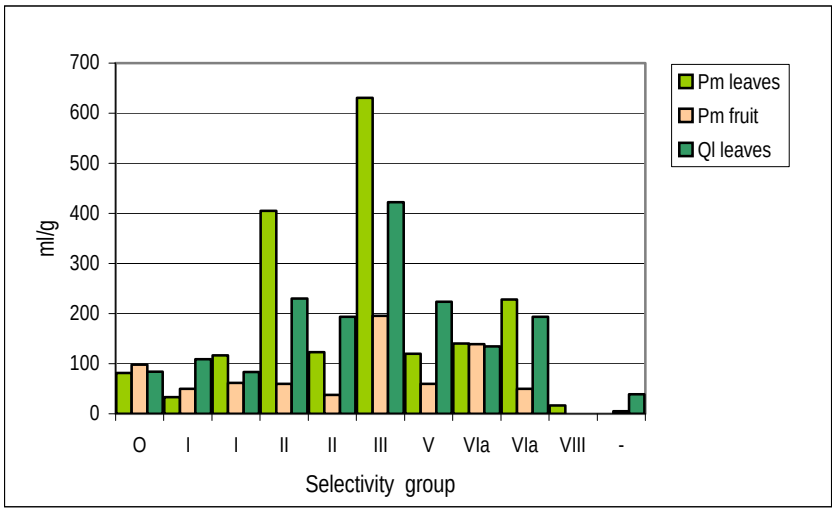


Figure 3.5. Graph of total activity for extractants in selectivity groups for each plant material type. (Pm = Pteleopsis *myrtifolia*, Ql = *Quisqualis littorea)*.

Extractants in selectivity groups II, III, V and VIa had good activity. These extractants had the required solvents strengths to extract active compounds from the plant materials used. These selectivity groups correspond to extractants with intermediate polarity.

MIC values of *Pteleopsis* leaves from different environments were determined and did not differ significantly (results not shown).

3.3.1.3 Bioautography

Bioautograms were made of the different extractants and bacteria to confirm MIC values, as well as to see if the same compounds (R_f values) were responsible for antibacterial activity for the different bacteria (Figures 3.6, 3.7 and 3.8). In each of Figures 3.6-3.8, the top row is the plant material of *P. myrtifolia* leaves, the middle row is *P. myrtifolia* fruit and bottom row is *Q. littoria* leaves. In Figures 3.6-3.8, the white areas indicate growth inhibition of bacteria and thus antibacterial activity of plant substance on the specific area (R_f) of the TLC.

Areas of growth inhibition were not the same for all bacteria. In figure 3.6 the R_f (of the areas of growth inhibition (1-4)) value in brackets behind each bacterium's name, for the hexane extract of *P. myrtifolia* leaves, differed for the bacteria *S. aureus* ((1) no R_f), *E. faecalis* ((2a) 0.04 and (2b) 0.28), *P. aeruginosa* ((3) 0.28), and *E. coli* ((4) 0.58). This indicated that (except for some similarities) different compounds were responsible for antibacterial activity for the different bacteria. In cases where the R_f values are the same, it would indicate one compound having a broad antibacterial spectrum. It could also be seen that the *n*-hexane extract of leaf material (*Pteleopsis* and *Quisqualis*) showed no or slight inhibition of growth of *S. aureus* and the water extract of *Pteleopsis* leaves and fruit showed no or slight inhibition of growth of *P. aeruginosa.*

For all plant material types, the CEF eluent system indicated areas of bacterial growth inhibition the clearest. In some cases (e.g. *E. coli* with *P. myrtifolia*) there is a good correlation between low MIC and clear areas of inhibition. In other cases (e.g. *E. coli* with *Q. littorea*) however, the clear area of inhibition on the bioautogram, is not as large as one would expect from a low MIC value. This can be explained if the active compounds are volatile, then they may have

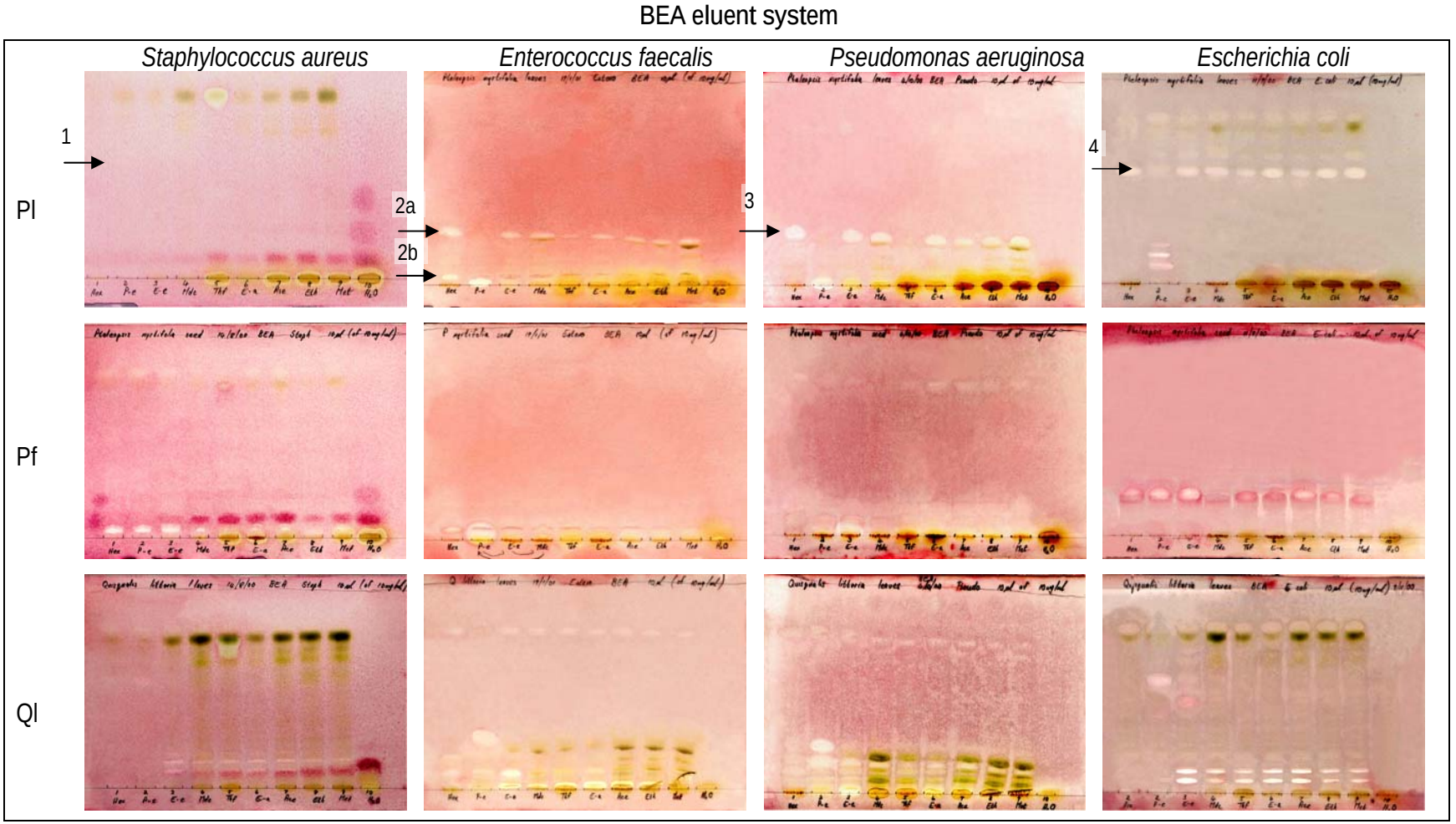


Figure 3.6. Bioautograms of Pl (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and Ql (*Quisqualis littorea* leaves) developed with the BEA eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H_2O = water or H_2-A = water: acetone. (BEA = benzene: ethanol: ammonia (36:5.4:4)).

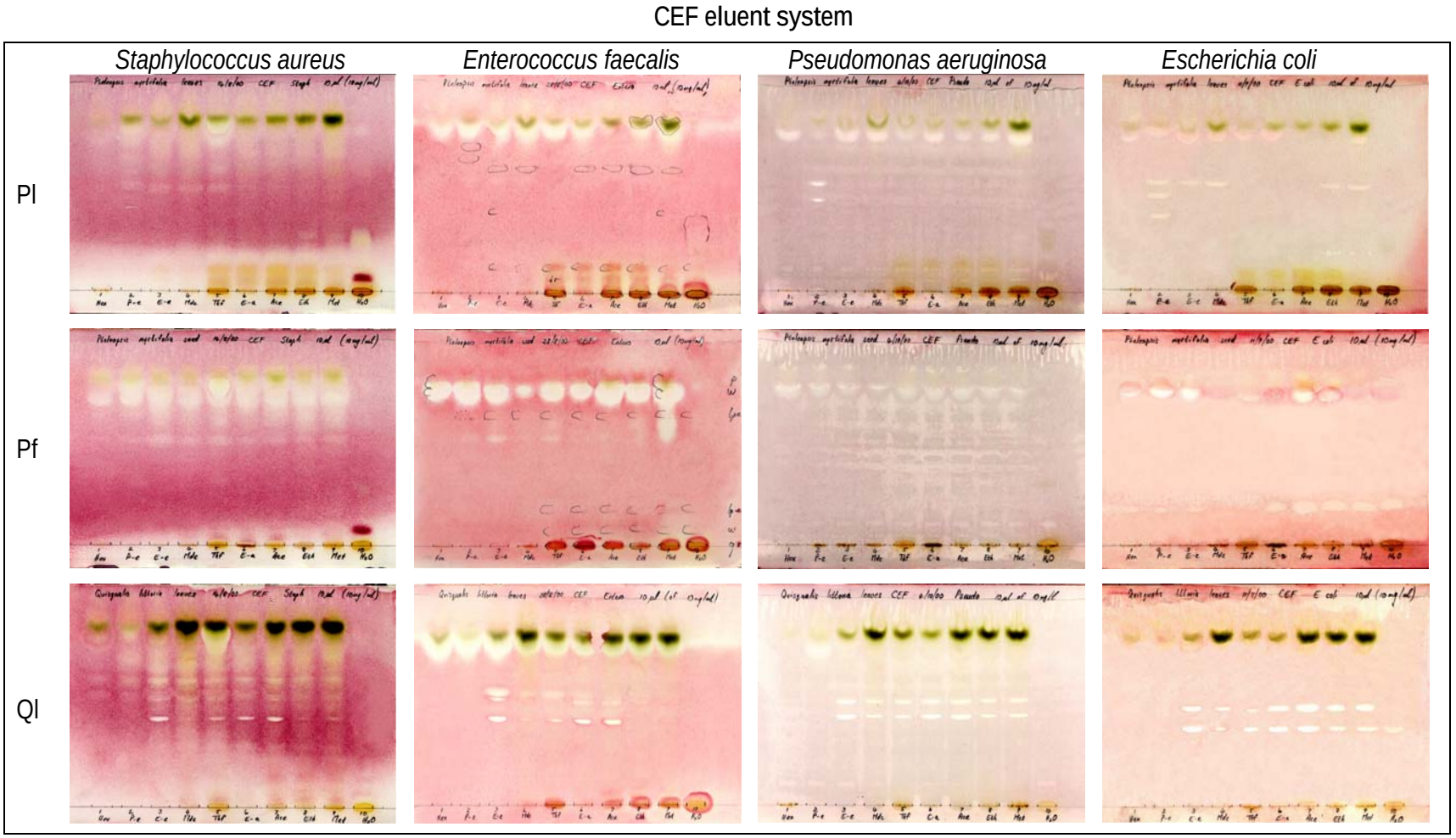


Figure 3.7. Bioautograms of Pl (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and Ql (*Quisqualis littorea* leaves) developed with the CEF eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H_2O = water or H_2-A = water: acetone. (CEF = chloroform: ethyl acetate: formic acid (5:4:1)).

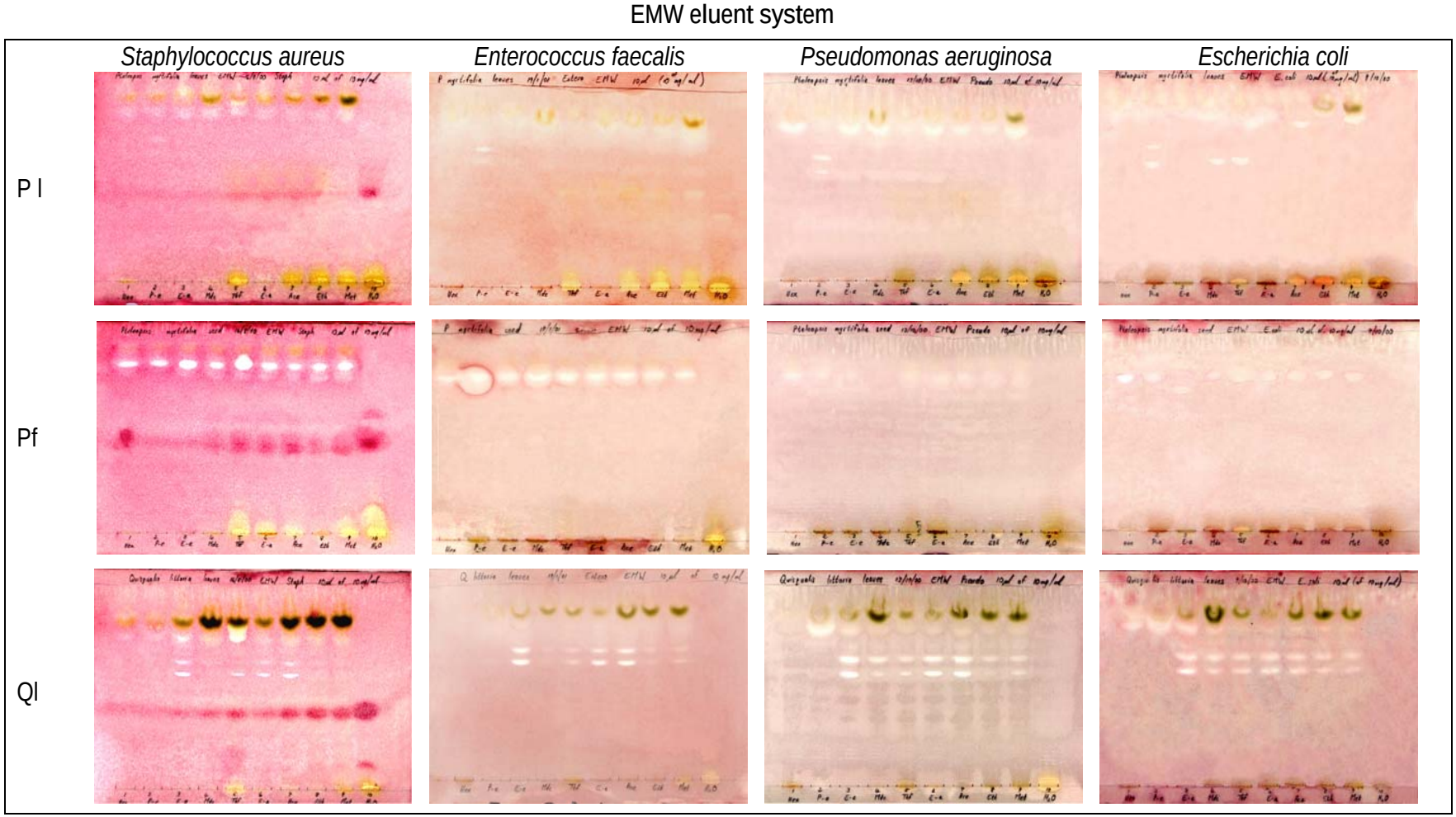


Figure 3.8. Bioautograms of Pl (*Pteleopsis myrtifolia* leaves), Pf (*Pteleopsis myrtifolia* fruit) and Ql (*Quisqualis littorea* leaves) developed with the EMW eluent system, sprayed with bacteria (columns) and 2.0 mg/ml p-iodonitrotetrazolium violet solution. For each bioautogram, lanes from left to right are: Hex = *n*-hexane, P-e = *di*-isopropyl ether, E-e = *di*-ethyl ether, Mdc = methylene dichloride, Thf = tetrahydrofuran, E-a = ethyl acetate, Ace = acetone, Met = methanol, H_2O = water or H_2-A = water: acetone. (EMW = ethyl acetate: methanol: water (40:5:4.4)).

evaporated from the overnight drying of the chromatograms before treatment with bacteria.

The bacterium *S. aureus* also indicated areas (on all three of the eluent systems, clearer with BEA and EMW (Figure 3.6 and 3.8)) of growth promotion (areas that stained darker red than the rest of the plate) and all plant materials. For *S. aureus* the MIC values obtained may be an average of the growth inhibitory and growth promoting areas.

Above-mentioned areas of bacterial growth inhibition (clear, but show white, as the TLC plate is white) may not be the only ones. Brown areas formed after application (on the baseline of the plates) of the more polar extracts like tetrahydrofuran, ethyl acetate, acetone, ethanol, methanol and water or 50% water and 50% acetone. In Figure 3.7 it can be seen that they were not in all cases (like with *E. faecalis*) clearly covered by a red formazan colour after sprayed with INT, and they probably contribute to the extracts' antibacterial activity.

Areas of antibacterial inhibition were tabulated according to R_f values. While tabulated, it was almost impossible to make a reasonable interpretation about the R_f values. The R_f values were therefore plotted in graphs to find visual presentations of antibacterial activity. Figures 3.9 and 3.10 are visual presentations of some R_f values found for the CEF and EMW respectively. For the CEF system the R_f values of the methylene dichloride, acetone and ethyl acetate extracts are represented in Figure 3.9, and for the EMW system the *di*-isopropyl ether, acetone and methanol extracts are represented in Figure 3.10. Similar R_f values (within one eluent system), indicate which extracts isolated the same compound, for example, the methylene dichloride, acetone and ethyl acetate extractants all isolated the compounds '1' and '2' in Figure 3.9, and compounds '3' and '4' in Figure 3.10. Compounds with similar R_f values that inhibited more than one bacterium, indicate compounds with broad antibacterial spectra, for example, "a" and "e" from *Pteleopsis* leaves, "b" and "c" from *Pteleopsis* fruit, and "d" from *Quisqualis* leaves (Figures 3.9 and 3.10). In Figure 3.9, only methylene dichloride isolated a

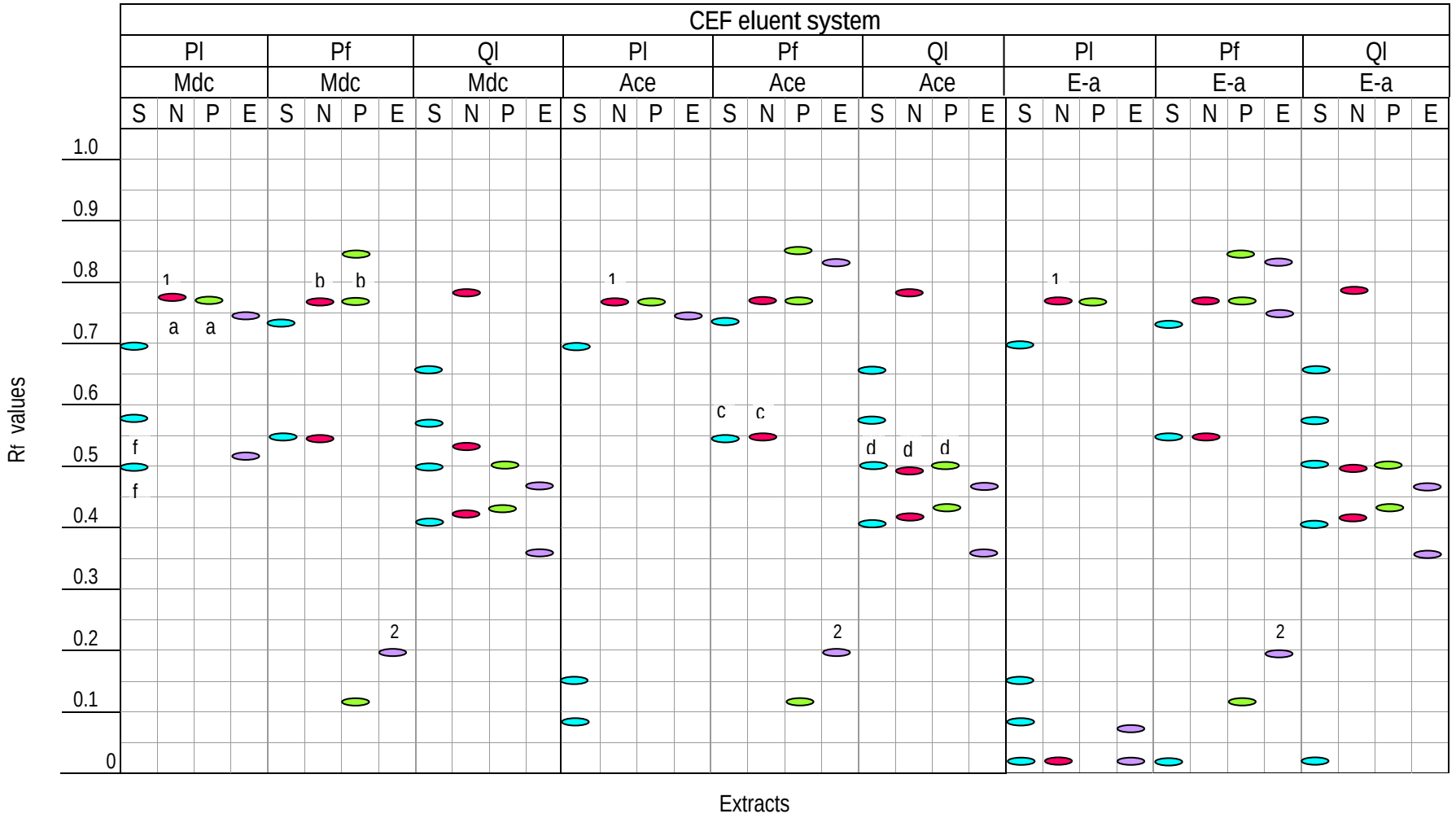


Figure 3.9. Visual presentation of R_f values of the methylene dichloride, acetone and ethyl acetate extracts on bioautograms developed with the CEF eluent system. Pl = *Pteleopsis* leaves, Pf = *Pteleopsis* fruit, Ql = *Quisqualis* leaves, S = *Staphylococcus aureus* (), N = *Enterococcus faecalis* (), P = *Pseudomonas aeruginosa* (), E = *Escherichia coli* (), Mdc = methylene dichloride, Ace = acetone, E-a = ethyl acetate, CEF = chloroform: ethyl acetate: formic acid (5:4:1).

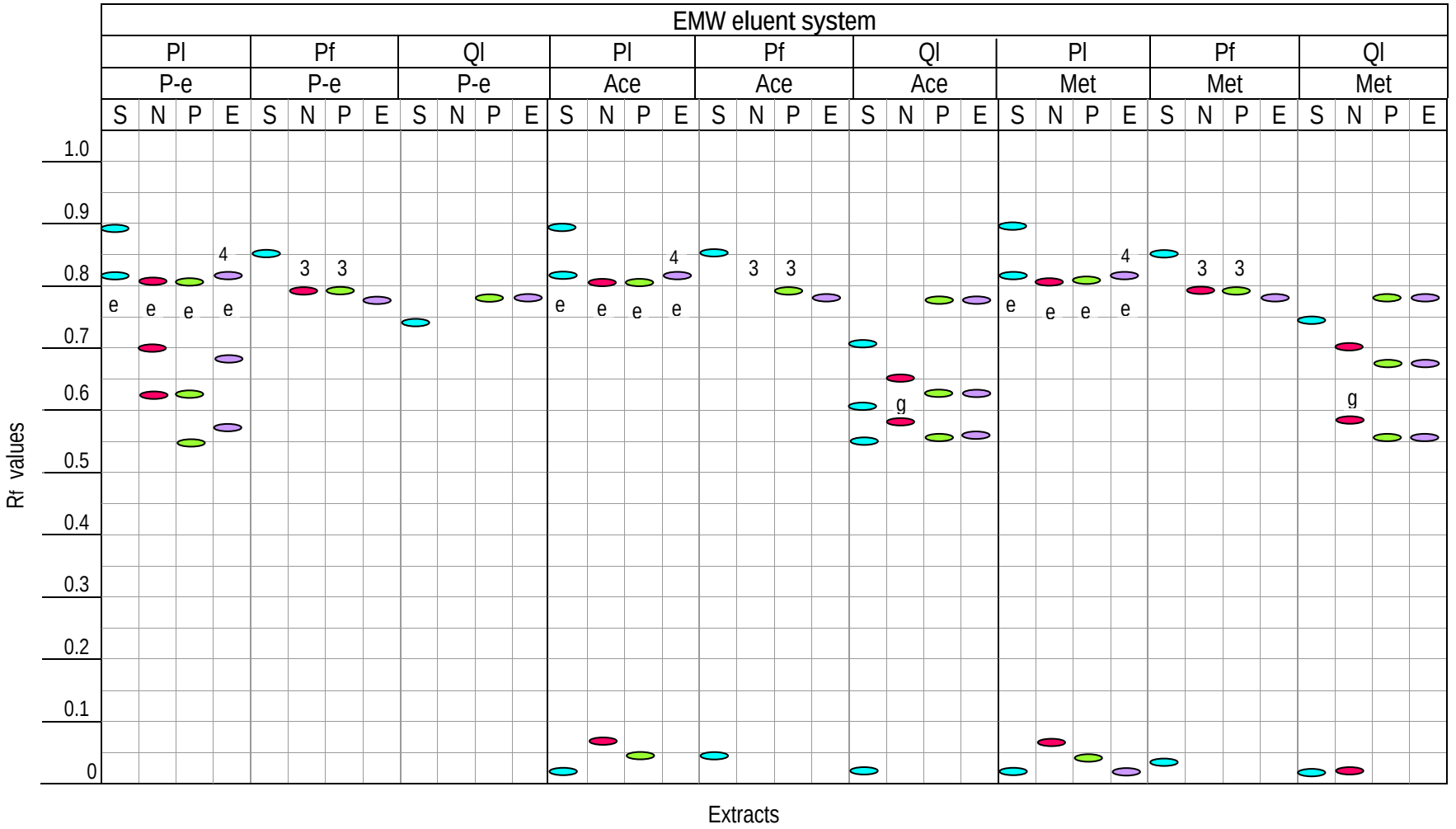


Figure 3.10. Visual presentation of Rf values of the *di*-isopropyl ether, acetone and methanol extracts on bioautograms developed with the EMW eluent system. Pl = *Pteleopsis* leaves, Pf = *Pteleopsis* fruit, Ql = *Quisqualis* leaves, S = *Staphylococcus aureus* (), N = *Enterococcus faecalis* (), P = P = *Pseudomonas aeruginosa* (), E = *Escherichia coli* (), P-e = *di*-isopropyl ether, Ace = acetone, Met = methanol, EMW = ethyl acetate: methanol: water (40:5:4.4).

compound indicated by " f ", from *Pteleopsis* leaves to which *S. aureus* was sensitive, and acetone and ethyl acetate did not. Similarly, only the acetone and methanol extracts isolated a compound indicated by " g ", from *Quisqualis* leaves to which *E. faecalis* was sensitive, and the *di*-isopropyl ether did (Figure 3.10).

3.3.2 Stability of extracts over time

MIC values of *P. myrtifolia* leaf extracts that were determined when freshly prepared, were compared to leaf extracts extracted and redissolved in acetone 8 months previously. The amount extracted was taken into account and total activity was calculated. For the extractants investigated: tetrahydrofuran, ethyl acetate, ethanol and methanol, the total activity for *S. aureus* was reduced within 8 months. The total activity for *E. faecalis*, *P. aeruginosa* and *E. coli* increased for tetrahydrofuran, ethyl acetate, acetone and ethanol after an 8-month period (Figure 3.11).

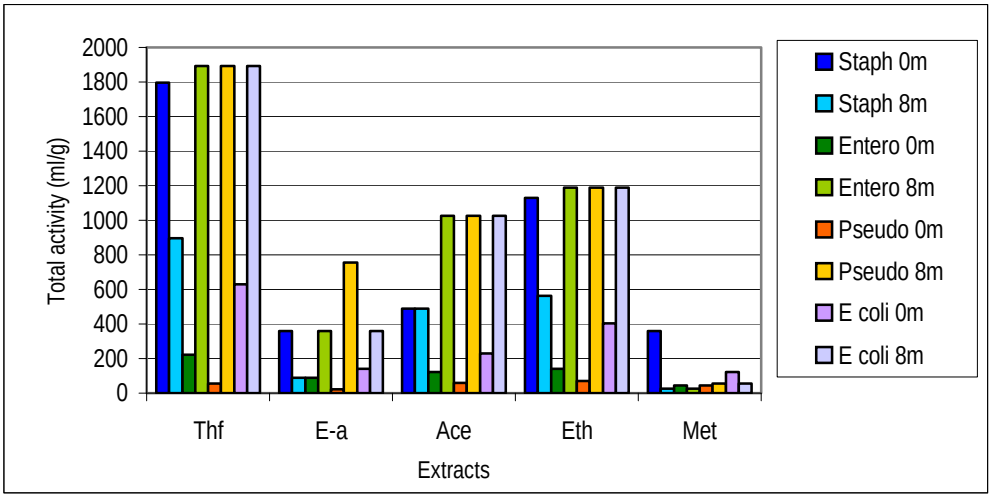


Figure 3.11. Total activity of *Pteleopsis myrtifolia* leaves against *Staphylococcus aureus* (Staph), *Enterococcus faecalis* (Entero), *Pseudomonas aeruginosa* (Pseudo) and *Escherichia coli* (E coli) and the extractants tetrahydrofuran (Thf), ethyl acetate (E-a), acetone (Ace), ethanol (Eth), and methanol (Met) at 0 and 8 months.

The loss of activity of certain extracts after storing in the cold could have been due to chemical modification of active compounds or to their precipitation over time. Since the extracts were kept in screw-capped containers and the volume was checked and evaporation losses corrected before testing their antibacterial activity, the surprising increased antibacterial activity could be explained if some inhibitory compounds were volatile or unstable over time, and this would explain the increased activity. The mechanism of the enhancement of potency and subsequent stability should be investigated (Eloff, 1999).

3.3.3 A test for toxicity of tetrahydrofuran

The CP grade of tetrahydrofuran (which was used for the assays) gave only 100 μl of a 10 mg/ml final concentration and when tested, MIC values were not increased, compared to a control where only water was added to the wells with bacteria (*S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli*). A bigger volume than 15 ml did increase the MIC values and there was therefore decided not to use this extractant for a large extract. If one would use tetrahydrofuran for large extracts, activity might be mistakenly ascribed to its measurable toxicity in larger volumes.

3.4 Conclusions

Results obtained in this study clearly show that *P. myrtifolia* leaves and fruit and *Q. littorea* leaves contain several antibacterial compounds. The majority of plants do not have activity against Gram-negative bacteria (Vlietinck *et al*., 1995), but *Q. littorea* leaf extracts have good activity against the Gram-negative bacterium, *E. coli*.

Establishing measurable toxicity to the test organisms when more than 15 ml tetrahydrofuran extractant is used, further indicate (except the reasons discussed in Chapter 2) that acetone is the extractant of choice for antibacterial assays and will be used in future investigations

(Chapter 4).

Further research to isolate pure compounds from antibacterial active fractions, as well as establishing toxicities of extracts of this plant, may offer medicinal uses for the indigenous population (at little cost) or offer a structure (of a pure compound) for Pharmaceutical development. If extracts would be used on a large scale for medicinal purposes, it is of utmost importance that cultivation and conservation of this plant accompany the use for medicinal purposes.

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Chapter 4

Solvent-solvent separation

Abstract

The skilful application of fractionation methods exploits the fact that an extract can be separated into groups of compounds sharing similar physico-chemical characteristics. Acetone was chosen as an initial extractant for leaves of *Pteleopsis myrtifolia* because many polar and non-polar compounds are soluble in acetone and the test organisms (bacteria) are not sensitive to 25% acetone in a serial dilution assay. Bioassay-guided fractionation identified that all fractions: n-hexane, carbon tetrachloride, chloroform, 35% water in methanol, n-butanol and water had antibacterial activity against the Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*. The chloroform and 35 % water in methanol extracts were active against the Gram-negative bacteria, *P. aeruginosa* and *E. coli.* The chloroform fraction was antibacterial to all bacteria tested, and had the largest amount of inhibition areas (seven), and thus amount of bacterially active compounds. This fraction was chosen for further purification and to isolate pure compounds from it in future research

4.1 Introduction

4.1.1 Initial extractant for solvent-solvent separation

A variety of extractants was examined for their ability to solubilise antimicrobial compounds from plants. The focus of the study was to provide a more standardised extraction method for a wide variety of researchers working in diverse settings (Eloff, 1998b). Although it is not one of the more frequently used extractants in studies published to date, acetone received the highest overall rating. Acetone had the advantage that it dissolved hydrophilic and lipophylic components from the plants used, was miscible with water, was volatile and had a low toxicity to

the bioassay used. In the table that Eloff (1998b) published, it could be seen from the number of inhibitors that each solvent extracted, that the most active components were not water-soluble. Cowan (1999) referred to the solvents (acetone, methylene dichloride, methanol, ethanol, water) that Eloff (1998b) examined and pointed out that in a review of 48 articles describing the screening of plant extracts for antimicrobial properties in recent years of the *Journal of Natural Products*, the *Journal of Ethnopharmacology*, and the *International Journal of Pharmacognosy*, only one study used acetone as an extractant. That the solvents used most often for initial extractions, like ethanol and methanol, may not demonstrate the greatest sensitivity in yielding antimicrobial chemicals on an initial screening and advised that this disparity should be addressed as the search for antimicrobials intensifies.

4.1.2 Solvent selection to find potentially active phytoconstituents

Physical properties of solvents (availability, detector compatibility, solvent reactivity, boiling point, viscosity, miscibility and safety) to be used for extractions and fractionations need to be carefully considered to make sure the desired compounds are extracted or separated. The total interaction of a solvent molecule with a sample molecule is the result of four interactions: dispersion, dipole, hydrogen bonding and dielectric. The larger these dispersion, dipole, hydrogen bonding and dielectric interactions are in combination, the stronger is the attraction of solvent and solute molecules (Snyder & Kirkland, 1979). Various solvents can be grouped according to their selectivity on a triangular diagram (without considering intermolecular effects) (Snyder & Kirkland, 1979). Solvents are then grouped into clusters of similar selectivity (Figure 4.1).

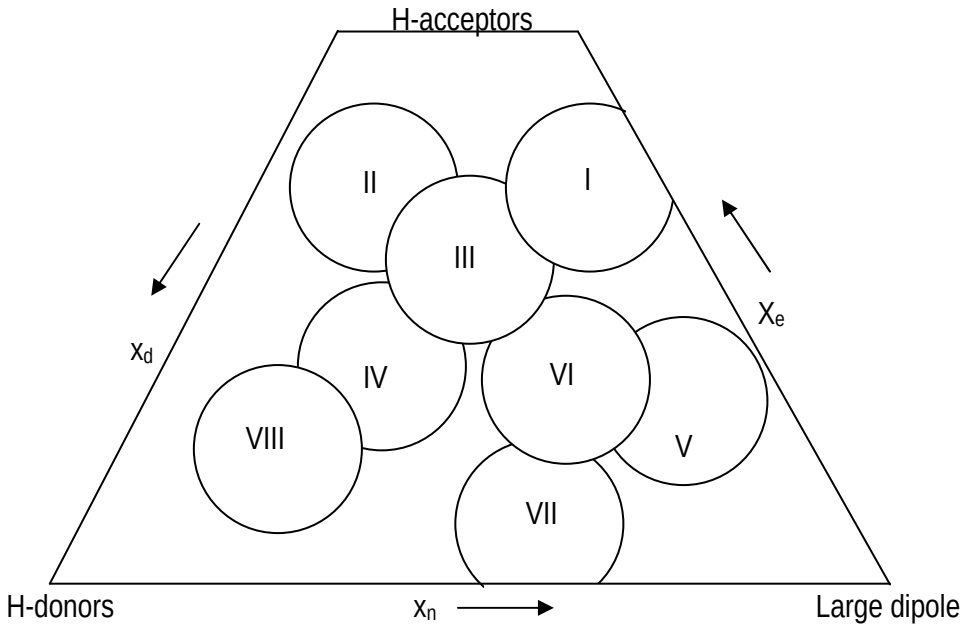


Figure 4.1 Solvents grouped according to selectivity in a selectivity triangle (Snyder & Kirkland, 1979).

4.1.3 Fractionation

The components of a mixture, such as an extract from a living organism, can be separated into groups of compounds sharing similar physico-chemical characteristics. This process is called fractionation and can be carried out in various ways, each of, which group compounds according to one or more particular features. Solubility, size, shape, electrical charge and several other features may influence grouping (Houghton and Raman, 1998). The skilful application of fractionation methods exploits these differences so that, when two methods are used in sequence, many fractions can be obtained each containing only one or two components. The initial fractionation may be based on solubility differences (e. g. solvent-solvent separation) while the second may utilise molecular size (e. g. Sephadex column chromatography). After a specific plant's activity has been established, the plant extract is fractionised by solvent-solvent separation and activity of different fractions established with bioassay guided fractionation. Utilising this method in initial purification, enables the isolation of

an active fraction and improve the probability to find pure compounds with similar activity above that of working with a crude extract only (Gailliot 1998; Houghton and Raman, 1998).

Acetone was chosen as an initial extractant because many polar and non-polar compounds are soluble in acetone and the test organisms (bacteria) are not sensitive to 25% acetone in a serial dilution assay (Eloff, 1998a). The aim of this investigation was to simplify a leaf extract of *Pteleopsismyrtifolia* by fractionation. Antibacterial activity of the different fractions will be established and quantified. One or more fractions with antibacterial activity will be used in a further investigation to isolate pure compounds from.

4.2 Material and Methods

4.2.1 Plant material

Leaves of *P. myrtifolia* (the same tree that has voucher specimens number 24/2000 in Lowveld NBI herbarium) were collected in the National Botanical Garden of Nelspruit during March 2000 and 2001 and prepared as described in 2.2.1 of Chapter 2.

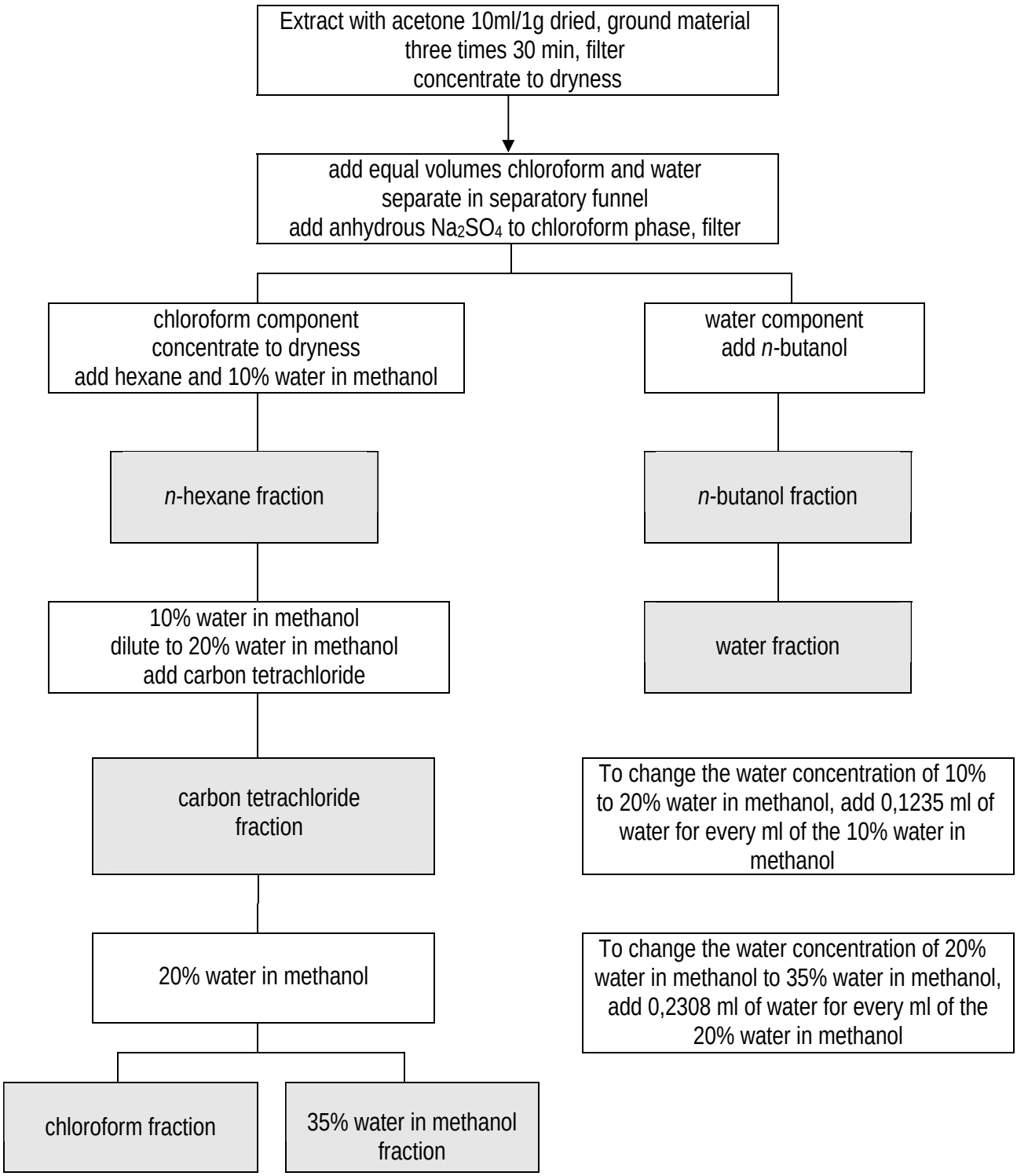
4.2.2 Solvent-solvent separation

The solvent-solvent separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) with a slight variation was used. Finely ground air-dried material (1 kg) of leaves were placed in a 10-liter glass bottle and filled (1:3) w/v with acetone. The bottle with ground leaves and acetone was then carefully shaken on a Labotec model 202 orbital shaker for one hour. Care had to be taken to secure the bottle on the rotary shaker, to avoid momentum and weight to move the bottle because of rotating motion. After an hour, the liquid was removed and filtered through Whatman No 1 quantitative filter paper. Fresh acetone was again added to the ground leaves. The container with leaf material was again shaken for one hour and the supernatant filtered. This was repeated for a third time. The

filtered liquid was dried by vacuum distillation in a Büchi rotary evaporator and the dry weight of the extract determined. The solvent-solvent separation method was followed (diagram below).

A schematic diagram of the method of the solvent-solvent separation procedure is shown in 4.2.2.1.

4.2.2.1 Schematic diagram of method of solvent-solvent separation procedure:



4.2.3 Thin layer chromatography (TLC)

The different fractions, namely: *n*-hexane, carbon tetrachloride, chloroform, 35 % water in methanol, *n*-butanol and water were each separated by thin layer chromatography (TLC) (10 µl of a 10 mg/ml final concentration) on Merck Silica gel F_{254} plates. The same eluent systems (selected to separate high, intermediate and low polarity) as in Chapter 2 were used. Separated compounds were examined under visible and ultraviolet light, marked and sprayed as described in Chapter 2.

To determine antibacterial activity, fractions were used to develop minimum inhibitory concentrations. Duplicate sets of thin layer chromatograms were used to develop bioautograms.

4.2.4 Determining antibacterial activity

4.2.4.1 Minimum inhibitory concentration (MIC)

MIC values were determined as described in 3.2.4.1 of Chapter 3.

4.2.4.2 Bioautography

For bioautography, all the fractions (10 µl of a 10 mg/ml final concentration) were applied on thin layer chromatograms as described in 3.2.4.3 of Chapter 3.

4.3 Results and Discussion

4.3.1 Fractions of group separation

After the third extraction with acetone, most of the green colour was washed out. The large amount of bubbles that originated between two phases of more polar fractions (without movement of the separating funnel) could be an indication of the presence of saponins. Dry

weight of each fraction was (listed in brackets): *n*-hexane (8.7 g), carbon tetrachloride (4.2 g), chloroform (9.8 g), 35% water in methanol (4.1 g), *n*-butanol (11.8 g) and water (5.6 g).

4.3.2 Thin layer chromatography (TLC)

Chromatograms developed by TLC, indicated the complexity of the material (each of the fractions contained several compounds) (Figure 4.2).

4.3.3 Antibacterial activity

4.3.3.1 Minimum inhibitory concentration (MIC) and total activity

MIC values indicated that all fractions had antibacterial activity (Table 4.1). Total activity values were calculated (as described by Eloff (2004) and explained in 3.2.4.2 of Chapter 3) and are listed in Table 4.1.

Table 4.1. Minimum inhibitory concentrations and total activity of *Pteleopsis myrtifolia* leaf fractions from liquid-liquid separation for the bacteria *Staphylococcus aureus* (S), *Enterococcus faecalis* (N), *Pseudomonas aeruginosa* (P) and *Escherichia coli* (E).

Fractions from *P. myrtifolia*	mg/pm from 1 g	MIC (mg/ml)					Tot al activity (ml/g)				
		S	N	P	E	Ave	S	N	P	E	Ave
n-hexane	8.7	0.01	0.002	0.005	0.02	0.009	870	4350	1740	435	1848.8
carbon tetrachloride	4.2	0.02	0.002	0.08	0.02	0.031	210	2100	52.5	210	643.1
chloroform	9.8	0.005	0.004	0.005	0.02	0.009	1960	2450	1960	490	1715.0
35 % water in methanol	4.1	0.01	0.002	0.08	0.002	0.024	410	2050	51.25	2050	1140.3
n-butanol	11.8	0.005	0.002	0.16	0.002	0.042	2360	5900	73.75	5900	3558.4
water	5.6	0.005	0.002	0.08	0.01	0.024	1120	2800	70	560	1137.5
Average	7.4	0.009	0.002	0.034	0.012	0.023	1155	3275	657.9	1607.5	1673.9

P. myrtifolia = *Pteleopsis myrtifolia,* pm = plant material, MIC = minimum inhibitory concentration, Ave = average, S = *Staphylococcus aureus,* N = *Enterococcus faecalis* , P = *Pseudomonas aeruginosa*, E = *Escherichia coli*.

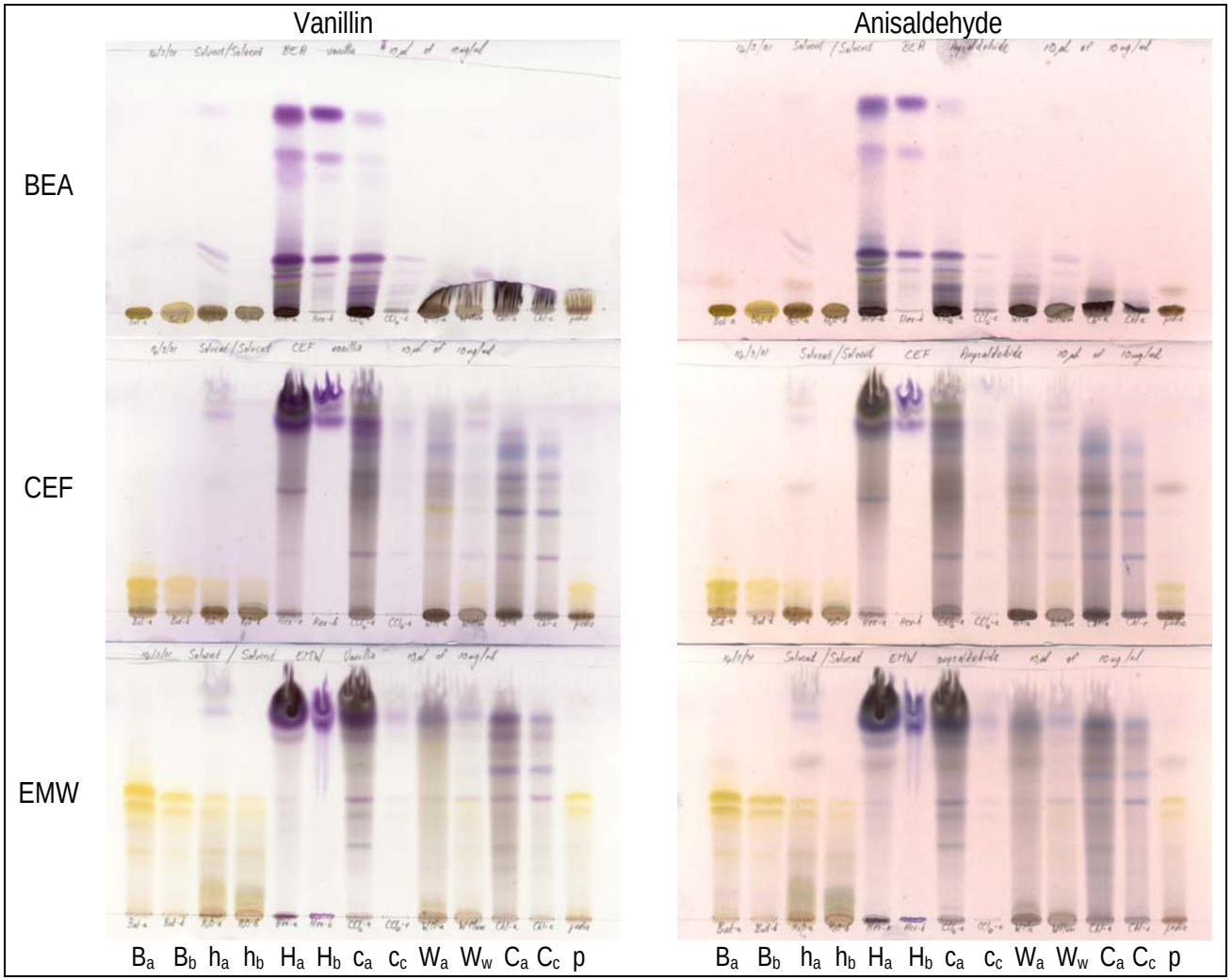


Figure 4.2. Thin layer chromatograms of solvent-solvent fractions from an acetone leaf extract of *Pteleopsis myrtifolia,* developed by the BEA solvent system (top), CEF solvent system (middle) and EMW solvent system (bottom) sprayed with vanillin (left) and anisaldehyde (right). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicates chloroform redissolved in chloroform). BEA = benzene: ethanol: ammonia (36:5.4:4), CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4) .

From the average MIC values of a fraction for all the bacteria (seventh column of Table 4.1), the chloroform fraction had the lowest MIC value, followed by the *n*-hexane fraction. The average MIC values of a bacterium for all the fractions (last row of Table 4.1), *E. faecalis* (N) had the lowest and *P. aeruginosa* (P) the highest MIC values. The average total activity for each fraction (all bacteria together) was calculated and the *n*-butanol, *n*-hexane and chloroform fractions had highest activities. The *n*-hexane and chloroform fractions had the best activity against all the bacteria tested.

4.3.3.2 Bioautography

From the bioautograms developed with different eluent systems and bacteria, the chloroform fraction from the EMW eluent system showed antibacterial activity for all four bacteria tested (Figures 4.3 and 4.4 and Figures 4.5 and 4.6).

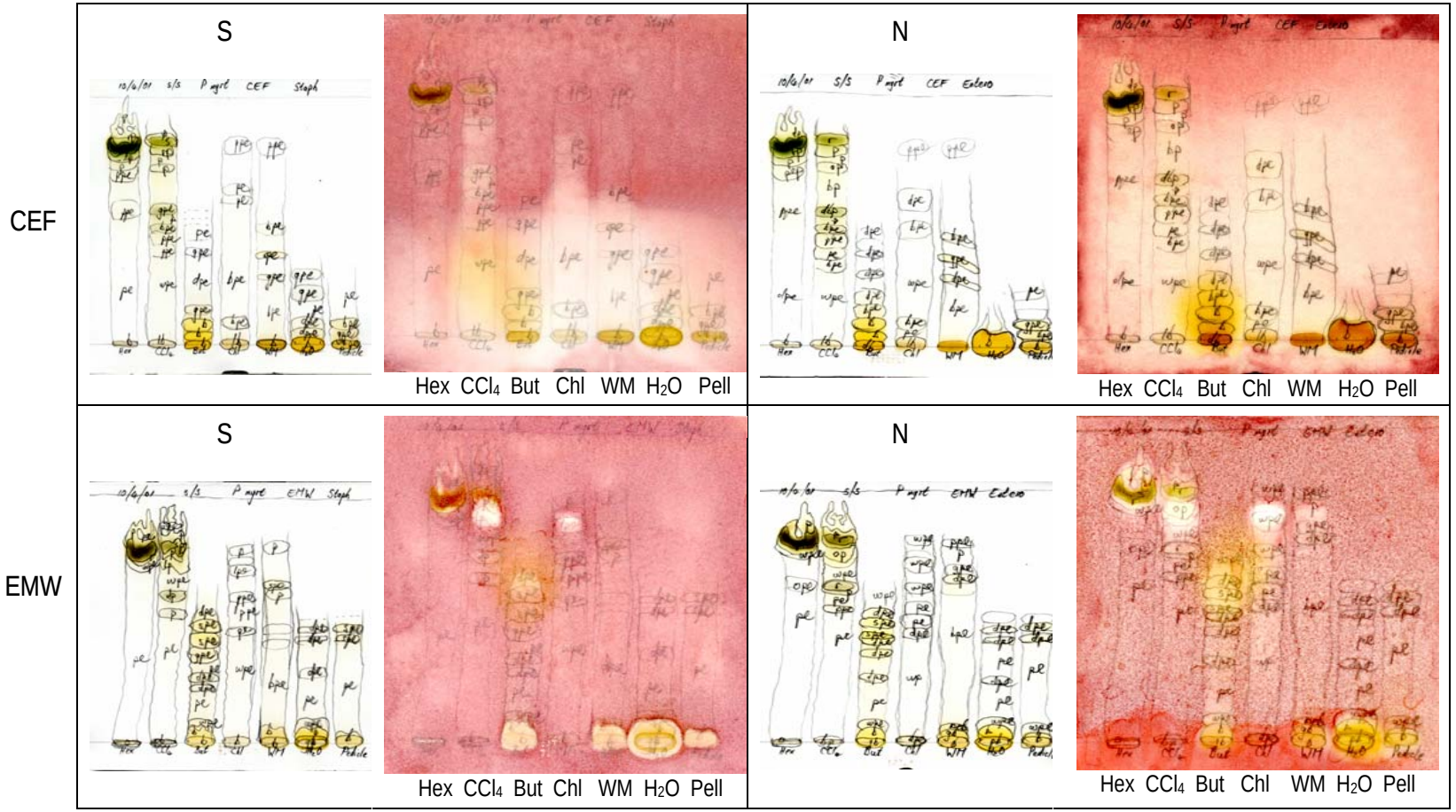


Figure 4.3. Bioautographs of fractions from *Pteleopsis myrtifolia* leaves and the bacteria *Staphylococcus aureus* (S) (left) and *Enterococcus faecalis* (N) (right). In each set, the top chromatogram was developed with the CEF eluent system and the bottom one by an EMW eluent system, and the one to the left, without bacteria. Fractions applied from lanes left to right were: Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35% water in methanol, H_2O = water, Pell = pellicle or foamy layer between phases of polar fractions. CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4).

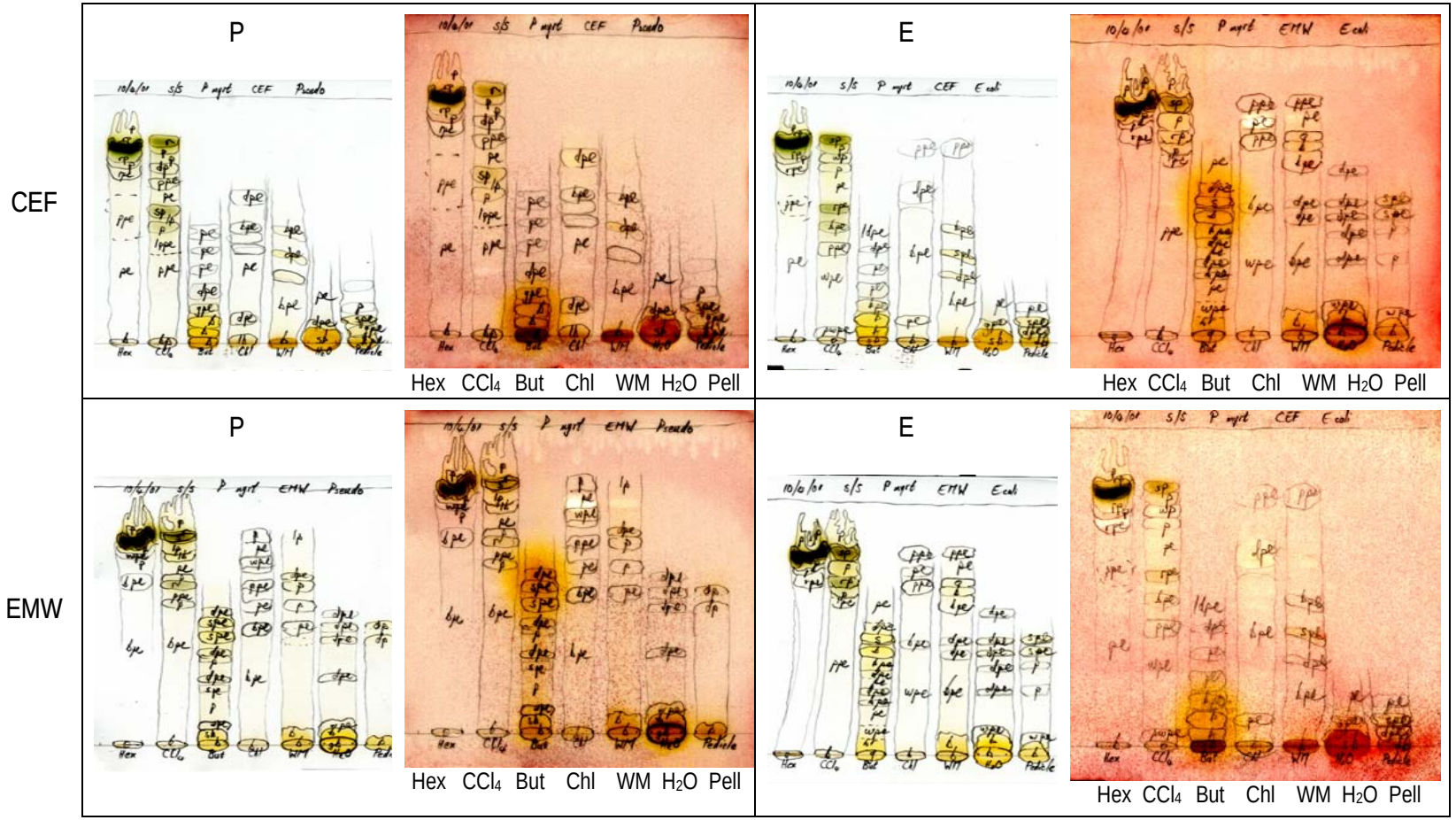


Figure 4.4. Bioautographs of fractions from *Pteleopsis myrtifolia* leaves and the bacteria *Pseudomonas aeruginosa* (P) (left) and *Escherichia coli* (E) (right). In each set, the top chromatogram was developed with the CEF eluent system and the bottom one by an EMW eluent system, and the one to the left, without bacteria. Fractions applied from lanes left to right were: Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35 % water in methanol, H_2O = water, Pell = pellicle or foamy layer between phases of polar fractions. CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4).

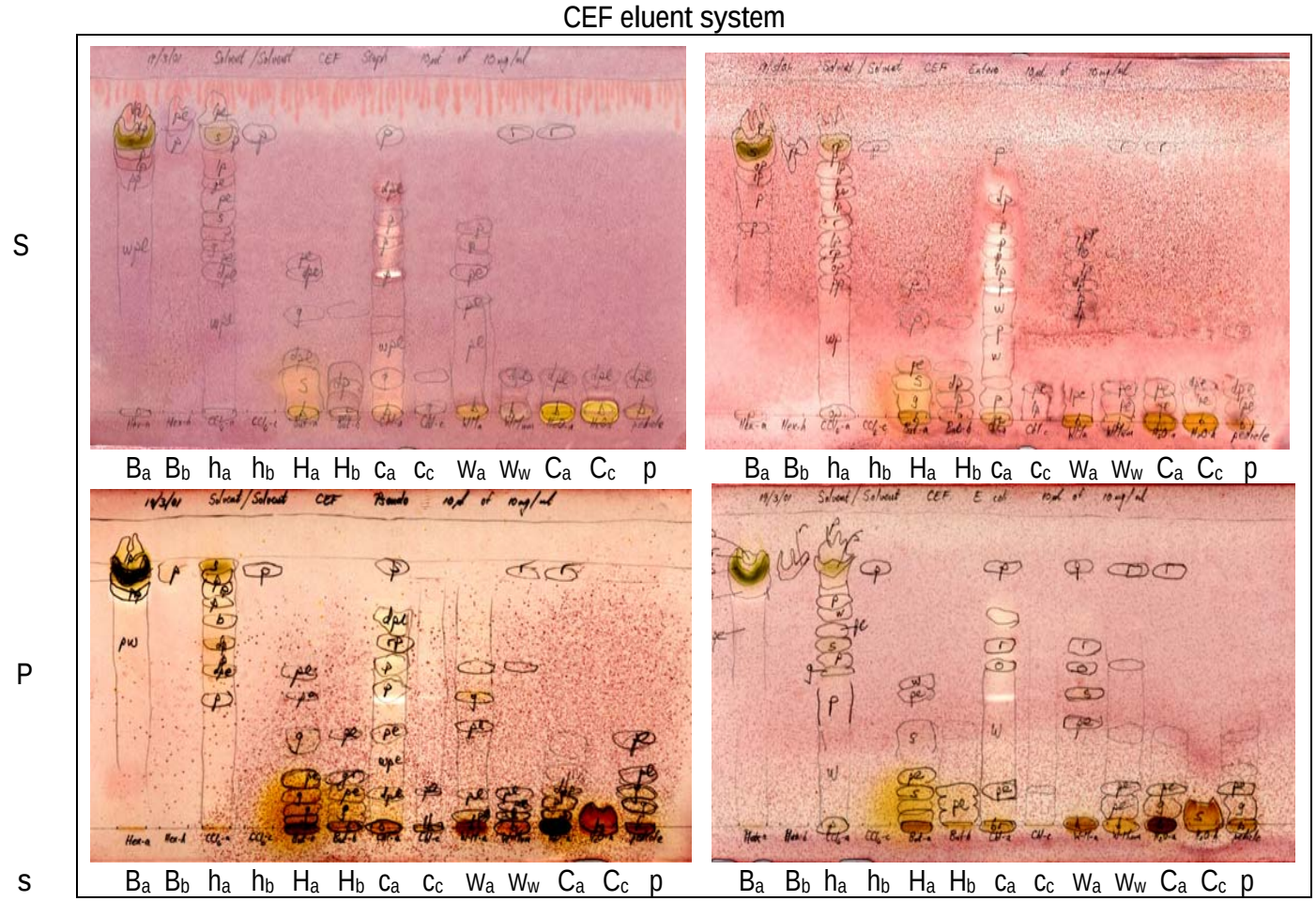
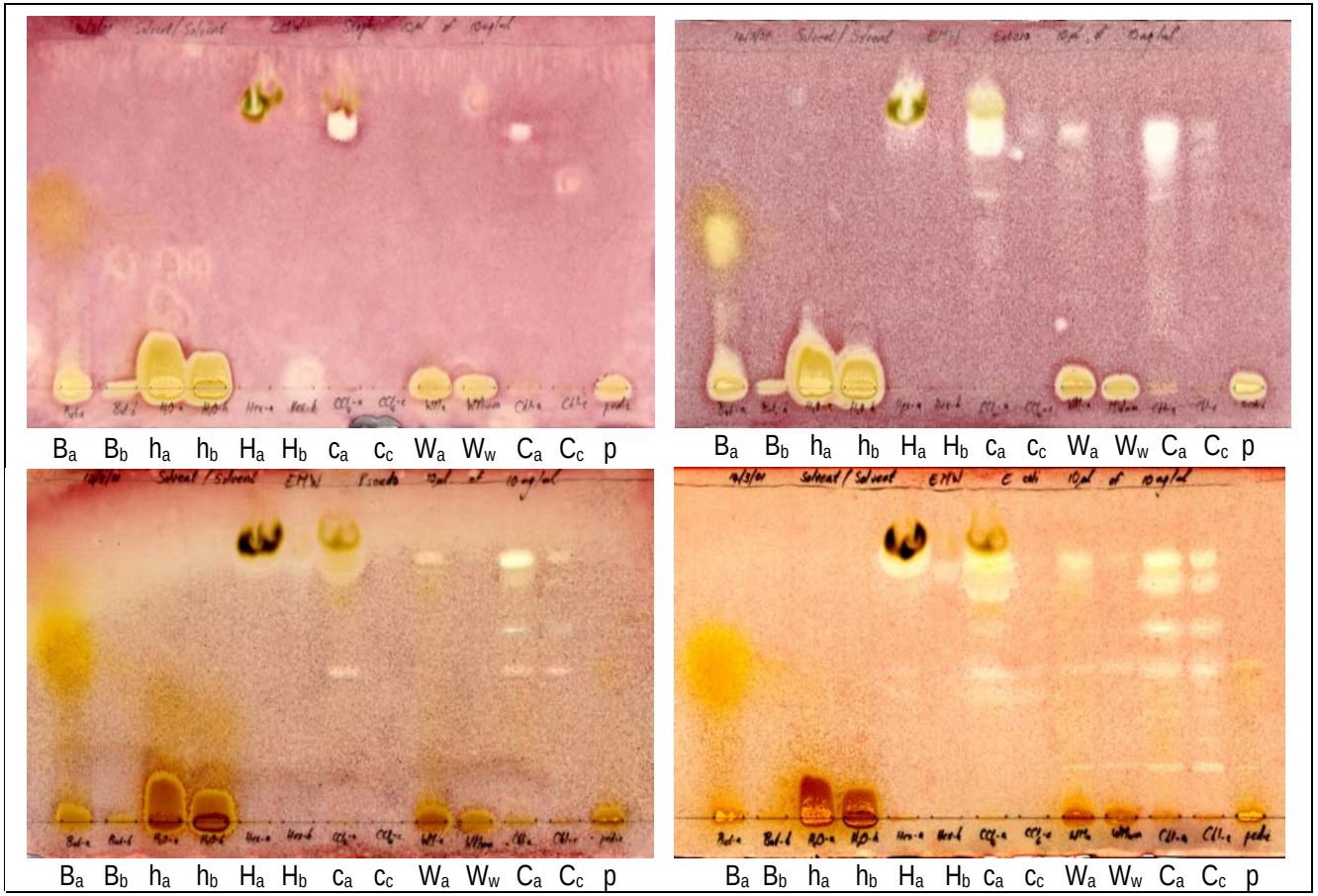


Figure 4.5. Bioautograms of *Pteleopsis myrtifolia* leaf fractions developed with the CEF eluent system and sprayed with bacteria, top left: *Staphylococcus aureus* (S), top right: *Enterococcus faecalis* (N), bottom left: *Pseudomonas aeruginosa* (P) and bottom right: *Escherichia coli* (E). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction solvent itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicate chloroform redissolved in chloroform). (CEF = chloroform: ethyl acetate: formic acid (5:4:1)).

EMW Eluent system

S

N



P

E

Figure 4.6. Bioautograms of *Pteleopsis myrtifolia* leaf fractions developed with the EMW eluent system and sprayed with bacteria, top left: *Staphylococcus aureus* (S), top right: *Enterococcus faecalis* (N), bottom left: *Pseudomonas aeruginosa* (P) and bottom right: *Escherichia coli* (E). For each thin layer chromatogram, the lanes from left to right were: But-a (B_a), But-b (B_b), H2O-a (h_a), H_2O-h (h_b), Hex-a (H_a), Hex-b (H_b), CCl_4-a (c_a), CCl_4-c (c_c), WM-a (W_a), WM-wm (W_w), Chl-a (C_a), Chl-c (C_c), pell (p). But = butanol, H_2O = water, Hex = *n*-hexane, CCl_4 = carbon tetrachloride, WM = 35% water in methanol, Chl = chloroform, pell = pellicle. Each fraction was redissolved in acetone (indicated by the suffix –a), as well as in the fraction itself (indicated by the first letter the fraction consists of (e.g. Chl-c indicate chloroform redissolved in chloroform). (EMW = ethyl acetate: methanol: water (40:5:4.4)).

The fact that the inhibition areas were better developed with the first set of bioautograms could be explained if the growth of the bacterial cultures differed for the two experiments. It is interesting to note that the fractions that were redissolved in acetone had more colour bands compared to fractions redissolved in the original extractant (for example the *n*-hexane, water and carbon tetrachloride fractions redissolved in acetone had 4, 5 and 5 colour bands respectively, compared to the *n*-hexane, water and carbon tetrachloride fractions redissolved in chloroform, which had 3, 1 and 3 colour bands respectively (The EMW eluent's chromatograms spayed with vanillin in Figure 4.2)). This confirmed the fact that acetone is miscible with polar and non-polar compounds and a larger variety of compounds, which led to more and clearer inhibition areas (e.g. the chloroform fraction of Figure 4.6 redissolved in acetone had clear areas of inhibition for *E. faecalis* and *P. aeruginosa* compared to the chloroform fraction of Figure 4.6 redissolved in chloroform where the inhibition areas for *E. faecalis* and *P. aeruginosa* were not clear at all.

All fractions had antibacterial activity against the Gram-positive bacteria, *S. aureus* and *E. faecalis* (bottom row of Figure 4.3 and top row of Figure 4.6). The chloroform and 35 % water in methanol extracts were active against the Gram-negative bacteria, *P. aeruginosa* and *E. coli* (bottom row of Figure 4.4 and bottom row of Figure 4.5). The largest amount of inhibition areas (seven) was observed with the chloroform fraction and *E. faecalis* and *E coli*. The chloroform fraction was antibacterial to all bacteria tested, and had the largest amount of inhibition areas, and thus amount of bacterially active compounds.

4.4 Conclusions

Bioassay-guided fractionation identified that all fractions: n-hexane, carbon tetrachloride, chloroform, 35% water in methanol, n-butanol and water had antibacterial activity against at least two of the bacteria tested. The chloroform fraction was antibacterial to all bacteria tested,

and had the largest amount of inhibition areas (seven), and thus amount of bacterially active compounds. This fraction was chosen for further purification and to isolate pure compounds from it in future research (Chapter 7 and 8).

4.5 Literature references

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Chapter 5

Cytotoxic activity of *Pteleopsis myrtifolia* leaf extracts

Abstract

There is considerable interest and need for the discovery of additional novel natural products and their semisynthetic analogs as potential cancer chemotherapeutic drugs. Since other researchers found that a methanolic extract of leaves of a Tanzanian *Pteleopsis myrtifolia* tree inhibited the growth of three human cancer cell lines, we investigated the cytotoxic activity of South African *P. myrtifolia* leaf extracts. Several leaf extracts (cold water, hot water, hot water without tannins (H-t), ethanol, methanol, methanol without tannins (M-t) and chloroform) of *P. myrtifolia* were tested against different human cell lines (MCF-12, MCF-7, H157, $WHCO_3$, HeLa). Standard cell culture techniques and crystal violet staining was used to measure cell growth spectrophotometrically. Graphs drawn from calculations made from spectrophotometer readings, indicated that for the non-cancerous human cell line, MCF-12A (breast), the growth was not inhibited at 10 μg/ml by all five extracts, and significantly inhibited at a concentration of 100 μg/ml by the hot water and methanol leaf extracts. The absence of extensive growth inhibition may indicate that the plant extracts are not toxic to the cell line at the concentrations used. The growth of the cancerous cell line $WHCO_3$ were inhibited by all plant extracts, (significant less growth inhibition occurred at 20 μg/ml than at 60 μg/ml), the growth of cell lines MCF-7 and H157 by all plant extracts except the M-t extract (with significant less growth inhibition at 20 μg/ml than at 60 μg/ml), and the growth of cell line HeLa by all plant extracts except the hot water and chloroform extracts ((with significant less growth inhibition at 20 μg/ml than at 60 μg/ml). When the hot water and methanol extracts were tested without tannins in a separate experiment, both the H-t and M-t extracts, inhibited growth of the H157 cell line more than the same extracts with tannin. These extracts without tannins inhibited growth less than

the extracts with tannins for the HeLa cell line. GI_{50} values were reached for all extracts at 100 μg/ml for the MCF-7 and $WHCO_3$ cell lines. No extracts reached GI_{50} values for the MCF-12A cell line, and only the hot water, methanol and chloroform extracts reached their GI_{50} values for the H157 cell line. LC values were reached for the cold water, hot water, methanol and ethanol extracts for the MCF-7, $WHCO_3$ and HeLa cell lines. No extracts reached constant lethal concentration values for the cell lines H157 and MCF-12. The cell lines differed in their sensitivity to the plant extracts and were most sensitive to the hot water and methanol extracts.

5.1 Introduction

5.1.1 Secondary metabolites from plants in the treatment of cancers

Today, natural products still represent more than 80% of pharmacological lead compounds. They constitute a practically endless source of novel substances able to enrich therapeutics. In the field of chemotherapeutics several substances were isolated, amongst which the Madagascan periwinkle, *Catharanthus roseus* that were the source of, vincaleukoblastine and leurocristine, marketed under the names of Velbe ®, and Oncovin ®, respectively. These two drugs have now been used for over thirty years in the chemotherapy of cancers and leukaemia. In 1974 Dr Lang Lois and Dr Portier managed to synthesize vincristine and vinblastine, as well as another original substance, Navelbine ® which exhibited therapeutic properties superior to the previous drugs (Portier *et al*., 1996).

A number of other secondary metabolites and their derivatives of plant origin, as well as natural products of marine and microbial origin are currently in preclinical and clinical trials as potential anticancer agents. One such is combretastatin from the Combretaceae plant family. It was isolated from the bark of the South African *Combretum caffrum* by Dr. Gordon Gragg in the laboratories of Prof. Pettit at Arizona State University. It had a very high affinity for tubulin. Since the yield was very low – 26.4 mg was isolated from 77 kg dry stem bark – several forms

were synthesised and tested. Experiments examining of the effect of combretastatin A4 and combretastatin A4 phosphate on murine tumors demonstrated that combretastatin A4 phosphate caused selective extensive vascular shutdown of tumors. The vascular shutdown was followed by large-scale cell death and necrosis within 24 h after administration (Chaplin *et al*., 1999).

Wall and co-workers (Wani *et al*., 1971) discovered a substance in the trunk bark of the yam (*Taxus* sp), which he called taxol. This substance remained unexplored for years, probably due to the facts that taxol can only be extracted from multi-centuried trees, the trees are very slow growing, the yield is very low and taxol not easily soluble. Several years later, the biological mode of action of taxol was discovered by Susan Horwitz (Schiff *et al*., 1979). Taxol belongs to a group of substances called “spindle poisons” and inhibits the disappearance of spindle after cell division by preventing the depolymerization of microtubules and thus cell progression.

In Tanzania, 47 plants were evaluated for cytotoxic activity by using methanolic extracts and three human cancer cell lines. From the nine plants traditionally used to treat cancer, only two exhibited a cytotoxic effect. From the 38 plants that are used to treat non-cancer diseases, 14 exhibited a cytotoxic effect. *Pteleopsis myrtifolia* was one of the plants not traditionally used to treat cancers, which had cytotoxic effects. At 100 μg/ml, 75-100% inhibition of growth was obtained *in vitro* for the HT29 (colon adenocarcinoma) and A431 (skin carcinoma), and 25-50% for HeLa (cervical carcinoma) cancer cell lines (Kamuhabwa *et al*., 2000).

5.1.2 Incidence of cancer

Cancer is the second leading cause of death amongst Americans. One out of every four deaths in the U.S. is due to cancer. In the United States in 1999, over 1500 people were expected to die of cancer each day. On a worldwide basis, figures for death rates for all cancer sites

combined, increased from 1990 up to 1994, decreased from 1994 up to 1998 and stabilised from 1998 through 2000. Increases in breast cancer amongst woman and prostate cancer amongst men are masked by a decrease in all cancer sites combined statistics. The Centres for Disease Control and Prevention (CDC), National Cancer Institute (NCI) and North American Association of Central Cancer Registries (NAACCR) released a United States Cancer Report in November 2003 (to date the most up to date available) with cancer incidence data up to the year 2000. The data covers 84% of the United States (US) population, over 60 primary cancer sites for men and woman, and provide specific information with regard to geographic area, race, sex and age:

- The most common childhood cancers are leukaemia (incidence highest amongst 1- to 4 - year- olds), lymphomas (incidence highest amongst 15- to 19-year-olds), cancer of the central nervous system and reticuloendothelial neoplasms.
- White men and woman are more often affected by melanomas of the skin and cancer of the brain than are black or Asian men and woman.
- Black men and woman are more affected by multiple myelomas than are white or Asian men and woman.
- Asian men and woman have higher incidence rates of liver and intrahepatic bile duct cancer and stomach cancer than do white or black men and woman (Centres for Disease control and Prevention, 2004).

During the 20th century, the leading cause of death in the U.S. shifted from infectious to chronic diseases. Seven out of every 10 U.S. residents who die each year, do so as a result of chronic (e. g., cancer, diabetes) disease. Chronic diseases can be prevented or controlled by adopting behaviours (eating nutritious foods, being physically active) (Medical News Today, 2004)

5.1.3 Underlying cause in many types of cancer

The underlying cause in many types of cancer seem to be free radical damage to the DNA of cells, triggering their altered behaviour. Since reactive oxygen radicals play an important part in carcinogenesis, it would be important to develop a course of action to prevent these free radicals from causing damage. Intake of antioxidant nutrients and supplements provide protection against free radical activity, therefore antioxidants present in consumable fruits, vegetables, neutraceuticals and beverages have received considerable attention as cancer chemopreventative agents (Muktar *et al*., 1994). Diet plays the most important preventative role in protection from cancer and Holford (1997) states that the balance between one's intake of antioxidants and exposure to free radicals may literally be the balance between life and death. Fruit, vegetables and seeds are top of the anti-cancer foods and are rich in phytochemicals, vitamins A and C, selenium, vitamin E, calcium and zinc. Plants make protective compounds (phytochemicals) to protect themselves from radiation, insects and humans. Ingestion of a plant rich diet, confers the chemoprotective effect of phytochemicals to man.

Currently the value of taking antioxidants during chemotherapy is researched. Cancer itself creates oxidative stress and impairs antioxidant status in the organism as a whole. Chemotherapy can overwhelm the antioxidant defence systems in the cell, which will lead to an increase in lipid peroxidation, which in turn leads to a decrease in cellular proliferation and therefore to a decrease in the effectiveness of chemotherapeutic agents. Patients with an impaired antioxidant status may become relatively resistant to chemotherapy, and there is evidence that antioxidants improve the antitumor response to antineoplastic agents. The value of antioxidants here is to overcome the growth inhibiting effects of oxidative stress and maintain responsiveness to chemotherapeutic agents (Drisko *et. al*., 2003)

5.1.4 Cytotoxic, antitumour, and antineoplastic activities

As mentioned in 1.1.9 of Chapter 1, the National Cancer Institute (NCI) has defined the following terms: 'cytotoxicity' refers to *in vitro* toxicity of tumour cells, while 'antineoplastic' and 'antitumour' should refer to *in vivo* activity in experimental systems (Ghisalberti, 1993). The NCI renamed the IC_{50} value, the concentration that causes 50% growth inhibition, to the GI_{50} value to emphasize the correction for the cell count at time zero (more detail in 5.2.6).

There is considerable interest in the discovery of additional novel natural products and their semisynthetic analogs as potential cancer chemotherapeutic drugs (Kinghorn *et al*., 1999). The aim of this research was to investigate leaf extracts from *P. myrtifolia* for their cytotoxic activity against one non-cancerous and four cancerous human cell lines.

5.2 Materials and Methods

5.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

5.2.2 Human cell lines

The following human cell lines of the American Tissue Culture Collection (ATCC) were chosen randomly from a standard battery of cell lines available at Department of Physiology. The ones chosen were a non-cancerous cell line - MCF-12A, and the cancerous cell lines MCF-7, H157, $WHCO_3$ and HeLa. They are described below in 5.2.2.1 and 5.2.2.2.

5.2.2.1 Non-cancerous human cell line: (limited number of cell divisions).

5.2.2.1.1 ATCC CRL- 10782 MCF–12A (mammary gland, human)

The MCF – 12A cell line was a gift from Professor Parker (Division of Medical Biochemistry, University of Cape Town). It is a non-tumourigenic epithelial cell line produced by long term

culture of non-cancerous mammary tissue in serum free medium with low Ca^{++} concentration. MCF–12A was derived from adherent cells in the population; are positive for epithelial cytokeratins, exhibit three-dimensional growth in collagen and form domes in confluent cultures.

It's current medium for propagation: 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 µg/ml insulin, 500 ng/ml hydrocortisone and 5% foetal bovine serum. They have a population doubling time of 19 hours in the above medium.

5.2.2.2 Cancerous human cell lines: (potential unlimited number of cell divisions)

5.2.2.2.1 ATCC HBT- 22 MCF– 7 (human breast carcinoma cell line)

The MCF-7 cell line was supplied by Highveld Biological (Sandringham, SA). For the derivation of this line, cells from the pleural effusion were seeded in Eagle's minimum essential medium with non-essential amino acids, 20 µg/ ml insulin and 20% bovine calf serum. It has retained several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

Current medium for propagation: Eagle's minimum essential medium (MEM), at 37 °C in a humidified atmosphere containing 5% CO_2. Media were supplemented with 10% heat inactivated foetal calf serum, penicillin (100 µg/l), streptomycin (100 µg/l) and fungizone (250 µg/l).

5.2.2.2.2 ATCC CRL- 5802 NCI-H157 (human squamous lung carcinoma)

The NCI – H157 is a non-small cell carcinoma cell line of the lung (NSCLC), originally derived by AF Gazdar, HK Oie, JD Minna and associates in 1979 from the pleural effusion of a 59-year-old male Caucasian with squamous cell carcinoma of the lung. It was given to Department of Physiology, University of Pretoria, as a gift from Dr. M de Kock (Dept. of Physiology, University

of the Western Cape). The cells express multiple markers of squamous differentiation. The line shows functional platelet-derived growth factor β (PDGF) receptors as well as mRNA expression of the 6-kb transcript for the PDGF β type receptor. The line expresses heterogeneous mRNA expression for PDGF A and B chain, transforming growth factor β and the epidermal growth factor.

NCI-H157 cells were grown as monolayers in the same medium as MCF-7.

5.2.2.2.3 $WHCO_3$ (human oesophageal cancer cell line)

The $WHCO_3$ cell line was a gift from Professor Thornley (Department of Zoology, University of the Witwatersrand, Johannesburg, South Africa). $WHCO_3$ cells were obtained through biopsies from a patient with squamous oesophageal carcinoma. The cells were poorly differentiated non-keratinising squamous cell carcinoma.

The $WHCO_3$ cells were grown and maintained as monolayer cultures in minimum essential medium, containing 10% heat inactivated foetal calf serum and a 10% mixture of 10 µg/ml Penicillin, 10 µg/ml streptomycin and 25 µg/ml fungizone at 37 °C in a humidified atmosphere containing 5% CO_2.

5.2.2.2.4 ATCC CCL-2 HeLa (human epitheloid carcinoma of cervix)

The HeLa cell line was purchased through Sterilab Services (Kempton Park, Johannesburg, South Africa) from the American Tissue Culture Collection (ATCC) (Maryland, USA). HeLa was the first aneuploid, epithelial-like cell line to be derived from human tissue and maintained continuously by serial cell culture. It was isolated by GO Gey, WD Coffman, and M.T. Kubicek in February 1951, from a carcinoma of the cervix of a 31-year-old Negro female (Cancer Res. 12: 264, 1952). In a recent re-examination of the original slides, Jones *et al*. (Obstet. Gynecol. 38: 945-949, 1971) diagnosed the tumour as an adenocarcinoma. Since its origin, it bas been

one of the most widely studied cell lines. HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences. It has to be handled as potentially biohazardous material under at least Biosafety Level 2 containment.

The current medium for propagation of HeLa cells is Eagle's MEM, in which they are grown as monolayers, at 37 °C in a humidified atmosphere containing 5% CO_2, with non-essential amino acids, 90% Earle's BSS, and 10% foetal bovine serum.

5.2.3 Plant extracts

The chloroform, ethanol, methanol and cold water extracts were prepared in the same way as described in 2.2.2 of Chapter 2. For the boiling water extract, dried, ground leaves of *P. myrtifolia* was boiled in distilled water (1:10) for 2 hours, cooled down and the supernatant centrifuged to separate any pieces of material from the extract. (For traditional use (venereal diseases), a concoction is made by boiling material for approximately 2 hours). Yield was determined and the dried extracts (from the extractants ethanol, methanol, cold water, and boiling water) were redissolved in distilled deionized water to a final concentration of 10 mg/ml and stored in a tightly sealed dark glass container at 5 °C. The dried extracts from chloroform were redissolved in dimethylsulfoxide (DMSO) to a final concentration of 1000 mg/ml (which served as a stock from which dilutions were made) and stored in a tightly sealed dark glass container at 5 °C. All final extracts were filter sterilised with 0.22 μm filters before use.

5.2.4 Statistical considerations

Statistical analysis of the data was conducted as discussed with Dr P. Becker of the unit for Biostatistics at the Medical Research Council (MRC). When exposing cells to the effects of plant extracts, biological variation was introduced within each cell line. The analytical variation

in the experimental procedures and biological variation within each cell line were analysed by determining ANOVA and students' t-test for paired samples, respectively. Quantitative experiments were repeated thrice and all experiments included an appropriate set of controls. $P < 0.05$ was considered significant.

Positive controls, such as vincristine or paclitaxel (taxol), were nor used due to budget constraints of the experiments. The NCI's values for vincristine or paclitaxel on human cell lines were compared to results of plant extracts (Table 5.2 in section 5.3).

5.2.5 Growing cell lines at required density in multiwell plates

A primary culture was grown to confluency in a 25 cm^2 tissue culture flask containing 5 ml tissue culture medium. Before the start of the experiments, all medium was removed from the culture. The adhering cell monolayer was washed once or twice with a small volume of 37 °C PBS to remove any residual foetal bovine serum (FBS) that may inhibit the action of trypsin. Enough 37 °C trypsin/EDTA solution was added to the culture to cover the adhering cell layer. The plate was placed in the incubator for 1 to 2 min. The bottom of the plate was tapped lightly on the countertop to dislodge cells. The culture was checked with an inverted microscope to make sure that cells were rounded up and detached from the surface. If cells were not sufficiently detached, the plate was returned to the incubator for an additional minute or two. As soon as cells were detached, medium-containing serum (to inhibit further trypsin activity that might damage cells) was added. The cell suspension was transferred to a tube or flask. Cells were counted using a haemocytometer and diluted to the desired density. Tryphan blue (0.1%) exclusion staining was used to test for cell viability. 5 ml fresh medium was added to the original flask and returned to the incubator.

Aseptic techniques were used throughout all cellular experiments and preparation of multiwell

plates were carried out in a laminar flow cabinet.

Cell cultures were seeded (at the correct density - 5000 cells/ well (in 96 wells)) in 200 µl and incubated at 37 °C for 48 hours (2 days) after which they were inspected to see of they were growing and adhering to the bottom of the MPW. The outside wells of the 96-MWP were not used for experimental readings, because the loss of moisture from them over the 5 day duration of the experiment, might have given incorrect values. They were filled with 200 µl of 0.2% Triton X-100. When cell cultures grew and adhered to the bottom of the MWP, the plant extracts were added to the wells containing cells and the MWPs were incubated for another 3 days.

Initially each cell line was tested at 10 and 100 µg/ml of plant extract (Figure 5.1).

Three days after the plant extracts were added, the medium was discarded from and 100 µl 1% gluteraldehyde added to the wells and incubated at room temperature for 15 min. After 15 min, the gluteraldehyde was discarded and 100 µl of 0.1% crystal violet stain added and left at room temperature for 30 min. After 30 min, the plates were immersed in running tap water for 15 min and then dried thoroughly. 200 µl of 0.2% Triton X-100 were added to each well to solubilize the crystal violet stain and the plates incubated at room temperature for 30 min. After 30 min 100 µl of the solution was transferred to a clean micrometer plate and the absorbance read at 570 nm. Optical density is linearly related to the number of adhering cells with a sensitivity of ca.500 cells; therefore, the technique is applicable to study agents that affect cell proliferation (Gillies *et al*. 1986). This method makes use of a technique allowing rapid and reproducible quantification of cell number in cultures grown in 96 microtitre plates. Quantification is possible by solubilizing the adsorbed dye into a solution of Triton X-100 and determining optical density

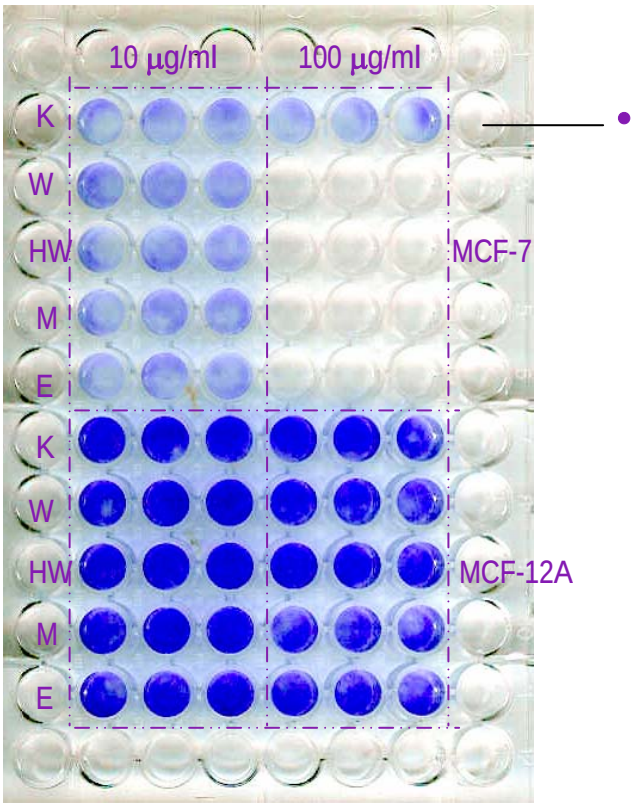

Figure 5.1. Scan of a 96-multiwell plate (MWP) with the human cell lines MCF-7 (top half) and MCF-12A (bottom half) at 10 (left) and 100(right) µg/ml of each of the plant extracts (rows marked: cold water (W), hot water (HW), methanol (M) and ethanol (E)). • The wells bordering the MWP contained only Triton X-100.

using a spectrophotometer. From the spectrophotometer readings, calculations were made and the graphs were drawn from the calculations to present the data in an easy to interpret format.

All plant extracts were also tested at 20, 40, 60 and 80 µg/ml. Figure 5.2 show the MCF-7 cell line at those concentrations.

The two plant extracts that inhibited the growth of the human cancer cell lines most were also tested without tannins. The tannins were removed from these extracts with the use of polyvinylpolypyrrolidone PVPP, described by Houghton & Raman (1998) and which is, according to these authors, useful for small-scale work.

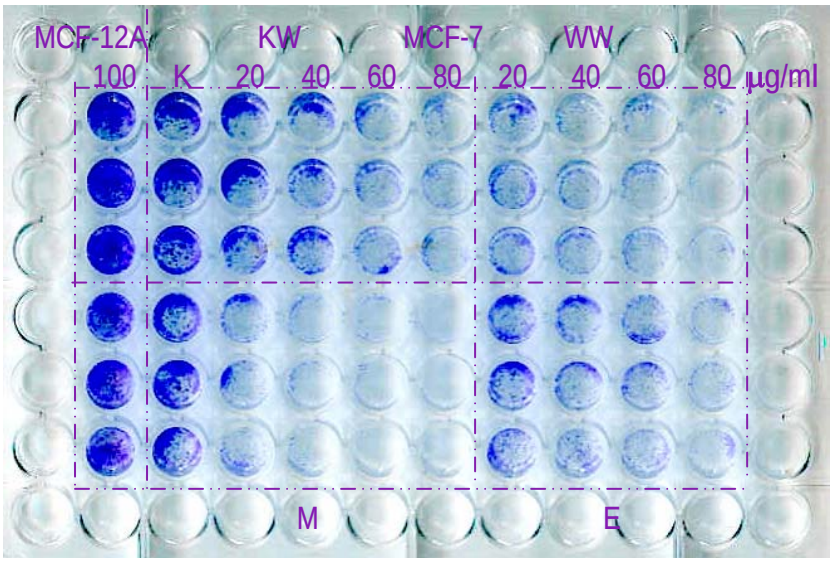

Figure 5.2. 96-MWP with the MCF-7 human cancer cell line and different plant extracts (KW = cold water, WW = warm water, M = methanol, E = ethanol) (columns) at concentrations 0, (0 = control = K), 20, 40, 60 and 80 µg/ml. The right column is the MCF-12A cell line at 100 µg/ml.

Time zero observations were made at the moment the plant extracts were added. The reason being that at this time the cancer cell cultures have already grown for 72 h. If this zero value is calculated, one is able to represent or calculate results in terms of growth inhibition by 50% (GI_{50}) and lethal concentration (LD) – and in addition, able to compare with other extracts tested by other researchers according to standards used by National Cancer Institute (NCI). To obtain this value a second set of controls was grown and at the moment the plant extracts were added to the first set, the second set of controls was fixated and stained. The value obtained here is subtracted from all wells. The control values (without plant extract) were taken as 100% cancer cell growth – and the different concentrations of plant extracts calculated as percentage cell growth inhibition.

5.2.6 Calculation of growth inhibition, GI_{50} and LC values

Graphs were drawn from the growth inhibitory effects of plant extracts for each cell line. For these graphs the control values were taken as 100% growth and the values obtained with plant

extracts, calculated as a percentage of the control. The 50% growth inhibition concentration (GI_{50}) as well as the lethal concentration (LC) formulas of NCI were applied and graphs drawn of resulting values. To calculate GI_{50}, the following formula was applied:

$$100 \times (T_{72}-T_0)/(K_{72}-T_0)$$

To calculate LC, the following formula was applied:

$$100 \times (T_{72}-T_0)/T_0$$

T_{72} is the spectrophotometer reading of the specific cell line after 72 hours and crystal violet staining, T_0 is the spectrophotometer reading of the specific cell line at time zero, at the moment the plant extracts are added*, K_{72} is the spectrophotometer reading of the control values (cells without any extract), of which an average is calculated and used. From each spectrophotometer reading, the average triton value is deducted. The graph values are the average of 3 repetitions.

* = A second set of controls (cells without any extract added) is used to obtain this value, since they are fixated and stained.

The TGI (total growth inhibition) is the concentration of plant extract or test drug where:

$(T_{72}-T_0)/(K_{72}-T_0) = 0$ and signifies a cytostatic effect.

LC50 is where $100 \times (T_{72}-T_0)/T_0 = -50$ and signifies a cytotoxic effect.

5.3. Results and discussion

After fixation and crystal violet staining, it was possible to see which plant extracts inhibited the cell lines extensively by the absence of colour in the wells of the MWP (Figure 5.3).

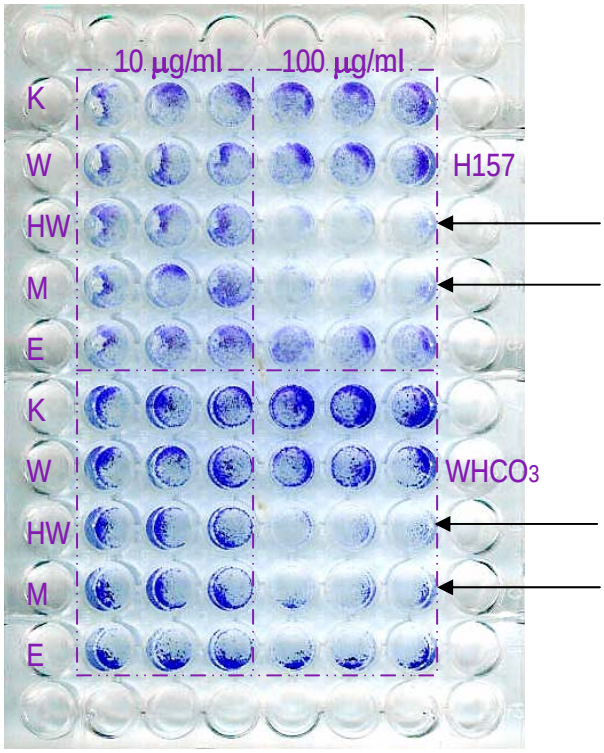

Figure 5.3. Scan of a MWP in which the inhibitory effects of the plant extracts hot water (HW) and methanol (M) against human cancer cell lines, (top half) H157 and (bottom half) $WHCO_3$, could be seen (indicated by arrows).

5.3.1 Non-cancerous human cell line MCF–12A

Graphs drawn from calculations made from spectrophotometer readings, indicated that for the non-cancerous human cell line MCF-12-A, the growth was not inhibited at 10 µg/ml for all extracts tested, and according to Figure 5.4a, slightly inhibited at a concentration of 100 µg/ml. Error bars, indicating standard deviation values, are present on all graphs. At 100 µg/ml, the MCF-12A cell's growth was inhibited significantly more than at 10 µg/ml only for the hot water and methanol extracts. Significant differences between concentration levels are indicated with stars at the higher concentration on graphs.

At the 100 µg/ml concentration, the cold water and methanol extracts' growth inhibition effects differed significantly. The same applied for the ethanol and methanol extracts. Extracts whose

growth inhibition effects were significantly different, are indicated with dots of the same colour in Table 5.1. For example, if the cold water and methanol extracts' growth inhibition effects differed significantly, each was given a dot of the same colour in Table 5.1. In addition, the ethanol and methanol extracts were given a dot of the same colour, a different colour than that of the cold water and methanol.

For the MCF-12A cell line the hot water and methanol extracts without tannins inhibited growth less (7% and 13%) than the extracts with tannins (27% and 38% respectively) (Figure 5.4b). This difference was not significant at a 5% confidence interval.

Formulas used by the National Cancer Institute for calculating 50% inhibitory concentration (GI_{50}) and lethal concentration (LC) were applied. The resulting values for the leaf extracts (cold and hot water, methanol, ethanol and chloroform and hot water and methanol extracts without tannins (H-t and M-t respectively)) were plotted; the 50% inhibitory concentration (GI_{50}) in Figure 5.4c, and the lethal concentration (LC) in Figure 5.4d. The GI_{50}-values (50% value of the control and y-axis at 50%) and the LC-values (no growth and y-axis below 0) were not reached.

The presence of growth inhibition only by the hot water and methanol extracts, may indicate that the plant extracts are not toxic to the cell line at the concentrations used. This is a very important result, since it indicates that the effects of the plant extracts are not due to general toxicity.

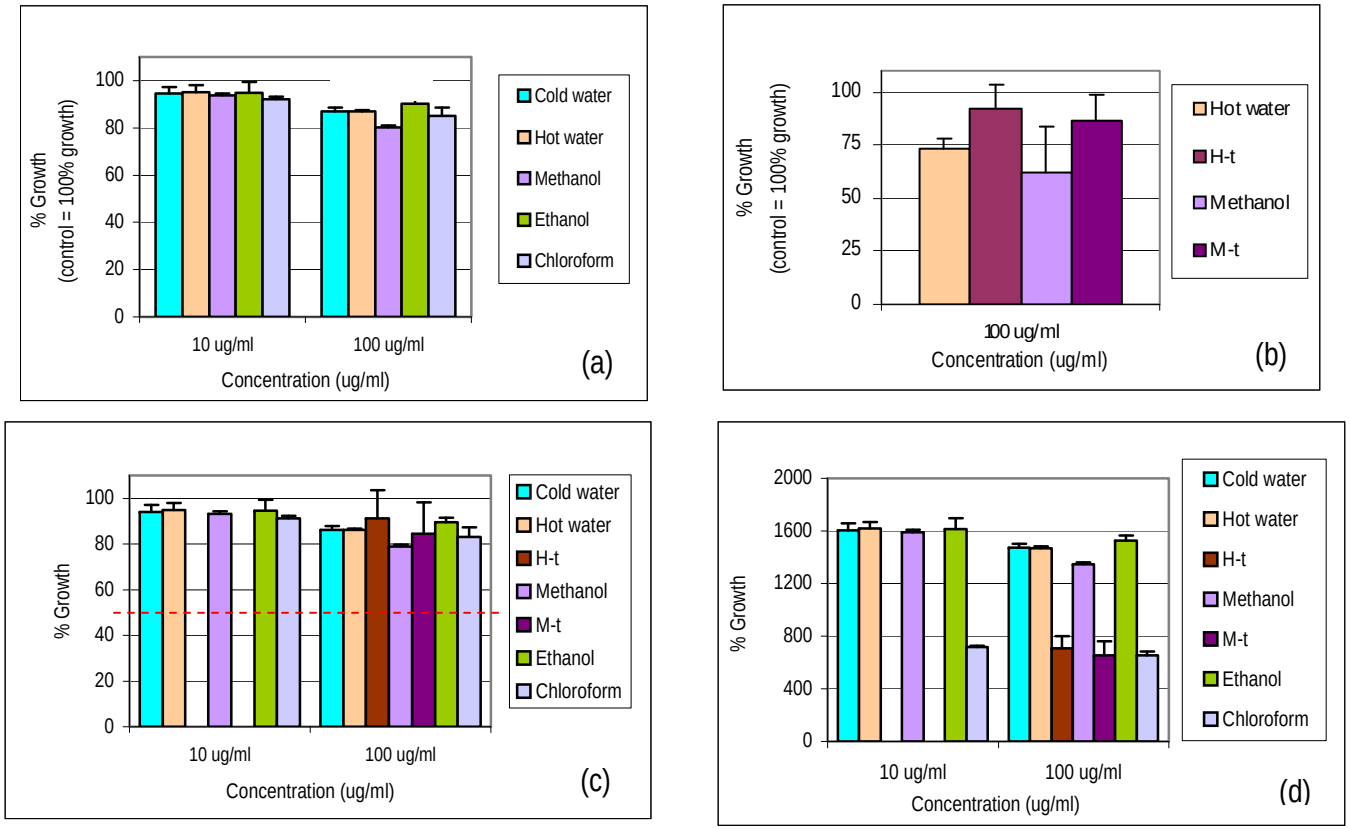

Figure 5.4. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the non-cancerous human cell line MCF-12A (breast), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values (GI_{50}) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

5.3.2 Cancerous human cell line MCF–7

The MCF-7 (breast) cancerous cells were inhibited by all plant extracts except the chloroform extract (Figure 5.5 top left). At 20 μg/ml, they were inhibited most by the H-t extract – 93% inhibition, and in decreasing order by the methanol, M-t, hot water, cold water, chloroform and ethanol extracts – 90%, 79%, 55%, 29%, 26% and 15% respectively. The effect in growth inhibition was significantly less at 10 μg/ml than 20 μg/ml for all extracts, less at 20 μg/ml than 40 μg/ml for all extracts except methanol, and less at 40 μg/ml than 60 μg/ml for all extracts except methanol and chloroform. In addition, the growth inhibition effect was significantly less at 60 μg/ml than 80 μg/ml only for the ethanol extract, and significantly less at 80 than 100 μg/ml only for the methanol extract. To simplify calculations and presentation of results, cell lines were analysed for significant differences of a specific extract's effect only for the 20, 60 and 100 μg/ml concentrations (at these concentrations the extracts without tannins were tested as well), and the difference in different extract's effect only at 100 μg/ml (where most cell lines had an effect). Growth inhibition was significantly less at the 20 μg/ml than the 60 μg/ml for the cold water, hot water, ethanol and chloroform extracts. So also, the growth inhibition was significantly less at 60 μg/ml than 100 μg/ml for the cold water, hot water, ethanol and methanol extracts. Significance is indicated by stars at the higher of the two concentrations at Figure 5.5a, as well as in Table 5.1.

At a concentration of 100 μg/ml, all leaf extracts except chloroform, inhibited growth by 90% or more. At 100 μg/ml, the difference in growth inhibition was significant between several extracts. These significant differences are indicated as dots of the same colour in Table 5.1. When the hot water and methanol extracts were tested without tannins in a separate experiment, the extracts without tannins inhibited MCF-7 cell's growth less (94.5% and 93.5%) than the extracts with tannins (98% and 93% respectively) (Figure 5b). This difference was significant for the hot

water and H-t extracts and is indicated with a star at the top of the extract without tannins in Figure 5b.

GI_{50} and LC formulas of NCI were applied and graphs drawn of resulting values; the GI_{50} values in Figure 5.5c, and the LC in Figure 5.5d. At 20 μg/ml the methanol, hot water and M-t were already below their GI_{50} values. At 100 μg/ml all the plant extracts, except chloroform, were below the GI_{50} values. The lethal concentrations were attained for all extract types except M-t.

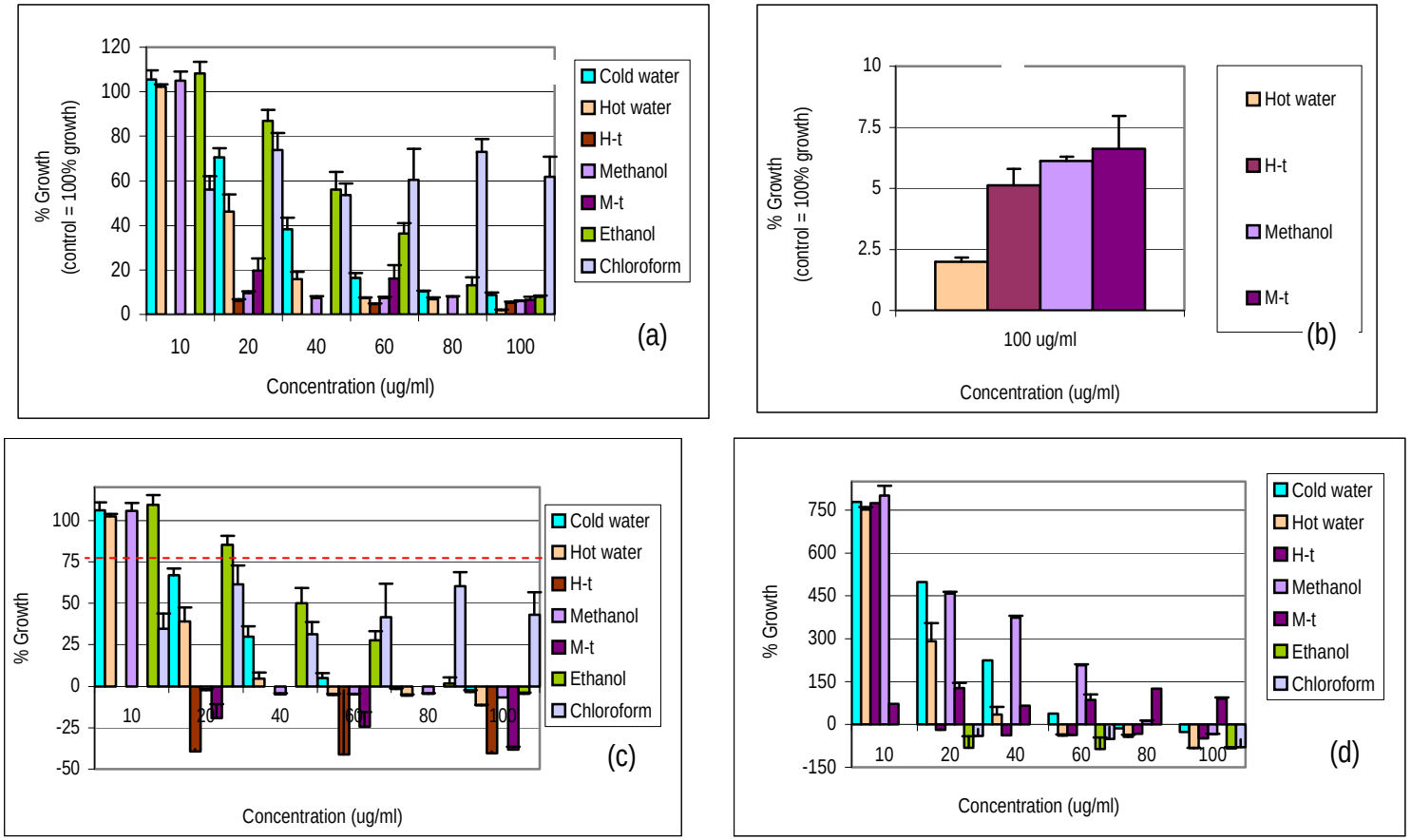

Figure 5.5. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the normal human cell line MCF-7 (breast), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values (GI_{50}) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

5.3.3 Cancerous human cell line H157

The H157 (lung) cells were least inhibited by the ethanol extract (Figure 5.6a). At concentrations of 100 μg/ml, growth was inhibited by 30% by the chloroform and 25% by the M-t extracts. The cold water and chloroform extracts inhibited growth significantly less at 20 μg/ml than 60 μg/ml, and significantly less at 60 μg/ml than 100 μg/ml. At 100 μg/ml, the difference in growth inhibition was significant for several plant extracts as indicated by dots of the same colour in Table 5.1. The hot water and methanol extracts without tannins inhibited growth more (8 and 30%) than the same extracts with tannins (no inhibition). This difference was significant for both extracts and is indicated with stars at the top of the extracts without tannins in Figure 6b.

The GI_{50} and LC formulas of NCI were applied and graphs drawn of resulting values; the GI_{50} values in Figure 5.6c, and the LC in Figure 5.6d. At 60 μg/ml, only the chloroform extract was below its GI_{50} concentration. At 100 μg/ml all the plant extracts, except H-t, M-t and ethanol, were below the GI_{50} values. The LC was not reached (to stay below zero) by any of the plant extracts in the 10 – 100 μg/ml concentration range.

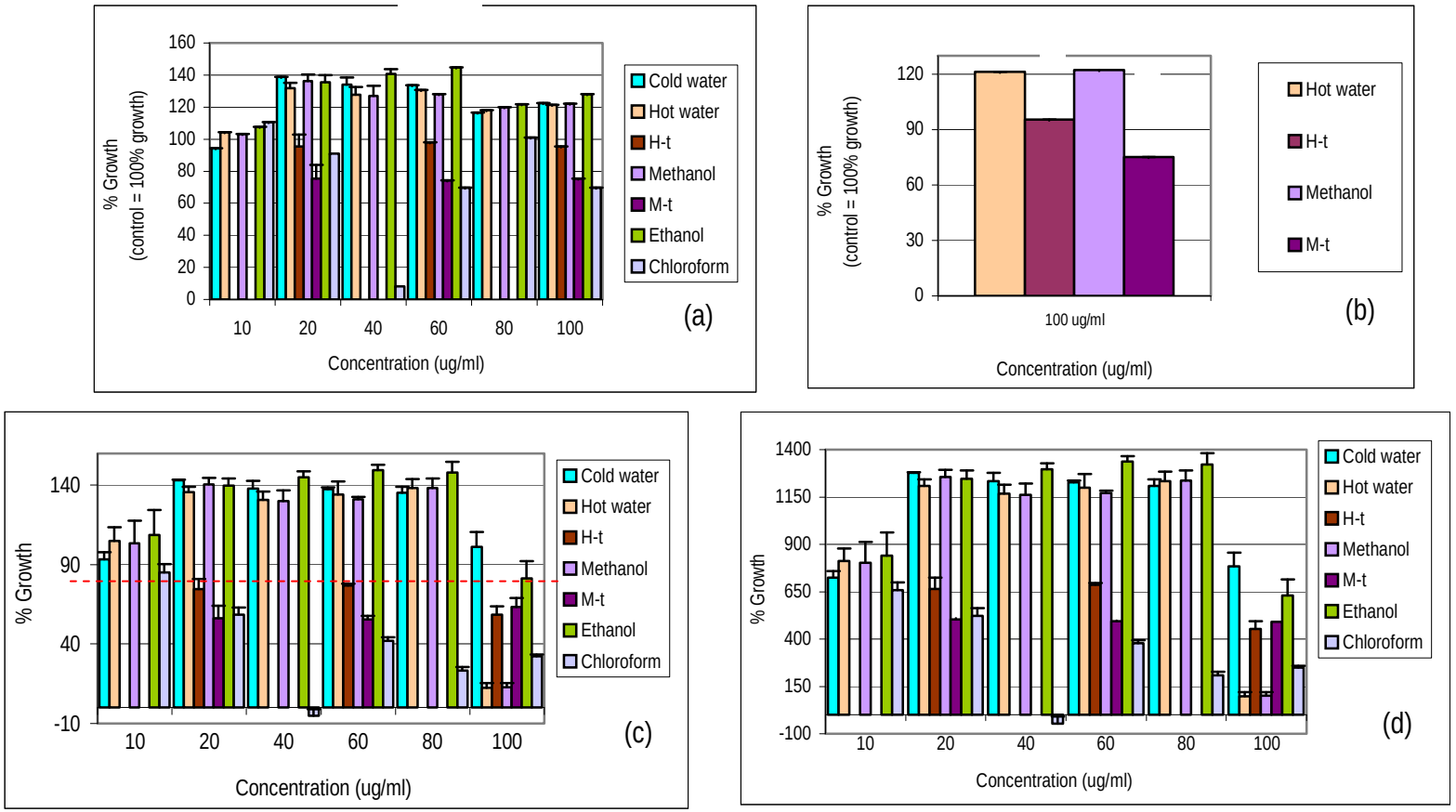

Figure 5.6. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the human cancer cell line H157 (lung), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values (GI_{50}) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

5.3.4 Cancerous human cell line $WHCO_3$

The cancerous cell line $WHCO_3$ (oesophagus), were inhibited by all plant extracts (Figure 5.7a and b). Growth inhibition was significantly less at the 20 μg/ml than at 60 μg/ml for all extracts. At 60 μg/ml, growth inhibition was significantly less than at 100 μg/ml for the cold water, hot water, methanol and chloroform extracts. Significance is indicated by stars at the higher of the two concentrations at Figure 5.7a, as well as in Table 5.1. At a concentration of 100 μg/ml, all leaf extracts except cold water, inhibited growth by 50% or more. At 100 μg/ml, the difference in growth inhibition was significant between several plant extracts as indicated by a dot of the same colour in Table 5.1. Although the hot water extract with tannins inhibited growth less (78%) than the same extract without tannins (84%) and the methanol extract inhibited growth more (75%) than the same extract without tannins (71%), this difference was not significant for a 5% confidence interval (Figure 5.7b).

GI_{50} as well as LC formulas of NCI were applied and graphs drawn of resulting values; the GI_{50} values in Figure 5.7c, and the LC in Figure 5.7d. At 20 μg/ml, the hot water, methanol, hot water and M-t extracts' growth inhibition were already below their GI_{50} values. At 80 and 100 μg/ml all the plant extracts were below the GI_{50} values. At 20 μg/ml, the hot water, methanol, and H-t have not yet attained their LC values. At 80 μg/ml all the plant extracts except methanol have not reached their LC values yet. Lethal concentrations were exhibited for all extract types except M-t at 100 μg/ml. Extracts that reach their GI_{50} values but not their LC values at a specific concentration, have the desired effect. The desired effect is for plant extracts or compounds are to inhibit cell growth, but not be toxic (lethal).

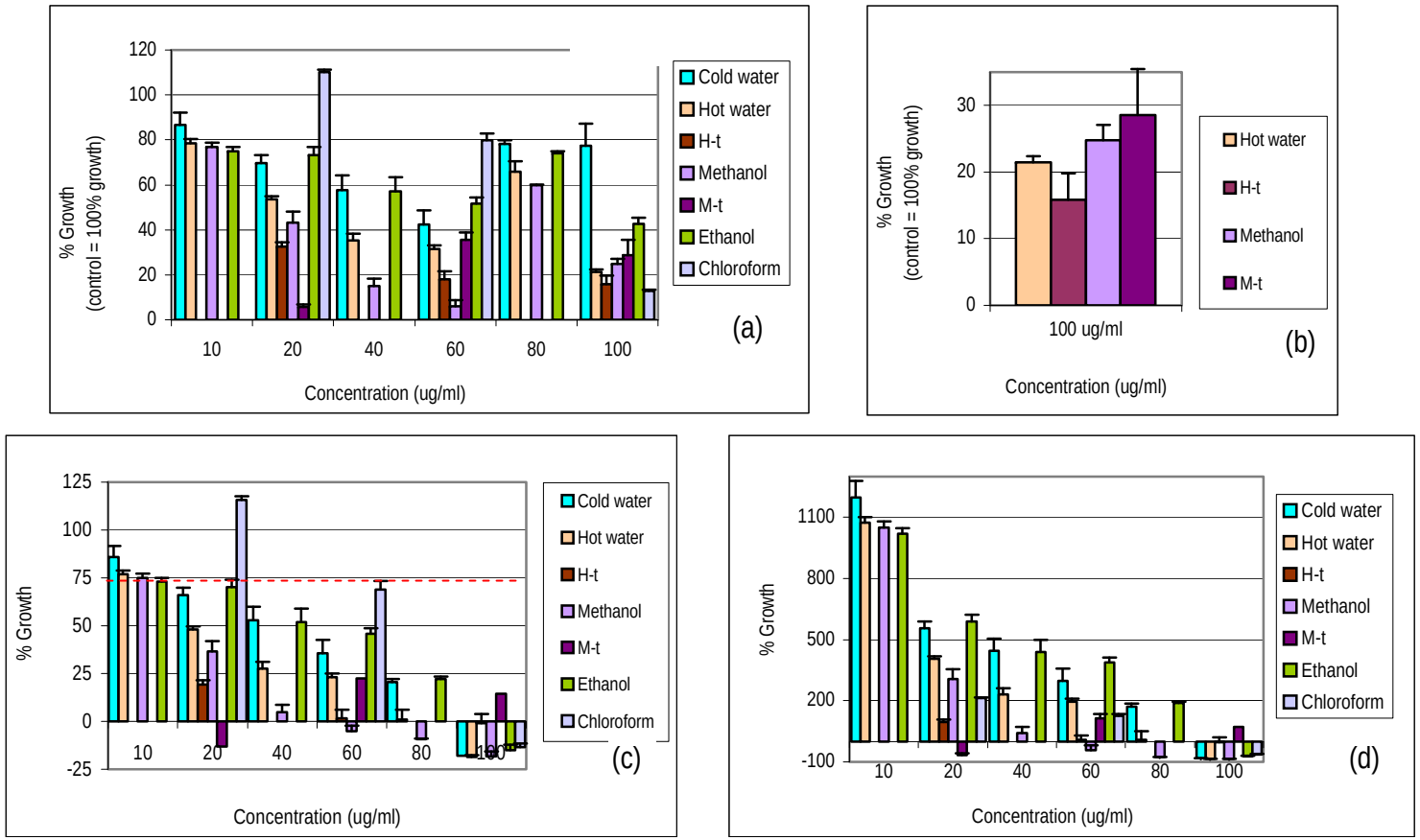

Figure 5.7. Effect of different *Pteleopsis myrtifolia* leaf extracts on the growth of the human cancer cell line $WHCO_3$ (oesophagus), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values (GI_{50}) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

5.3.5 Cancerous human cell line HeLa

The graphs of the HeLa (cervix) cell line indicated that these cancerous cells were inhibited by all extracts except chloroform (Figure 5.8a). At 60 μg/ml, all extracts except the chloroform extract, inhibited the HeLa cell line by 80% or more. All extracts, except the hot water and chloroform extracts, inhibited the HeLa cell line significantly less at 20 μg/ml than 60 μg/ml. Growth inhibition was significantly more at the 100 μg/ml than the 60 μg/ml only for the hot water and M-t extracts. Significance is indicated by stars at the higher of the two concentrations on Figure 5.8a, as well as in Table 5.1. At a concentration of 100 μg/ml, all leaf extracts except cold water, inhibited growth by 50% or more. At 100 μg/ml, the difference in growth inhibition was significant between several extracts as indicated by dots of the same colour in Table 5.1. The hot water and methanol extracts with tannins, inhibited growth more (95% and 89%) than the same extracts without tannins (84% and 41%) respectively (Figure 8b). This difference was significant for both extracts and is indicated with stars at the top of the extracts without tannins in Figure 5.8b.

The GI_{50} and LC formulas of NCI were applied and graphs drawn of resulting values; the GI_{50} values in Figure 5.8c, and the LC in Figure 5.8d. At 60 μg/ml the cold water, hot water, methanol, ethanol and H-t extracts were already below their GI_{50} values. At 100 μg/ml all the plant extracts except chloroform were below the GI_{50} values. The lethal concentrations were attained for all extract types except M-t and chloroform at 100 μg/ml.

The significant differences between the concentrations (20 and 60 μg/ml, as well as 60 and 100μg/ml) within one cell line as well as the between different plant extracts at 100 μg/ml for a specific cell line are listed in Table 5.1.

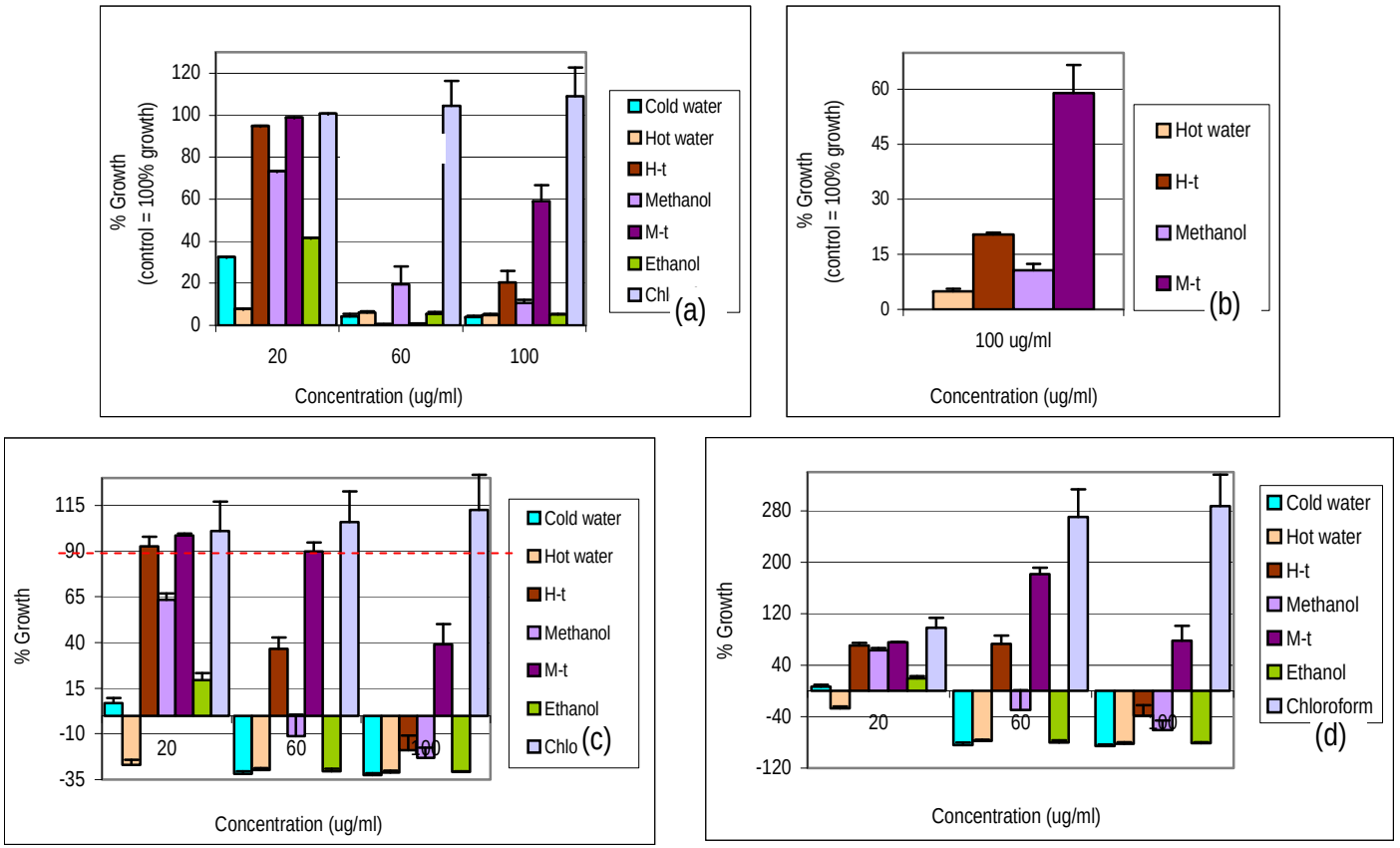

Figure 5.8. Effect of different *Pteleopsis myrtifolia* leaf extracts on growth of the human cancer cell line HeLa (cervix), for the extracts (a) cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively), and (b) hot water and methanol extracts – with and without tannins. Extracts for (c) 50% growth inhibition values (GI_{50}) and (d) lethal concentration values (LC) were: cold and hot water, methanol, ethanol, chloroform and hot water and methanol without tannins (H-t and M-t respectively).

Table 5.1. Significant differences between concentrations within one human cell line and between different leaf extracts of *Pteleopsis myrtifolia* at 100 µg/ml.

Cell line	Concentrations			Different plant extracts at 100 µg/ml						
MCF-12A	10	100		K	H	M	E	C	H-t	M-t
K				●		●●	●			
H										
M										
E										
C										
H-t										
M-t										
MCF-7	60 <	60	100	K	H	M	E	C	H-t	M-t
K				●●●	●●●●●	●●	●●	●●●●●	●●●	●●
H										
M										
E										
C										
H-t										
M-t										
H157	20	60	100	K	H	M	E	C	H-t	M-t
K				●●●●	●●●●	●●	●●●	●●●●●	●●●	●●●
H										
M										
E										
C										
H-t										
M-t										
$WHCO_3$	20	60	100	K	H	M	E	C	H-t	M-t
K				●●●●●	●●	●	●●●	●●●●	●●	●●●
H										
M										
E										
C										
H-t										
M-t										
HeLa	20	60	100	K	H	M	E	C	H-t	M-t
K				●●●	●●●	●	●●	●●●●	●●●●	●●●●●
H										
M										
E										
C										
H-t										
M-t										

K = cold water, H = hot water, M = methanol, E = ethanol, C = chloroform, H-t = hot water without tannins, M-t = methanol without tannins, ● indicate effects (growth inhibition at different concentrations) that differed significantly at a 5% confidence interval, ● indicate at which concentrations GI_{50} values, but not LC values, (the desired effect) was reached.

The log values of (the GI_{50} and LC growth inhibition values) the hot water and methanol extracts with and without tannins for the human cancer cell lines investigated, were compared with the log values of two extracts from the Combretaceae, as well two plant derived drugs, paclitaxel (taxol) and vincristine sulphate and human cell lines that were available from the NCI's trials (Table 5.2).

Table 5.2. Log values of (50% growth inhibition (GI50) and lethal concentration (LC) values) the hot water and methanol extracts with and without tannins and the human cancer cell lines MCF7, H157, $WHCO_3$ and HeLa, as well as the plant derived drugs paclitaxel (taxol) and vincristine sulphate and different human cell lines investigated by the National Cancer Institute*.

Human cell lines		MCF7 breast	H157 lung	$WHCO_3$ oesophagus	HeLa cervix
Extracts		µg/ml	µg/ml	µg/ml	µg/ml
Hot	GI_{50}	<1.30	<2.00	<1.30	<1.30
water	LC	<1.78	-	<2.00	<1.30
Methanol	GI_{50}	<1.30	<2.00	<1.30	<1.30
	LC	<1.30	-	<1.78	<1.78
H-t	GI_{50}	<1.30	-	<1.30	<1.78
	LC	<1.30	-	<2.00	<2.00
M-t	GI_{50}	<1.30	-	<1.30	<2.00
	LC	<1.30	-	-	-
Human cell lines		MCF7* breast	EKVX* non-small cell lung	K-562* leukemia	OVCAR-4* ovarian
Extracts		µg/ml	µg/ml	µg/ml	µg/ml
N10109*	GI_{50}	2.00	0.4	1.4	1.5
	LC				
N10009*	GI_{50}	1.5	-	1.4	1.2
	LC				
Drugs		µg/ml	µg/ml	µg/ml	µg/ml
Taxol*	GI_{50}	-8.0	-6.6	-8.0	-6.2
	LC				
Vincristine	GI_{50}	-9.0	-5.2	-9.0	-5.0
sulphate*	LC				

* Cell lines, extracts (form Combretaceae) and drugs marked with an asterisk are from the National Cancer Institute's trials (Screening services, 1990).

5.4 Conclusions

The growth of the cancerous cell line $WHCO_3$ was inhibited by all plant extracts, (the 20 µg/ml

had significant less growth inhibition than 60 μg/ml), the growth of cell lines MCF-7 and H157 were inhibited by all plant extracts except the M-t extract (at 20 μg/ml there was significant less growth inhibition than at 100 μg/ml), and the growth of cell line HeLa by all plant extracts except the hot water and chloroform extracts (the 20 μg/ml significantly less than 100 μg/ml).

Although Kamuhabwa *et al*. (2000) found 25-50% inhibition of growth *in vitro* for the HeLa (cervical carcinoma) at 100 μg/ml of a methanol extract, results found in this study indicated 95% and 89% growth inhibition *in vitro* for the hot water and methanol extracts. This difference could be ascribed to differences in experimental procedure or composition of plant or cell line material.

Extracts with tannins inhibited the growth of the cancer cell lines MCF-7 and HeLa more than the same extracts without tannins. For the H157 cell line, extracts without tannins inhibited the growth more than extracts with tannins. Possible explanations could be that the H157 cells were more sensitive to tannins present than the other cell lines. After the tannins were removed, the active compound(s) were free to bring about more pronounced activity. This was not the case for the other cell lines, as they were less sensitive to tannins or responded to other compounds in the extract. Another explanation could be that the tannins bound to proteins concerned in the cell cycle and caused more inhibition of growth in the MCF-7 and HeLa cell lines.

GI_{50} values were attained for all extracts at 100 μg/ml for the MCF-7 and $WHCO_3$ cell lines. All except the chloroform extract reached GI_{50} values for the HeLa cell line. No extracts exhibited GI_{50} values for the MCF-12A cell line, and only the hot water, methanol and chloroform extracts reached their GI_{50} values for the H157 cell line. LC values were attained for the cold water, hot water, methanol and ethanol extracts for the MCF-7, $WHCO_3$ and HeLa cell lines. No extracts

reached constant lethal concentration values for the cell lines H157 and MCF-12. The cell lines differed in their sensitivity to the plant extracts, as they would as well to different chemotherapeutic drugs. They were most sensitive to the hot water and methanol (polar) extracts.

Often more differentiated cell lines (tumours) (like in this case $WHCO_3$, MCF-7 and HeLa) respond better to chemotherapy than less differentiated cell lines (like in this case H157). This was also seen in the response of these cell lines to plant extracts. The more differentiated cell lines showed more growth inhibition.

The result that the non-cancerous cell line MCF-12A is not extensively inhibited by the plant extracts, indicates that the growth inhibitory effects of cell lines are not the result of a general toxicity of leaf extracts. One can also argue that the non-cancerous cells grow slower than the cancerous ones and that the cancerous ones' cell cycle will therefore be more often affected by drugs than the non-cancerous cells. Within a 48 h test period – the time that the extracts were in contact with the cells (and is also the method the NCI uses for assays) the effect on cell cycle may not have been so pronounced as with longer periods. With most extracts, the $WHCO_3$ cell line reached GI_{50} concentrations, but not LC at the 40, 60 and 80 μg/ml concentrations. Examples of other cell lines and plant extracts that reached GI_{50} but not LC, were: the HeLa cell line and the hot water extract without tannins at 60 μg/ml as well as the methanol without tannins extract at 100 μg/ml, the MCF-7 cell line and the hot water extract at 20 μg/ml, as well as the cold and hot water extracts at 40 μg/ml, the H157 cell line and the chloroform extract at 60, 80 and 100 μg/ml, as well as the hot water and methanol extracts at 100 μg/ml (indicated by ~ at the concentrations that are in Table 5.1). In above-mentioned cases the desired effect has been reached; i.e. an effect that inhibits growth but are not lethal (toxic).

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Chapter 6

Antioxidant activity of *Pteleopsis myrtifolia* leaf extracts

Abstract

Recently there has been increasing interest in free radicals in biological systems and their implied role as causative agents in the aetiology of a variety of pathological physiologies. Antioxidants' potential in preventing damage associated with free radicals and their implied role in disease has encouraged the search for compounds with potent antioxidant activity. This research investigated the antioxidant activity of *Pteleopsis myrtifolia* leaf extracts. The radical scavenger capacities of acetone, chloroform, ethanol, hot water, cold water and methanol leaf extracts were established by using a 340 ATC ELISA plate reader to measure the disappearance of the purple colour of 1,2-diphenyl-2-picrylhydrazyl (DPPH) at 515 nm. The radical scavenger activity was expressed as the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}). Compared to black tea, where one gram of dry weight, had a vitamin C equivalent of 0.126 mg, the cold water extract of *P. myrtifolia* leaves, had a vitamin C equivalent of 0.34 mg, more than twice that of black tea. Further, the vitamin C equivalent of the methanol and hot water extracts were 0.20 and 0.147 mg/g respectively. All *P. myrtifolia* leaf extracts gave positive scavenging capacity (antioxidant) with DPPH. The cold water, methanol and hot water's free radical scavenging or antioxidant activity were more than that of black tea.

6.1 Introduction

6.1.1 Antioxidants protect against oxidative damage

Human consumption of antioxidants has many health benefits, including the prevention of oxidative damage associated with free radical damage and their contribution to disease such as

cancer, the aetiology of aging, coronary heart disease, ischemia-reperfusion injury (Yang *et al*., 2001), multiple sclerosis, Parkinson's disease, senile dementia, autoimmune disorders, and asbestosis (Ng *et al*., 2000). Their protective role has prompted investigators to search for compounds with potent antioxidant activity. Oxygen, vital for the survival of the human species, is present in the atmosphere as a stable triplet biradical ($^3O_2$), in the ground state. Once inside the human body, it can be transformed by a four-electron reduction process to water, producing a super oxide radical (O_2-.), a hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2) as the reactive intermediates. Singlet oxygen ($^1O_2$) is formed from the excited state of various sensitizers (such as chlorophyll, acridine and other pigments) and primarily targets and damages cellular and extra cellular components, proteins, enzymes, lipids, DNA and RNA. It can also oxidize unsaturated fatty acid components of cell membranes (Ramarathnam *et al*., 1995). Free radicals can be described as unstable molecules with unpaired electrons that float freely through the body, seeking out completely healthy cells from with they can 'steal' electrons to rebalance themselves. This can cause a chain reaction in body cells and if left unchecked, can cause cellular damage. These unstable molecules are formed naturally in the body, through normal metabolic processes like oxidation, or they can be generated by car fumes, smoking, factory emissions, alcohol, pesticides, solvents and even from illness or infection. Antioxidant nutrients have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. Most plants have protective biochemical functions of naturally occurring antioxidants in the cells. Many secondary compounds and enzymes of higher plants have been demonstrated with *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Larson, 1988). Naturally occurring antioxidants in plant cells are:

i) enzymatic and peptide defence mechanisms (catalases, peroxidases, superoxide dismutases, glutathione and other proteins),

ii) phenolic defence compounds (vitamin E, flavonoids, phenolic acids and other phenols),

iii) nitrogen compounds (alkaloids, amino acids and amines),

iv) cartenoids, chlorophyll derivatives,

v) other compounds (vitamin C, ketones) and;

vi) synergism between more than one type of compound (for example: phenolic compounds and proteins) (Larson, 1988).

The required compounds would have potent antioxidant activity and low cytotoxicity. Antioxidants present in consumable fruits, vegetables and beverages have received considerable attention as cancer chemopreventative agents (Muktar *et al*., 1994). A study about dietary constituents as potential cancer chemoprotective agents found that resveratrol, sesamol, sesame oil and sunflower oil all had strong anti-tumour promoting effects in both *in vitro* and *in vivo* (mouse skin) models induced by a potent carcinogen. Only sesamol and resveratrol showed profound free radical scavenging activity in 1,2-diphenyl-2-picrylhydrazyl (DPPH) bioassays. The anti-oxidant capabilities of these compounds could not solely explain the observed anticancer characteristics (Kapadia *et al*., 2002). Studies on tea, the most popularly consumed beverage aside from water (associated with decreased risk of various proliferative diseases such as cancer and arteriosclerosis in humans), provided evidence that green tea catechins, in addition to their antioxidative properties, also effect the molecular mechanisms involved in angiogenesis, extracellular matrix degradation, regulation of cell death and multidrug resistance (Demeule *et al*., 2002). Apostolides (1995) found that food-borne carcinogens (produced during food preservation and preparation) were inhibited by tea polyphenols. Research (cell culture and animal models) found that tea's anti-carcinogenic activity was mediated through a range of mechanisms including antioxidant activity, enzyme modulation, gene expression, apoptosis, up regulation of gap junction communication and P-glycoprotein activation (Duthie *et al.*, 2000). A single cup of black or green tea induces a significant rise in the plasma antioxidant activity *in vivo* (Leenen *et al*., 2000).

The use of plant extracts to combat infections or illnesses may be explained by the presence of antimicrobial compounds, but there is also another explanation. If plant extracts contain antioxidant activity, it may clear-up free radicals from the body, and boost the immune system to handle the infection or illness effectively. In the previous chapters 3 and 4, it was shown that *P. myrtifolia* extracts have antibacterial activity.

The aim of research described in this chapter, was to investigate antioxidant activity in extracts from *Pteleopsis myrtifolia* leaves. Free radical scavenging activity was detected with stable DPPH and colour formation (Pezzuto *et al*., 1999).

6.2 Material and methods

6.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

6.2.2 Preparation of plant extracts

Because antioxidant compounds are frequently highly polar compounds, four polar: ethanol, methanol, cold water, and boiling water, as well as an extractant of intermediate polarity: chloroform, were selected. The extraction method described in 2.2.2 of Chapter 2 was used. Yield was determined and the dried extracts (from the extractants were redissolved in methanol. For the DPPH antioxidant assays and the dot-blot DPPH staining procedures, a final concentration of 10 mg/ml of each extractant was prepared by redissolving the dried extract in acetone. For the spectrophotometric assays, each extract was redissolved in methanol. A stock concentration of 1000 mg/ml was prepared from which dilutions were made. The prepared extracts were stored in a tightly sealed dark glass containers at 5 ºC. The extracts were prepared the day previous to the DPPH colour measurements.

6.2.3 DPPH qualitative antioxidant assay

10 µl aliquots of each of the cold water, hot water, ethanol and methanol extracts (10 mg/ml) were applied to Merck Silica gel F_{254} plates. The plates were developed with the chloroform: ethyl acetate: formic acid (5:4:1) (CEF) and ethyl acetate: methanol: water (40:5:4.4) (EMW) eluent systems and sprayed with a 0.4 mM DPPH solution in methanol. This technique indicates how many antioxidant compounds are present.

6.2.4 Dot-blot DPPH staining procedure

According to Soler-Rivas *et al*. (2000), the dot-blot test is an easy, fast and reliable way to compare radical scavenging capacity (RSC) of various food products (excluding oils). This assay was used to establish whether different extracts of *P. myrtifolia* leaves (acetone, chloroform, ethanol, hot water, cold water and methanol) had radical scavenging activity. All the extracts were dried and redissolved in acetone. Aliquots of 5 µl (of a 10 mg/ml final concentration) of each extractant were applied on Merck Silica gel F_{254} plates and allowed to dry for a few minutes. Drops of each sample were placed in a row. The sequence was according to decreasing quantity: 20 µg (bottom row), 10 µg (middle row) and 5 µg (top row). The top four spots were applied in their original extractant. The staining of the TLC plates was done according to the method of Takao *et al*. (1994) with modifications. A 0.4 mM DPPH solution in methanol was sprayed on the plates until they were evenly covered.

6.2.5 Spectrophotometric assays

Extracts of *P. myrtifolia* leaves were prepared with the different extractants (cold water, hot water, ethanol, methanol and chloroform). Not all of the chloroform extract could be redissolved. The 1 g/ml concentration was used as a stock concentration for each extractant and series of dilutions were made separately for each extract. Vitamin C was prepared as a 1

mg/ml solution by adding boiling hot water then filtering it. Black tea was prepared separately as 10 mg/ml by placing 1 g of black tea in 70% ethanol for an hour (Du Toit *et al.*, 2001).

A 96-multi-well plate (MWP) was used as an experimental unit to lie out the different concentrations in triplicate. 180 µl of AR-grade 100% methanol was placed in each well and 20 µl of each sample to be tested was added. 50 µl of a 90 µM solution of DPPH in methanol was added to each well. The plate was covered with aluminium foil and left to stand at room temperature for 1 h before spectrophotometer readings were made.

6.2.6 Spectrophotometer readings

The radical scavenging capacities of the samples were established by using a 340 ATC ELISA plate reader to measure the disappearance of purple colour of DPPH at 515 nm. The radical scavenging activity was expressed as the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Du Toit *et al.*, 2001). The EC_{50} values of the standards and samples were determined and plotted as the percentage disappearance of DPPH as a function of the sample concentration in µg/ml for the standard and mg/ml for the samples.

6.2.6.1 Calculation as a percentage:

The EC_{50} is the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%. The results are expressed as the mg vitamin C equivalents/ g dry weight and are calculated as follows:

EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = X mg vitamin C equivalents/ g dry weight

Zero mg/ml were taken as 100%.

6.3 Results and discussion

6.3.1 DPPH antioxidant assay

Where the Merck silica gel F_{254} TLC plates were sprayed with a DPPH solution in methanol, the regions where substances with antioxidant capacity occurred stained yellow and the remainder of the plate stained purple. Figure 6.1 shows TLC plates of the fractions of liquid-liquid separation cold water, hot water, ethanol and methanol extracts of *P. myrtifolia* leaves developed with the CEF and EMW eluent systems and sprayed with a 0.4 mM DPPH solution in methanol.

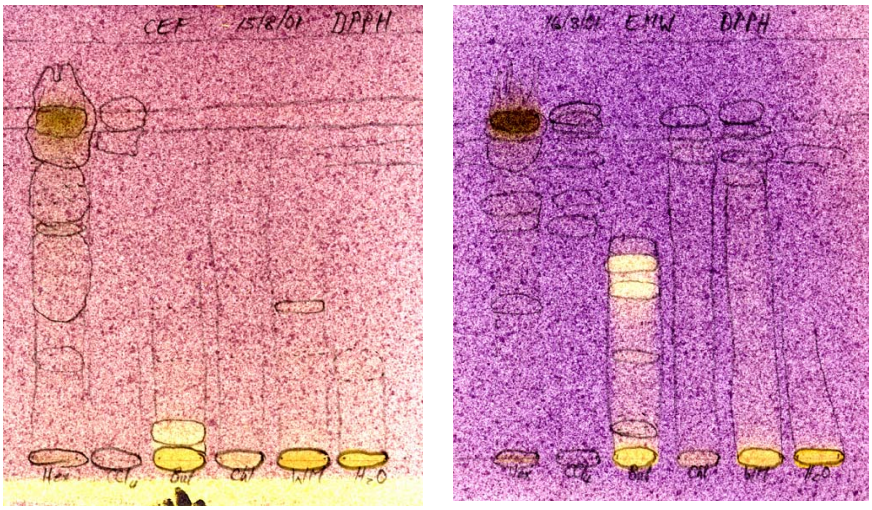


Figure 6.1. Chromatograms of thin layer chromatography plates with fractions from liquid-liquid separation (Hex = *n*-hexane, CCl_4 = carbon tetrachloride, But = *n*-butanol, Chl = chloroform, WM = 35 % water in methanol, H_2O = water) developed with the CEF (left) and EMW (right) eluent systems and sprayed with a 0.4 mM DPPH solution in methanol. (CEF = chloroform: ethyl acetate: formic acid (5:4:1), EMW = ethyl acetate: methanol: water (40:5:4.4), DPPH = 1,2-diphenyl-2-picrylhydrazyl).

6.3.2 Dot-blot DPPH staining procedure

The results of the dot-blot assay showed coloured spots where the aliquots of different extracts were placed in rows. The purple area on the plate indicates no free radical scavenging (antioxidant) activity and the yellow areas indicate free radical scavenger or antioxidant activity.

The more intense the yellow colour, the greater the antioxidant activity is (Figure 6.2). The yellow colour can be masked by the green of chlorophyll.

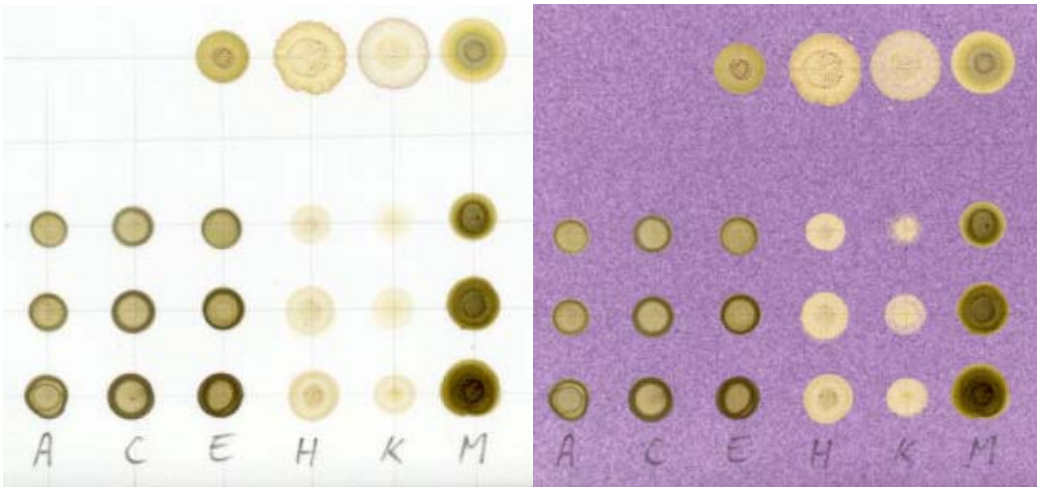


Figure 6.2. Scan of a dot-blot test of a thin layer chromatography (TLC) plate sprayed (right) with a 0.4 mM solution of 1,2-diphenyl-2-picrylhydrazyl in methanol after extracts A, C, E, H, K, and M was applied (A = acetone extract, C = chloroform extract, E = ethanol extract, H = hot water extract, K = cold water extract and M = methanol extract). The dot blots were 20 μg (bottom row), 10 μg (middle row) and 5 μg (top row). The top four spots were dots applied in their original extractant as a 20 μg quantity. Left TLC plate not sprayed yet.

6.3.3 Spectrophotometer readings

The different extract's colour reactions with DPPH in the MWPs were measured by a multiwell plate reader (Figures 6.3 – 6.5).

Vitamin C

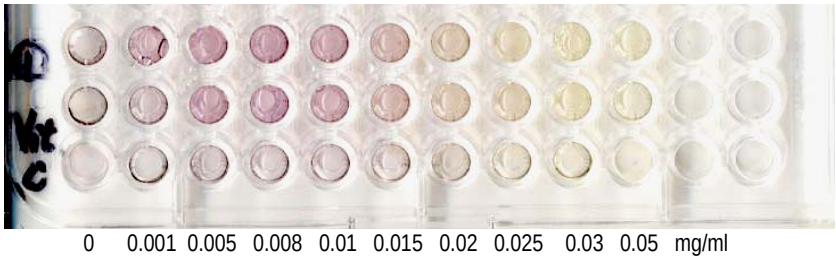


Figure 6.3. Part of a 96-Multiwell plate, showing the gradual colour change with the vitamin C at different concentrations, one hour after addition of 1,2-diphenyl-2-picrylhydrazyl (DPPH).

Ethanol extract (E)

Cold water extract (K)

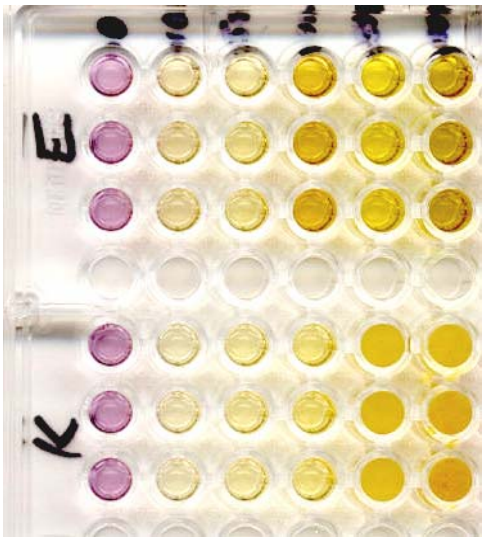


Figure 6.4. Part of a 96-Multiwell plate, showing the colour reaction after addition of 1,2-diphenyl-2-picrylhydrazyl (DPPH) to the initial concentrations (< 50 mg/ml) of cold water and ethanol extracts of *P. myrtifolia* leaves.

Colour formation with DPPH is indicative of a balance between antioxidants and free radicals. Yellow is indicative of antioxidants in excess (and that the concentration of the plant extracts is too high, like in the right hand side of Figure 6.4) and pink of free radicals in excess (and that the concentration of the plant extracts is too low). Therefore a concentration range is sought where the yellow colour just disappears or becomes translucent before pink appears.

Chlorofrom Extract (C)

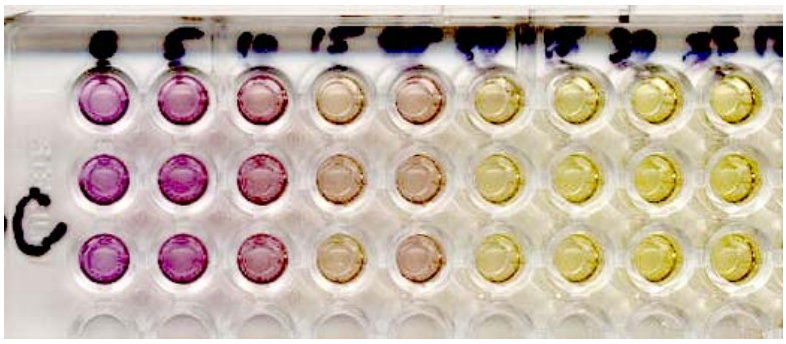
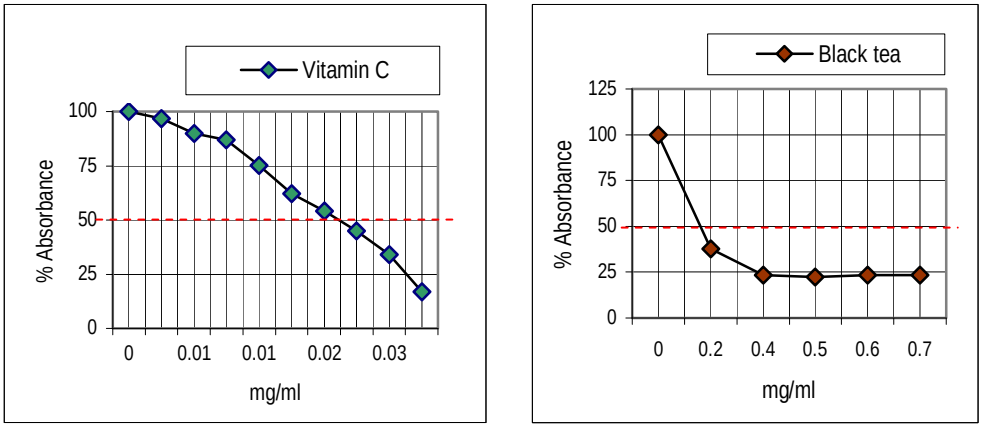


Figure 6.5. Part of a 96-Multiwell plate, showing the colour formation after 1,2-diphenyl-2-picrylhydrazyl (DPPH) was added to the chloroform extract of *P. myrtifolia* leaves.

The colour reaction shows a gradual change from yellow to pink and indicates that the optimum concentration range has been reached.

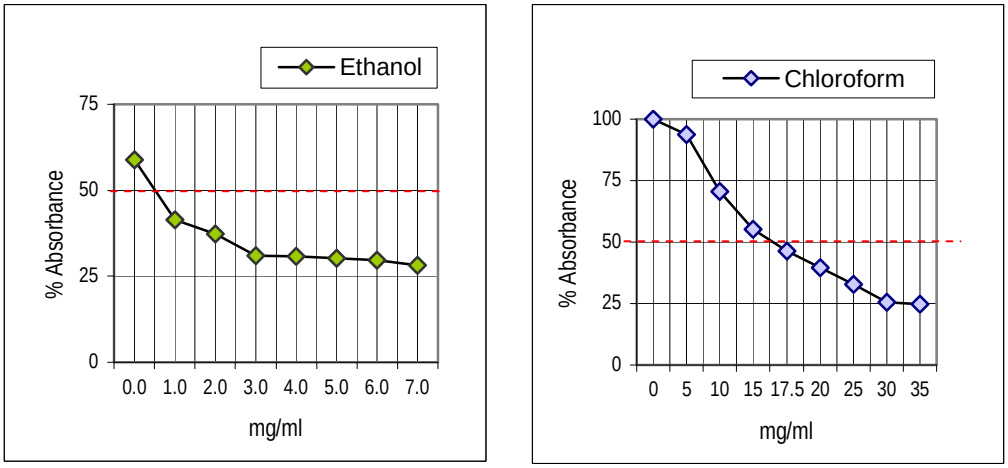
Results of the different extracts are shown in Figures 6.4 and 6.5 and the results that were of the same magnitude were combined and are shown in Figure 6.6. Custom error bars (calculated from three repetitions) are present on all graphs.

The % absorbance of all the graphs (Figures 6.6-6.8) decreased as the amount of extract or Vitamin C increased.



(a) (b)

Figure 6.6. Graphs of (a) vitamin C and (b) black tea plotted against absorbance as measured spectrophotometrically by the colour of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), which gives an indication of free radical scavenger or antioxidant activity.



(c) (d)

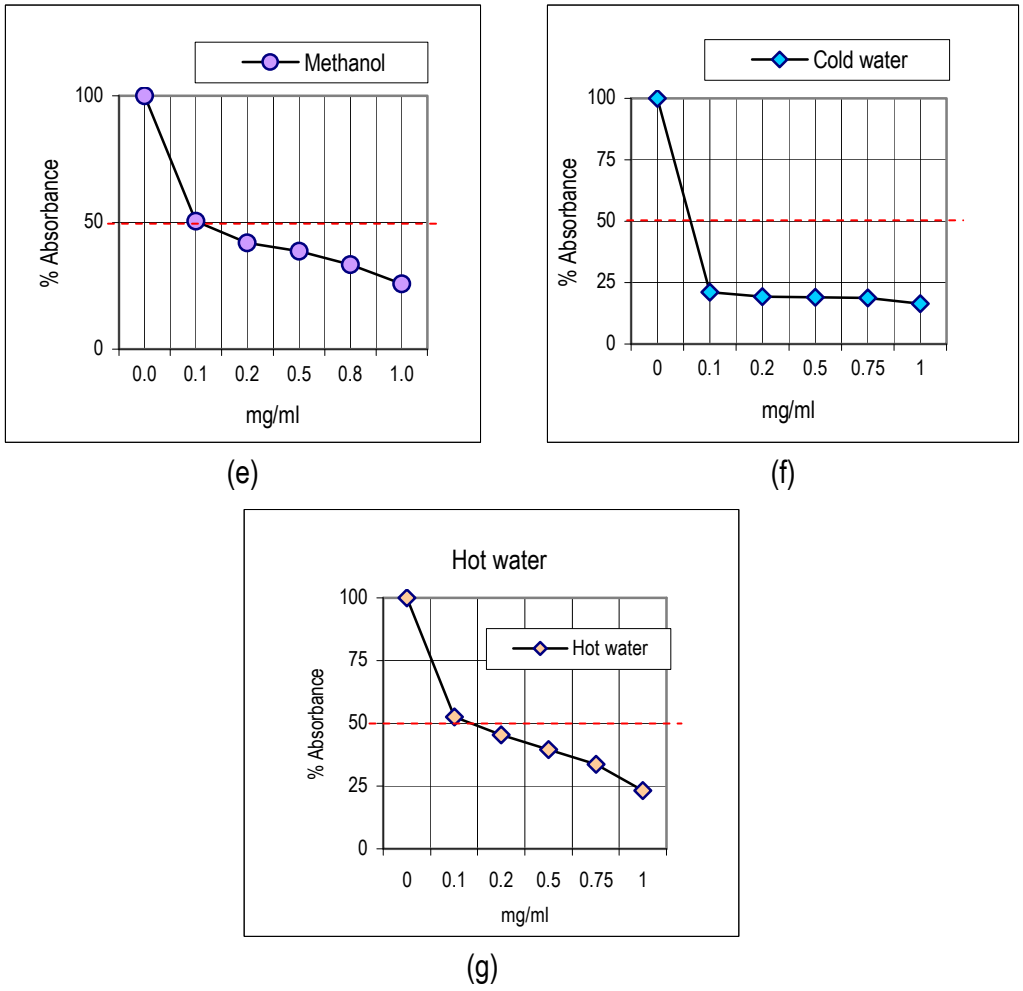


Figure 6.7. Graphs of different *Pteleopsis myrtifolia* leaf extracts (c) ethanol (d) chloroform (e) methanol (f) cold water and (g) hot water plotted against absorbance as measured spectrophotometrically by the colour of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), which indicates free radical scavenger or antioxidant activity.

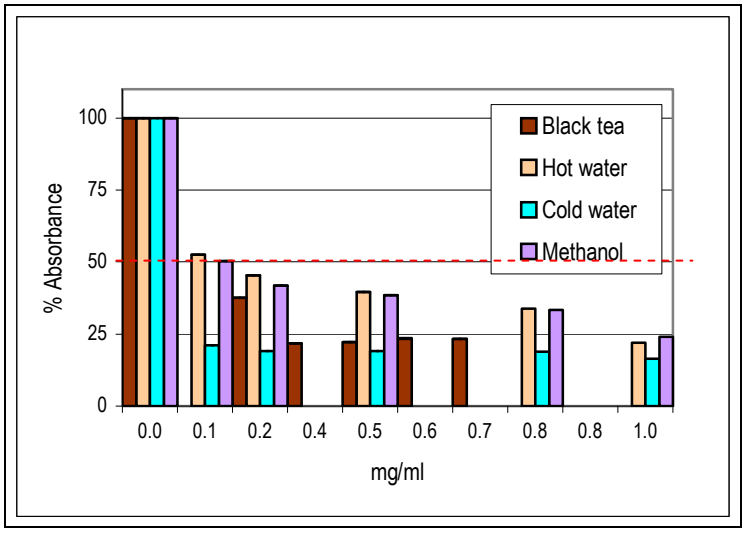


Figure 6.8. Graph of black tea and *Pteleopsis mytifolia* leaf extracts (hot water, cold water and

methanol) plotted against absorbance as measured spectrophotometrically by the colour formation of added 1,2-diphenyl-2-picrylhydrazyl (DPPH), indicating free radical scavenger or antioxidant activity. Error bars for a 5% confidence interval are present.

Expressing plant extracts' antioxidant activity in mg vitamin C equivalent has the benefits that the antioxidant activity is quantified and different plant extracts are comparable. The EC_{50} values (the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%) were calculated (EC_{50} Vit C mg/ml/EC_{50} sample (g/ml) = X mg vitamin C equivalents/ g dry weight) and are listed in Table 6.1.

Table 6.1. EC_{50} values for the different *P. myrtifolia* leaf extracts and black tea (Vitamin C's EC_{50} = 0.22 mg/ml).

Extracts	Mg vitamin C equivalents/ g dry weight (EC_{50} value)
Black tea	0.126
Ethanol	0.0138
Chloroform	0.00135
Methanol	0.20
Cold water	0.34
Hot water	0.147

Standard deviation values (present as error bars on graphs) were all ≤ a 1.3% confidence interval.

6.4 Conclusions

The antioxidant activity of the different extracts differed. Compared to black tea, where one gram of dry weight, had a vitamin C equivalent of 0.126 mg, the cold water extract of *P. myrtifolia* leaves, had a vitamin C equivalent of 0.34 mg, more than twice that of black tea. Further, the vitamin C equivalents of the methanol and hot water extracts were both more than

that of black tea, 0.20 and 0.147 mg/g respectively. All *P. myrtifolia* leaf extracts gave positive scavenging capacity (antioxidant) with DPPH.

With these results, it should be taken into account that the *in vitro* free radical scavenging potential of a substance or extract is related to its chemical properties in the medium tested and doesn't necessary reflect the *in vivo* activity.

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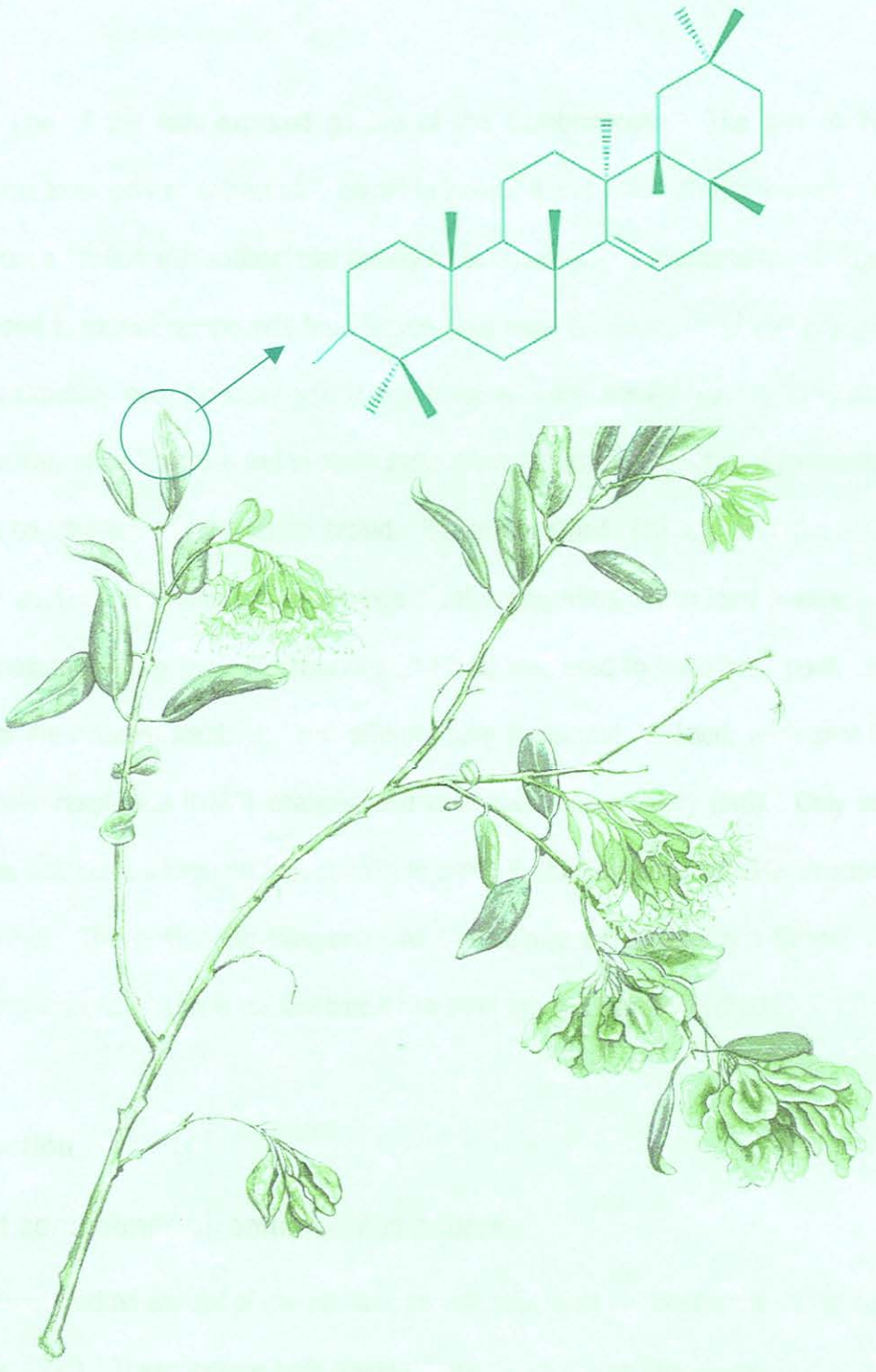
Chapter 7



Chapter 7

Column chromatography and isolation of pure compounds from *Pteleopsis myrtifolia*

Abstract

Pteleopsis is one of the less explored genera of the Combretaceae. The aim of this investigation was to simplify an extract of *P. myrtifolia* leaves, thereby facilitating the isolation of compounds from a fraction with antibacterial activity by bioassay-guided fractionation. Acetone was initially used to extract compounds from leaves (that were previously dried and ground). Liquid-liquid extraction and bioassay-guided fractionation were carried out to determine antibacterial activity of the fractions and to purify those that had activity. The chloroform fraction was found to be inhibitory to all bacteria tested. It was quantified (10.38g) and chromatographed over a silica gel column, using a range of solvent mixtures, starting with hexane and ending with methanol. Thin layer chromatography (TLC) was used to investigate purity and composition of the different fractions. The different pure compounds isolated, were sent for nuclear magnetic resonance (NMR) analysis, and later mass spectrometry (MS). Only one compound was isolated in a large enough quantity to clarify the two-dimensional NMR structure: 14-Taraxeren-3-ol. This pentacyclic triterpene was first isolated by Burrows and Simpson in 1938 from *Taraxacum* root. This is the first time it has been isolated from P. *myrtifolia*.

7.1 Introduction

7.1.1 Plant compounds as antimicrobial sources

Plants have developed an arsenal of chemicals to survive attacks by microbial invasion (Grayer and Harborne, 1994). These include both physical barriers as well as chemical ones, i.e. the

presence or accumulation of antimicrobial metabolites. These metabolites are either preformed in the plant (prohibitins) or induced after infection (phytoalexins). Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as 'antibiotics' formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors (Martini, 2002). When infection or damage to a plant takes place, a number of processes are activated and some of the compounds produced become activated immediately whereas phytoalexins are produced after two to three days. Sometimes it is difficult to determine whether the compounds are phytoalexins or prohibitins especially as the same compound may be a preformed antimicrobial substance in one species and a phytoalexin in another. The chemical classes in which these substances can be found vary greatly (Cowan, 1999).

In recent studies, several antibacterial compounds were isolated in the Combretaceae plant family, some for the first time in this family. One of these studies reported the isolation of the stilbene 2',3,4-trihydroxyl,3,5,4'-trimethoxybibenzyl (combretastatin B5) from *C. woodii*. This is the first report of antimicrobial activity of combretastatin B5 (Famakin, 2002).

Pteleopsis myrtifolia Engl. et Diels (Combretaceae) is distributed in KwaZulu-Natal and Limpopo (in South Africa), as well as in Botswana, Zambia, Malawi, Tanzania and other parts of Africa. In West Africa, a decoction of *P. hylodendron* 's root is popularly used for venereal diseases (Ngounou *et al.*, 1999). In folk medicine, *P suberosa* is used for its antiulcer properties (De Pasquale *et al.*, 1995). A decoction of the bark is taken orally three times a day; or sometimes the powdered bark is chewed and swallowed. The methanolic extracts had antimicrobial activity against some microorganisms that are responsible for skin infections (*Staphylococcus aureus*, *S. capitatis*, *S. epidermidis*, *S. saprophyticus*, *Bacillus subtilus*, *Pseudomonas aeruginosa*, *P. cepacia*, *Cochlospermum tinctorium* but not against *Escherichia coli*, *Proteus vulgaris* and *P. mirabilis*). The antibacterial activity ascertained, might justify the

traditional use of these plants in folk medicine for treatment of skin diseases (Bisignano *et al.*, 1996).

Antibacterial activity in leaf extracts from *P. myrtifolia* was described in Chapter 3. An acetone extract of *P. myrtifolia* leaves was separated into fractions (by group fractionation) of which the antibacterial activity was determined (Chapter 4). The chloroform fraction (which showed antibacterial activity against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli*) was chosen for further investigation. The aim of this investigation was to isolate antibacterial compounds from the chloroform fraction via column chromatography, and to elucidate the structure thereof by nuclear magnetic resonance (NMR) and mass spectrometry (MS).

7.2 Materials and Methods

7.2.1 Plant material

Plant material was collected and prepared as described in 2.2.1 of Chapter 2.

7.2.2 Column chromatography

The chloroform fraction of a liquid-liquid separation of *P. myrtifolia* leaves (Chapter4) was found to have antibacterial activity against *Staphylococcus aureus* American Type Culture Collection [ATCC29213], *Enterococcus faecalis* [ATCC29212], *Pseudomonas aeruginosa* [ATCC 25922] and *Escherichia coli* [ATCC 27853].

This fraction was dried, quantified (10.38 g) and placed on a silica gel column. The column was packed according to the ratios (120-80 g/1 g plant material) described by Houghton and Raman (1998) and 8 g silica gel was used for each gram dry plant material. The silica gel was weighed, wettened with hexane, poured into a glass column and left overnight to settle. Elution

of the column was started with 100% *n*-hexane. 5% chloroform was added to the *n*-hexane, then 10%, followed by 20%. The percentage of chloroform was gradually increased to 100%. Methanol was added to the eluent (chloroform) in the same manner as chloroform was added to *n*-hexane, with gradual increments. At an eluent mixture of 40% methanol (and 60% chloroform), silica gel started eluting as well and the column was stopped. Each fraction's original volume, which varied between 100 and 400ml, was documented.

The fractions were air dried at room temperature. As they became more concentrated, two layers formed, often with slightly different colours. The two layers were separated into separate containers and tested for pure compounds on TLC plates. Thereafter, the dried fractions were each washed in hexane, methanol and chloroform and each fraction obtained from these washes were tested on TLC plates again. In total, there were 93 fractions. Since most of these were each separated into two sub fractions, approximately 186 test tubes were dried and each washed with hexane, methanol and chloroform. Crystals from test tubes were also separated according to where they dried, on the sides or bottom of the tubes, and kept in separate tubes. Each of these approximately 558 tubes' contents was tested for purity by TLC. Test tube washes that had single bands and the same R_f values were combined. These gave two pure compounds, which were dissolved in deuterated chloroform and sent for analysis with nuclear magnetic resonance (NMR). NMR data were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard.

From chromatograms, fractions with similar separation patterns on TLC plates (more than one band) were recombined to form 13 recombined fractions (Figure 7.3). Each of these was each run on a smaller Sephadex LH-20 column with the same eluents as the silica gel column. The fractions from Sephadex columns were again washed with hexane, methanol and chloroform and tested for its composition with TLC.

From the smaller Sephadex columns, fractions or fraction combinations that gave single bands (representing possible pure compounds), 17 in total, were sent for NMR analysis.

A second crude column was run from 500mg of dried leaf material. This time, Sephadex G 100 was used as a packing material with the objective to obtain a larger amount of pure compound in the fractions. The Sephadex column was eluted with acetone and gradually methanol. The 31 fractions were recombined into 10 fractions and each developed again in smaller Sephadex columns.

Tables were drawn from ^{13}C NMR results and compared to other natural compounds described in literature. The pure compounds were sent for mass spectrometry.

7.3 Results and Discussion

7.3.1 First crude column

As the column's elution started, compounds started to be separated and moved down the column at different rates (Figure 7.1).

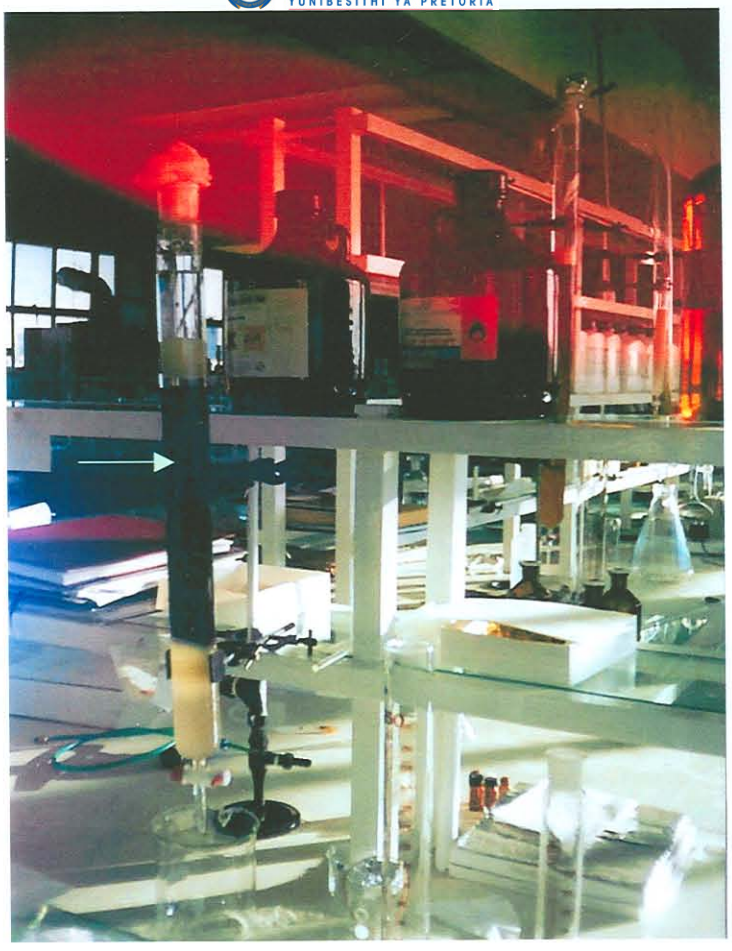


Figure 7.1. Photo of a silica gel column while eluting the chloroform fraction of *Pteleopsis myrtifolia* leaves with a mixture of hexane and chloroform (4:6).

None of the original column fractions were pure. Some of the *n*-hexane, methanol and chloroform washes (of each fraction) were pure.

When a thin layer chromatogram of every tenth fraction from the column fractions was developed, one could see that the non-polar fractions were less complex than the ones of intermediate polarity and the polar ones. The fractions of intermediate polarity and polar ones, gave several bands with TLC (Figure 7.2).

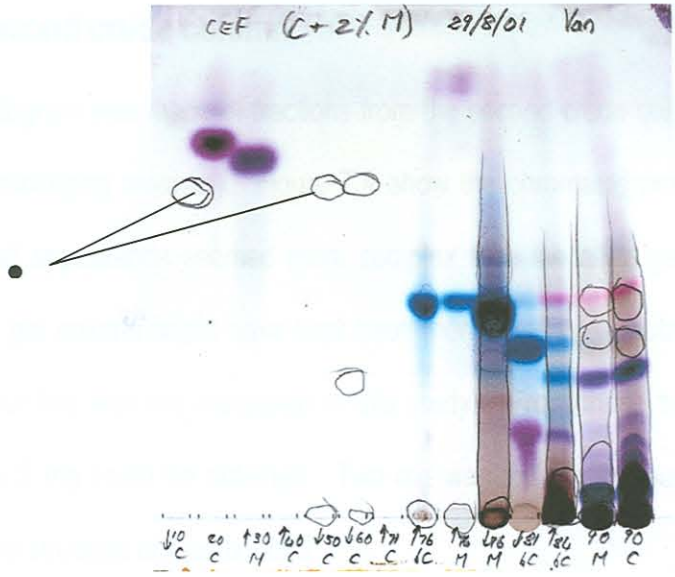


Figure 7.2. Thin layer chromatogram of very tenth fraction of column, sprayed with 0.34% vanillin in 3.5% sulphuric acid in methanol. ● = Bands that did not stain with the vanillin spray.

The 13 recombined fractions (Figure 7.3) each given an alphabet number; from left (non-polar) to right (polar), they were: E, D, F, G, H, I, J, K, L, M, N, C, B, A.

Figure 7.3. Thin layer chromatogram of the 13 combined fractions of *Pteleopsis myrtifolia* leaves, sprayed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

7.3.2 Second crude column

A chromatogram was made of fractions from the second crude column, (where Sephadex was used as packaging material). Figure 7.4 show the chromatogram after sprayed with vanillin. The overall appearance seemed more complex than the silica gel column's chromatograms. The silica gel column might have kept back more hydrophilic substances than the Sephadex column (but this was not measured in this study). From these fractions, no pure compound more than 2 mg could be obtained. Two mg was too small a quantity to determine the true identity and structure of a compound.

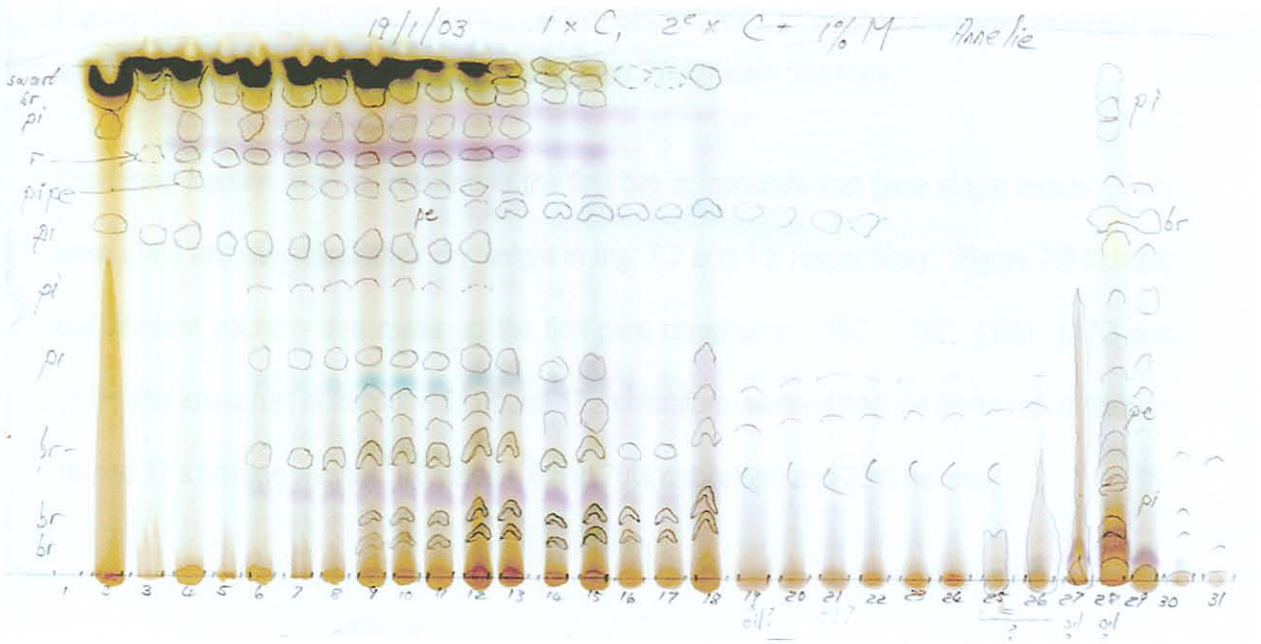


Figure 7.4. Thin layer chromatogram of fractions of second crude column (Sephadex), sprayed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

7.3.3 Pure and recombined fractions

The lower half of fraction 15, 16 and 17's chloroform washes and the lower half of fraction 16 and 17's hexane washes gave single bands with a R_f value of 0.2. So also, the upper half of fraction 31 and 32's chloroform washes gave single bands as well, with an R_f of 0.88 (Figure 7.5).

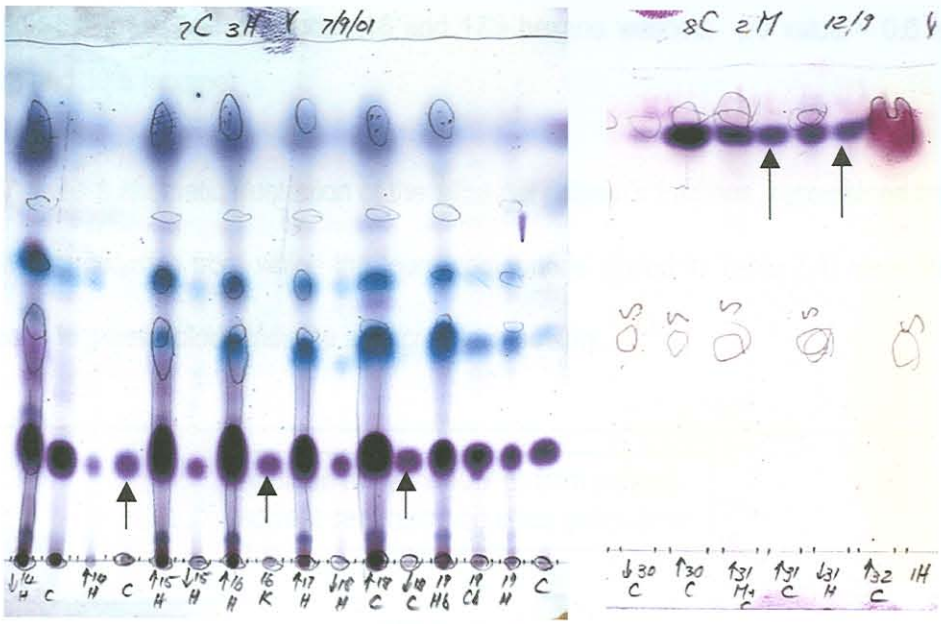


Figure 7.5. Thin layer chromatograms where single bands of washed fractions (indicated by arrows) of compound 1 (ar1) (left) and compound 2 (ar2) were first seen.

Combined fraction washes, resulted in the first two compounds that gave single bands. They were dried and quantified; their dry weight in mg: 7.7 and 1.8 respectively. Figure 7.6 indicate the different fractions that made up the first pure compound: ↓15C, ↓16C, ↓16H, ↓17C and ↓17H (the lower half of fraction 15, 16 and 17's chloroform washes and the lower half of fraction 16 and 17's hexane washes. (R_f value = 0.6 in 70% chloroform and 30% hexane)

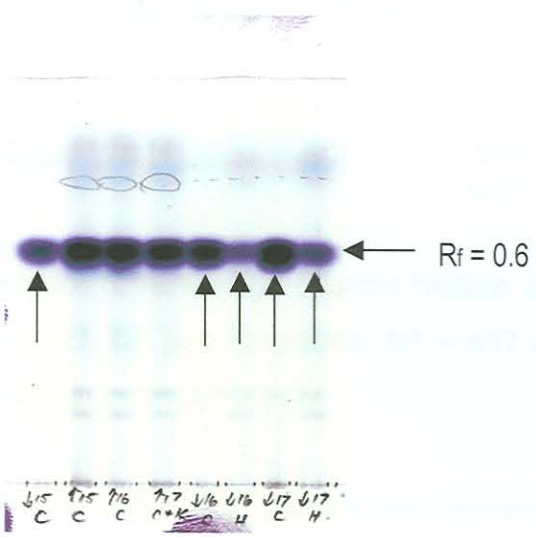


Figure 7.6. Chromatogram of bota ar**1**, developed from column fractions that were washed: ↓15C, ↓16C, ↓16H, ↓17C and ↓17H (the lower half of fraction 15, 16 and 17's chloroform

washes and the lower half of fraction 16 and 17's hexane washes. (R_f value = 0.6 in 70% chloroform and 30% hexane)

Figure 7.7 gives a shematic illustration of the silica gel column's fractions, recombined fractions and Sephadex columns from which the 'pure compounds' (listed in Table 7.1) were isolated. The increase in green colour, indicate an increase in polarity.

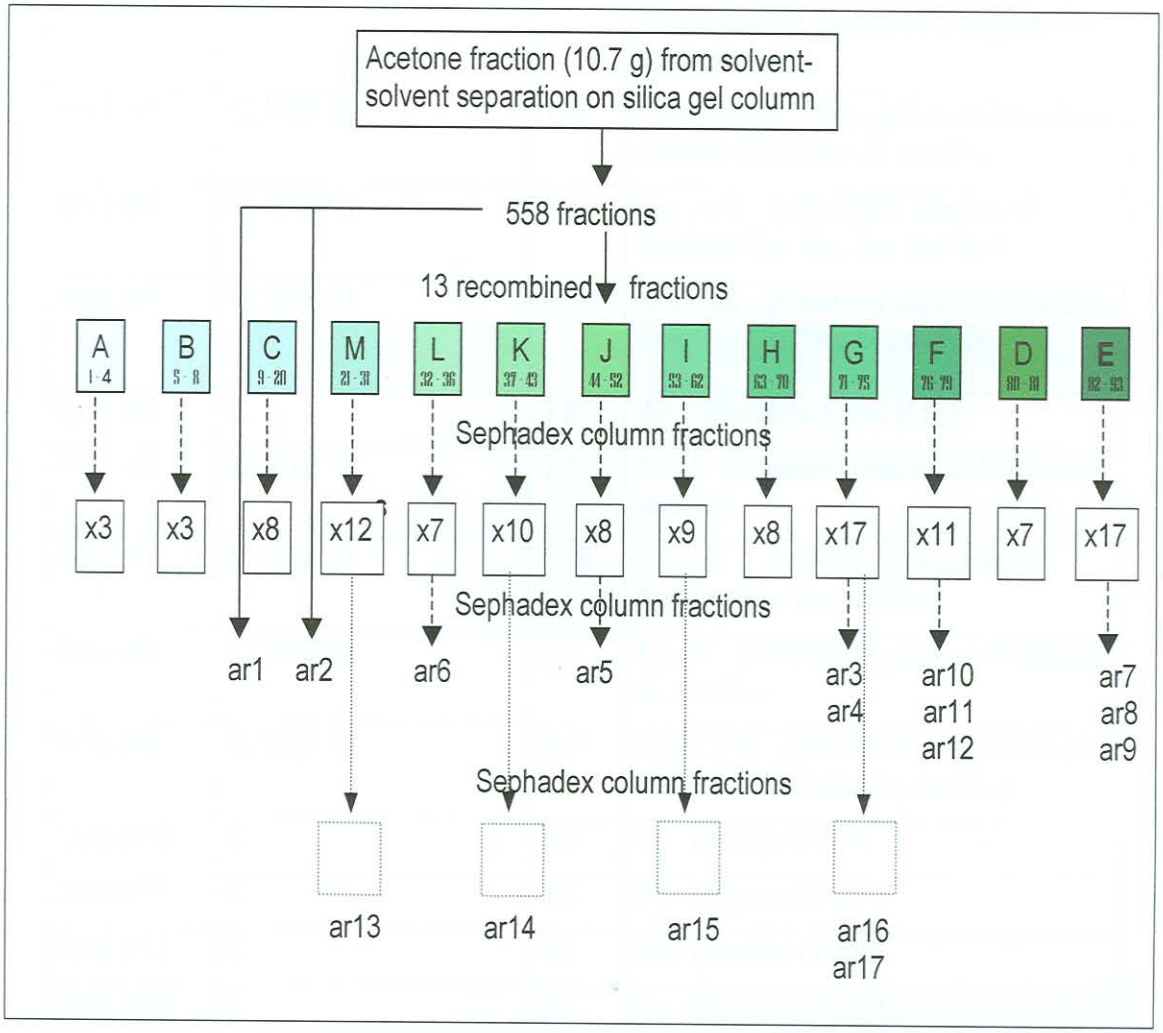


Figure 7.7. A schematic illustration of the silica gel column's fractions, recombined fractions and Sephadex columns from which the 'pure compounds' ar1 – ar17 were isolated. The increase in green colour, indicates an increase in polarity.

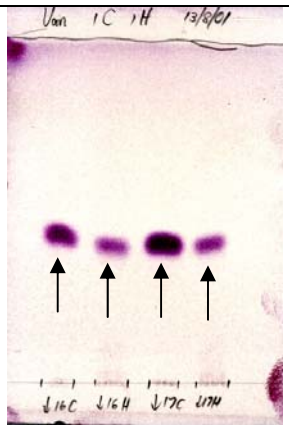
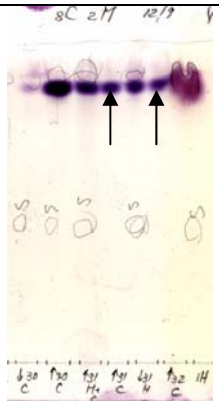
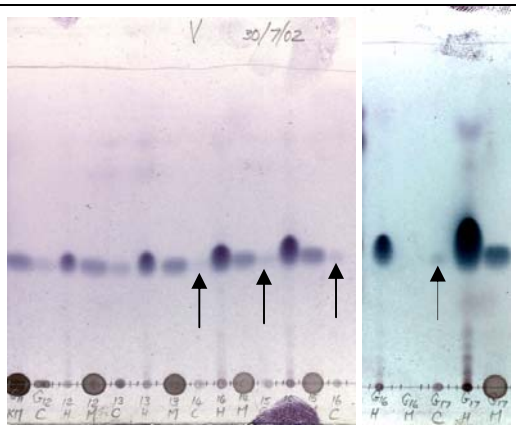
Table 7.1 lists and explains the composition the 17 possible pure compounds (from fractions of the crude column as well as from Sephadex columns run from recombined fractions) that were sent for NMR analysis, and Table 7.2 show their single bands.

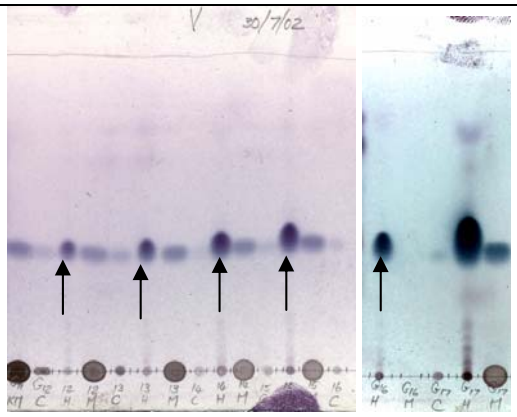
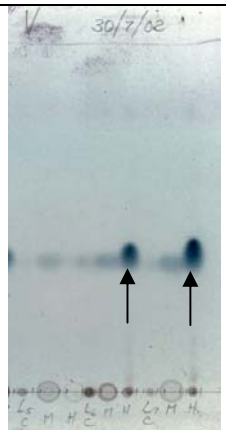
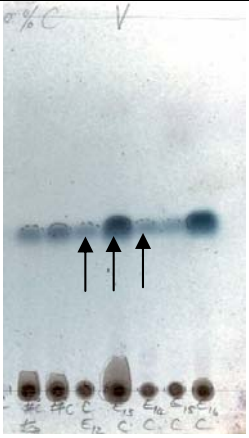
Table 7.1. Table of possible pure compounds, listed from left to right: the NMR tube number, the eluent ratio the chromatogram was developed with, milligram of material (m) and composition (fraction numbers). The deuterated solvent for all compounds was chloroform.

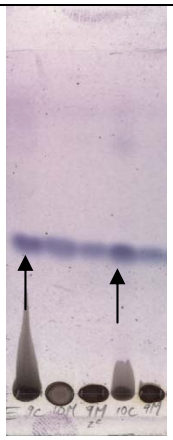
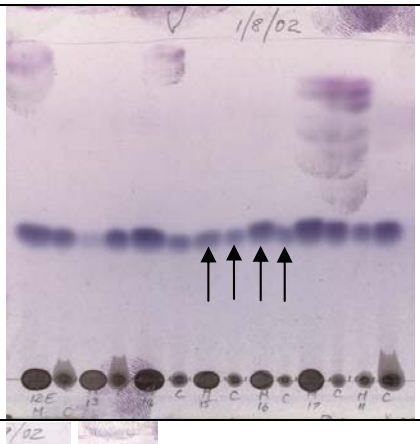
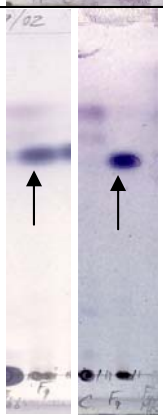
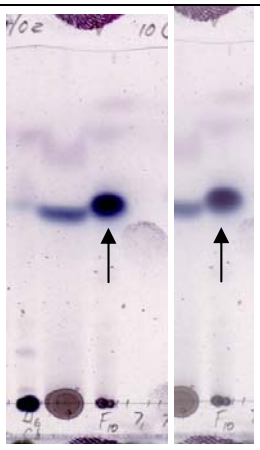
Tube number for NMR	Eluent (ratio) chromatogram was developed with:	Mg m	Fraction numbers
bota ar1*	C: M (8: 2)	7.7	↓15C↓16C↓16H↓17C↓17H (the lower half of fraction 15, 16 and 17's chloroform washes and the lower half of fraction 16 and 17's hexane washes)
bota ar2	C: M (8: 2)	1.8	↑31C↑32C (the upper half chloroform washes of fraction 30 and 31)
bota ar3	C: M (9: 1)	5.4	G_{14-17} C (chloroform washes of fractions G_{14}, G_{15}, G_{16} and G_{17})
bota ar4	C: M (9: 1)	4.3	G_{12-17}H (*n*-hexane washes of fractions G_{12}, G_{13}, G_{14}, G_{15}, G_{16} and G_{17})
bota ar5	C	2.0	$J_{8,9}$ (fractions 8 and 9 of J)
bota ar6	C: M (9: 1)	2.1	$L_{6,7}$H (*n*-hexane washes of fractions L_6 and L_7)
bota ar7	C: M (9: 1)	12.0	$E_{12\text{-}14}$ C (chloroform washes of fractions E_{12}, E_{13} and E_{14})
bota ar8	C: M (9: 1)	4.1	$E_{9,10}$ C (chloroform washes of fractions E_9 and E_{10})
bota ar9	C: M (9: 1)	9.3	$E_{15,16}$C+M (chloroform and methanol washes of fractions E_{15} and E_{16})
bota ar10	C	3.8	F_9 (fraction 9 of F)
bota ar11	C	3.6	F_{10} (fraction 10 of F)
bota ar12	C	2.6	F_{11} (fraction 11 of F)
bota ar13	C	2.7	4_1 (fraction 11 of F)
bota ar14	C	3.5	$6_{0,1,6}$ (fractions 0, 1 and 6 of 6)
bota ar15	C	3.9	7_2 (fraction 2 of 7)
bota ar16	C	3.4	$3_{6,7,8}$ (fractions 6,7 and 8 of 3)
bota ar17	C	3.3	$4_{5,6,7}$ (fractions 5, 6 and 7 of 4)

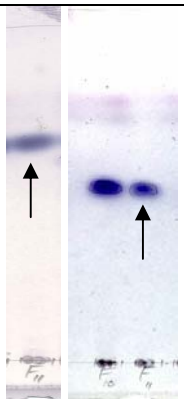
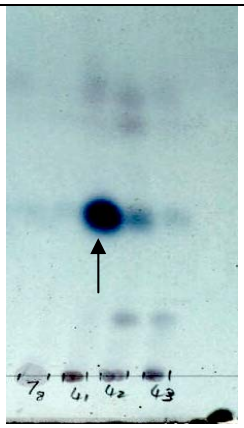
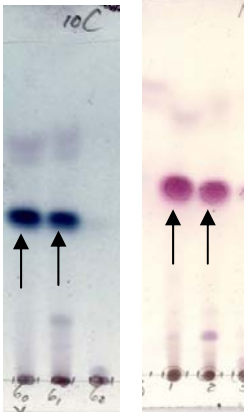
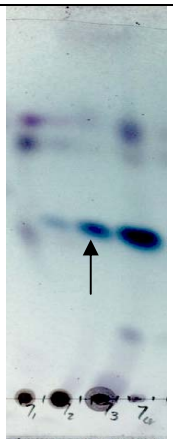
* Needle-like crystals drifted on top of the deuterated chloroform and dissolved with difficulty. (H = *n*-hexane C = chloroform, M = methanol).

Table 7.2. Table of possible pure compounds, listed from left to right: the NMR tube number and the eluent ratio the chromatogram was developed with (bottom label of each row's first column), milligram of material (m) and composition (fraction numbers). The deuterated solvent for all compounds was chloroform.

Tube no	Chromatogram	Mg m	Fractions
bota ar1 C: M (8: 2)	 C* *Needle like crystals dissolved with difficulty	7.7	↓15C↓16C ↓16H↓17C ↓17H
bota ar2 C: M (8: 2)		1.8	↑31C↑32C
bota ar3 C: M (9: 1)		5.4	G_{14-17} C

Tube no	Chromatogram	Mg m	Fractions
bota ar4 C: M (9: 1)		4.3	$G_{12-16}H$
bota ar5 C		2.0	$J_{7,8}$
bota ar6 C: M (9: 1)		2.1	$L_{6,7}H$
bota ar7 C: M (9: 1)		12.0	$E_{12-14}C$

Tube no	Chromatogram	Mg m	Fractions
bota ar8 C: M (9: 1)		4.1	$E_{9,10}$ C
bota ar9 C: M (9: 1)		9.3	$E_{15,16}$ C +M
bota ar10 C		3.8	F_9
bota ar11 C		3.6	F_{10}

Tube no	Chromatogram	Mg m	Fractions
bota ar12 C		2.6	F_{11}
bota ar13 C		2.7	4_1
bota ar14 C		3.5	$6_{0,1,2}$
bota ar15 C		3.9	7_2

Tube no	Chromatogram	Mg m	Fractions
bota ar16 C		3.4	3 $_{6,7,8}$
bota ar17 C		3.3	4 $_{5,6,7}$

The compounds were all send for NMR analysis - firstly the proton spectrum, then the ^{13}C spectrum and lastly 2-dimensional spectra. After each spectrum, a compound was re-evaluated for purity and structure type.

7.3.4 Nuclear magnetic resonance

Proton spectra of NMR analysis indicated structures of terpenoids for the first two (Figures 7.8 and 7.9) and twelfth (Figure 7.10) pure compounds. The ^{13}C NMR graph of the pure compound bota ar1 is shown in Figure 7.11. The two-dimensional NMR graphs, heteronuclear multiple quantum correlation (HMQC) and correlated spectroscopy (COSY) of bota ar1 is shown in Figures 1.12 and 7.13 respectively.

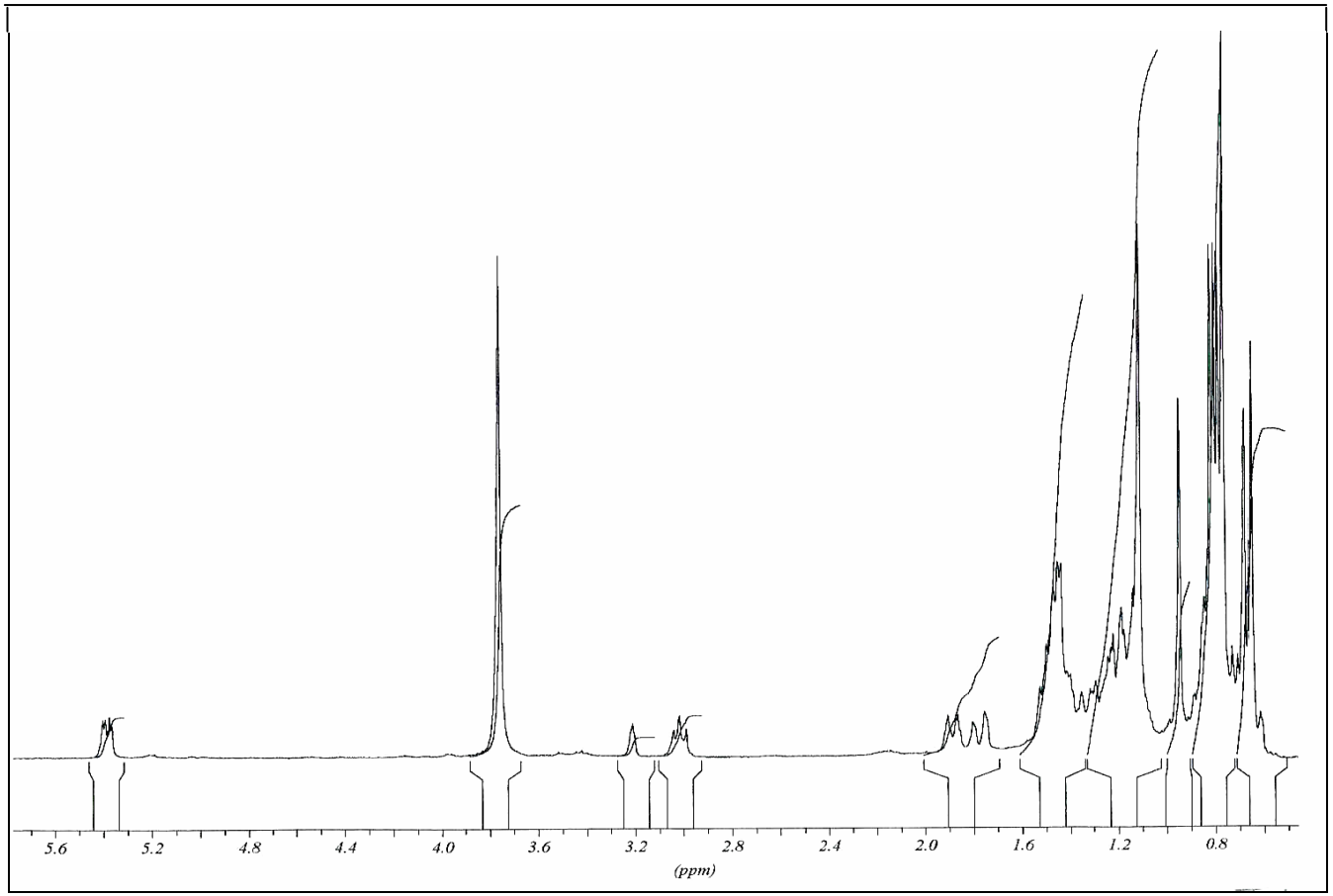


Figure 7.8. The proton spectra from NMR analysis of the first (bota ar1) pure compound.

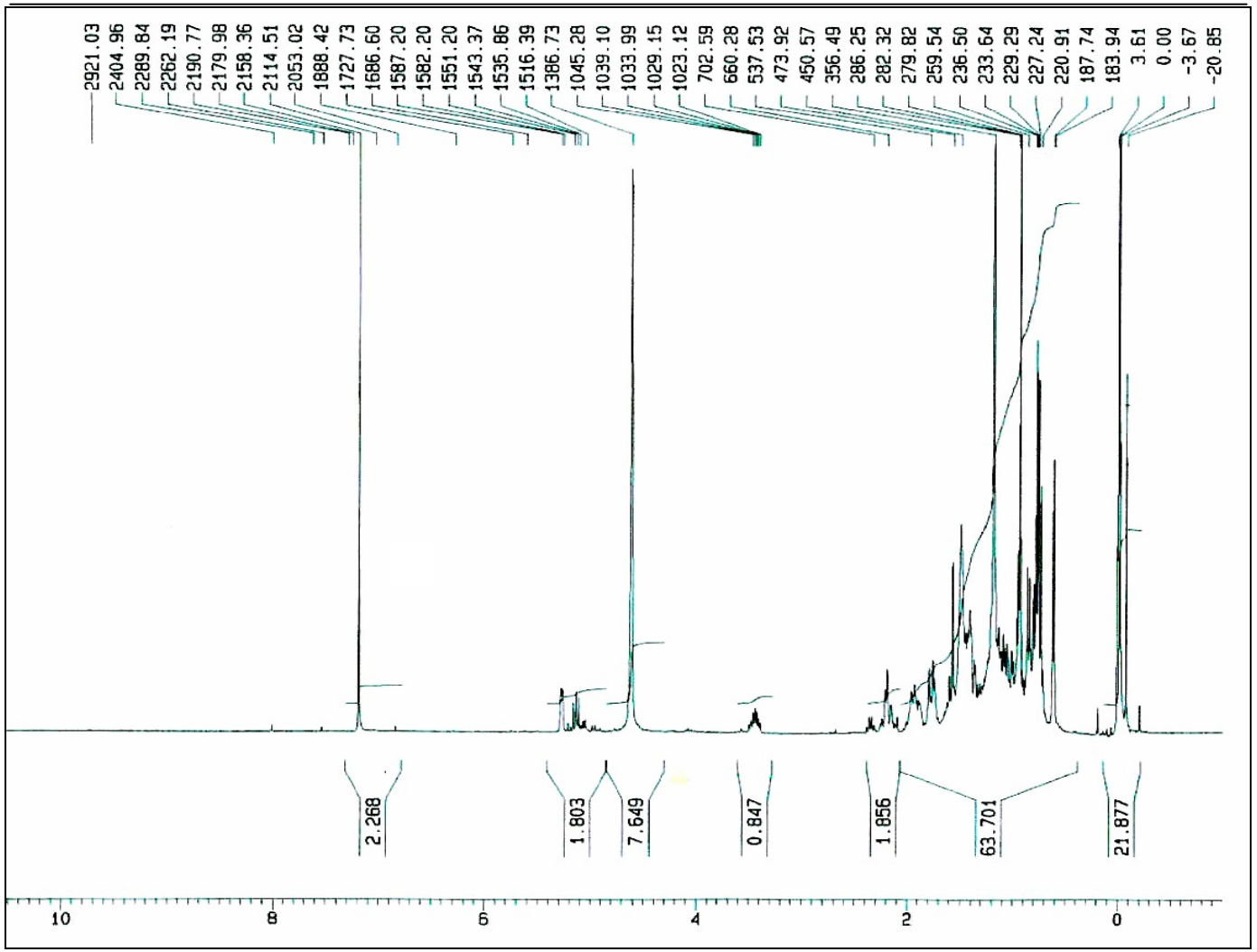


Figure 7.9. The proton spectra from NMR analysis of the second (bota ar2) pure compound.

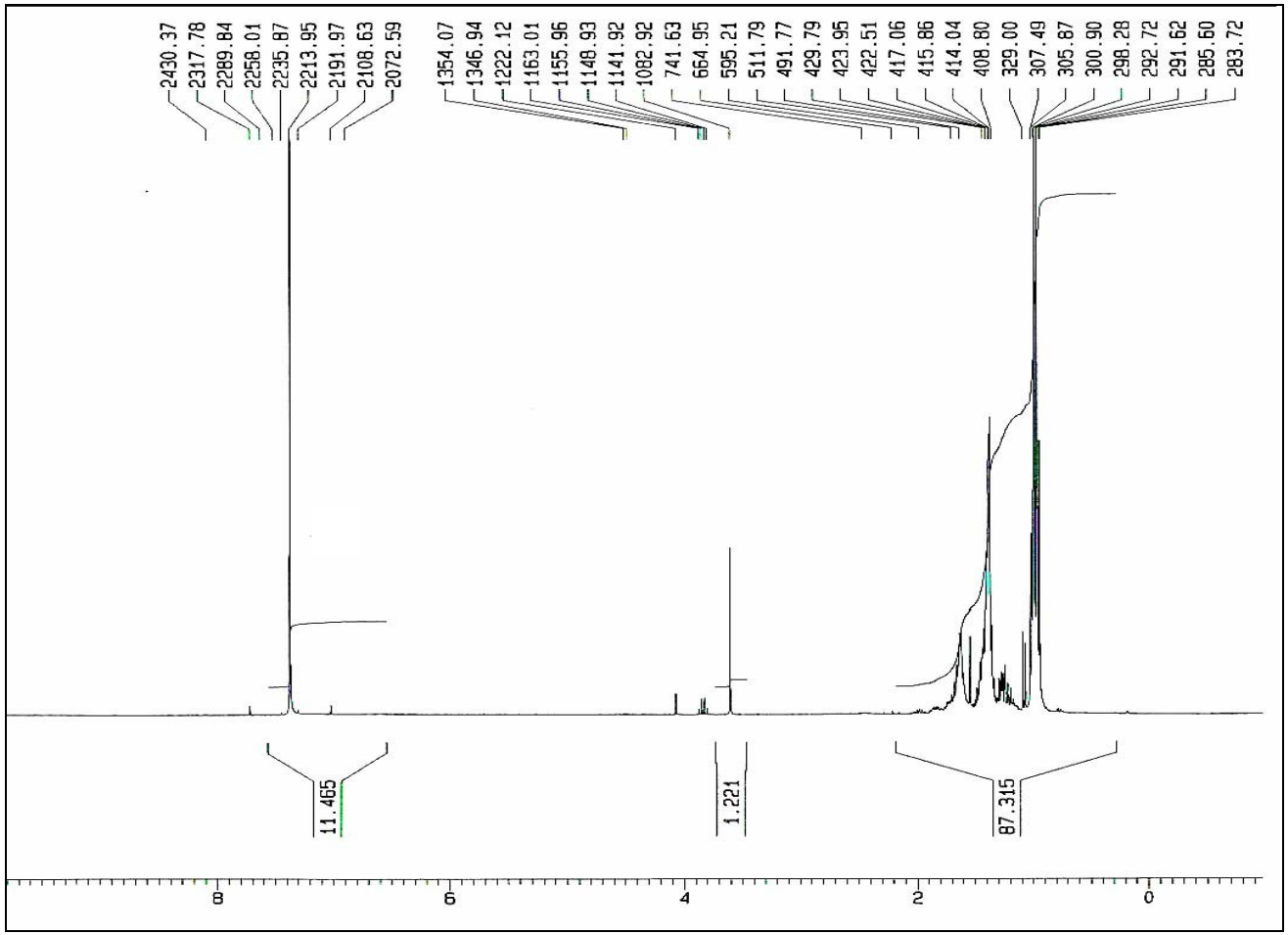


Figure 7.10. The proton spectra from NMR analysis of the twelfth (bota ar12) pure compound.

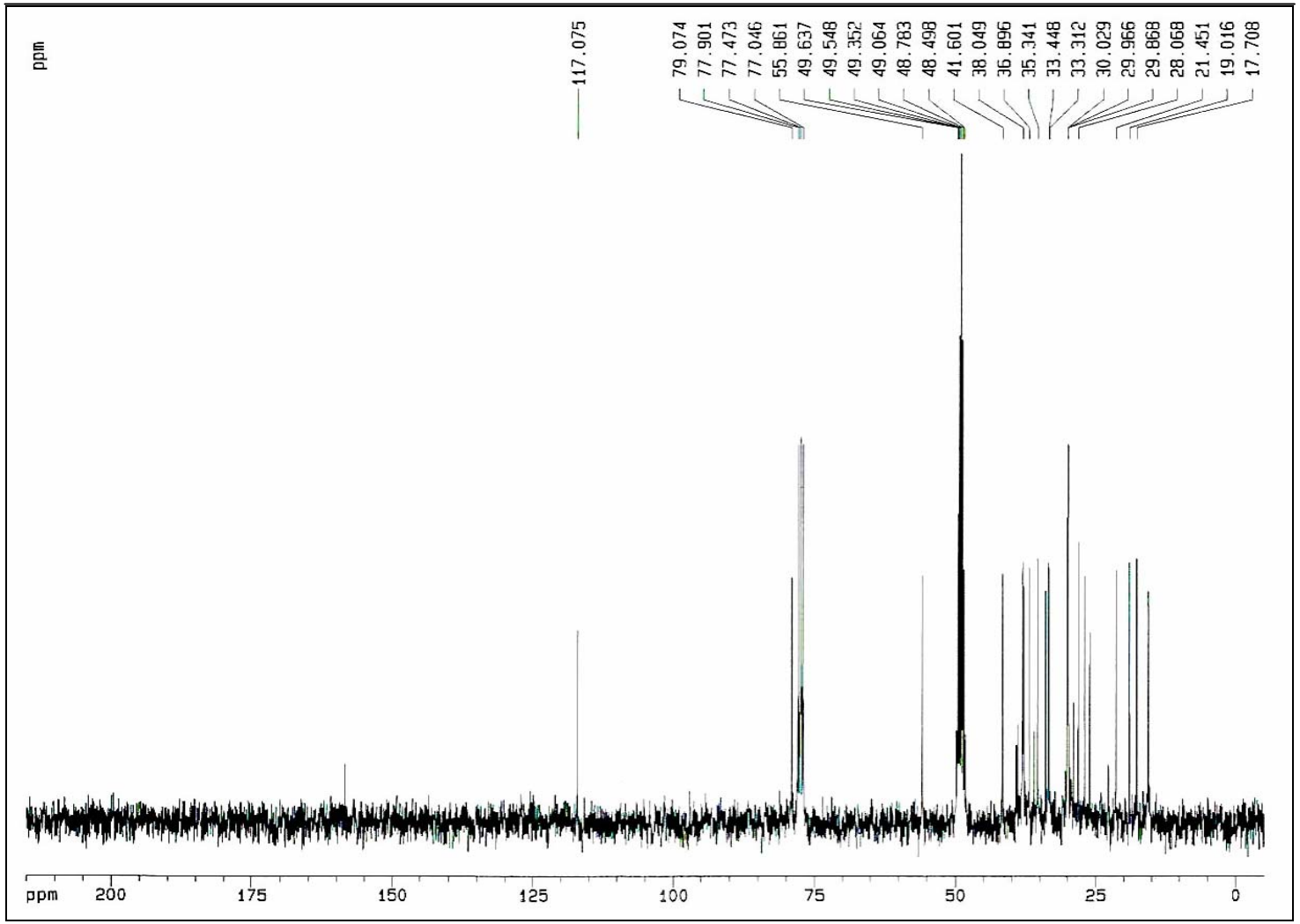


Figure 7.11. The ^{13}C NMR graph of the pure compound bota ar1.

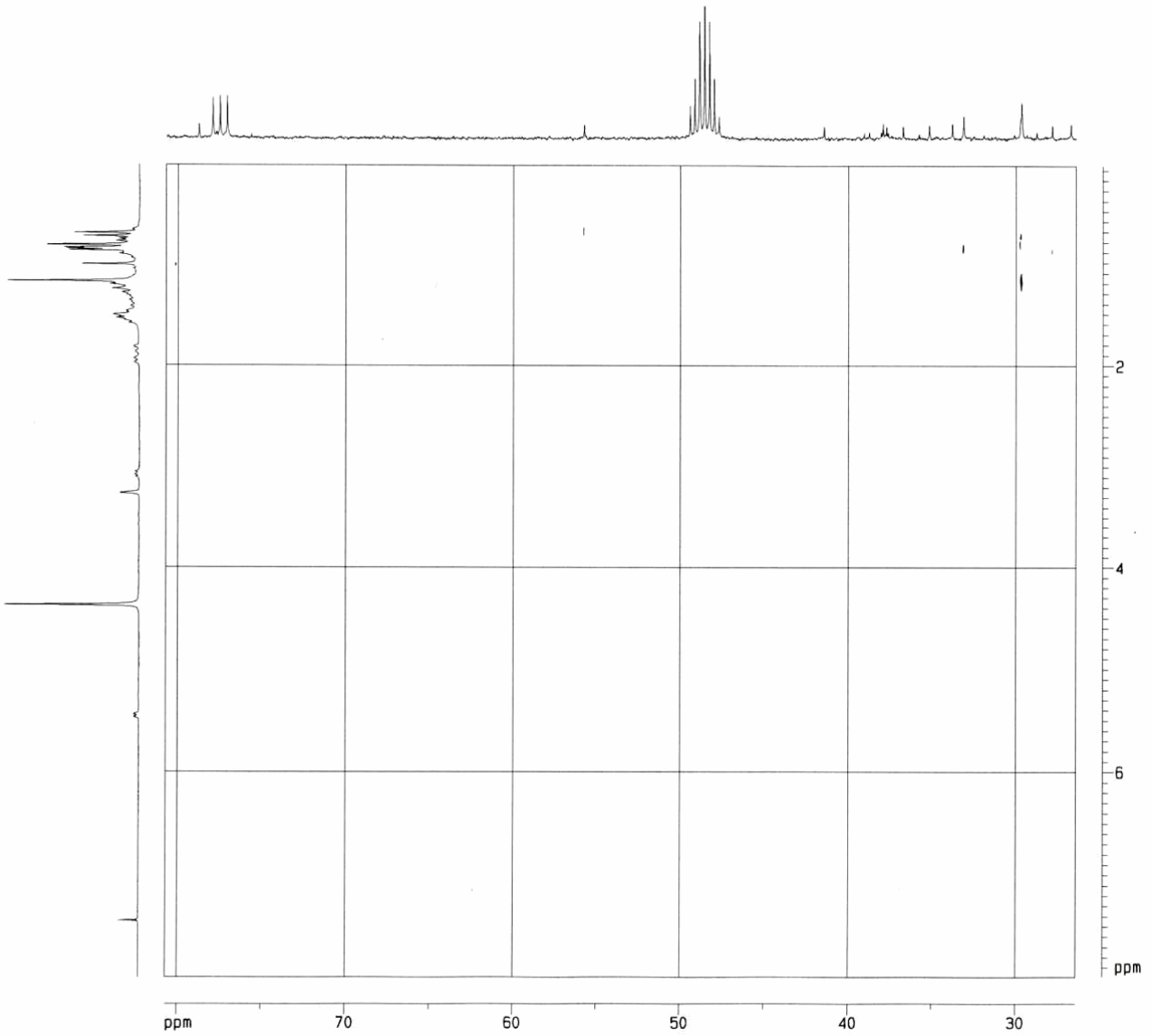


Figure 7.12. The two-dimensional heteronuclear multiple quantum correlation (HMQC) NMR graph of bota ar1.

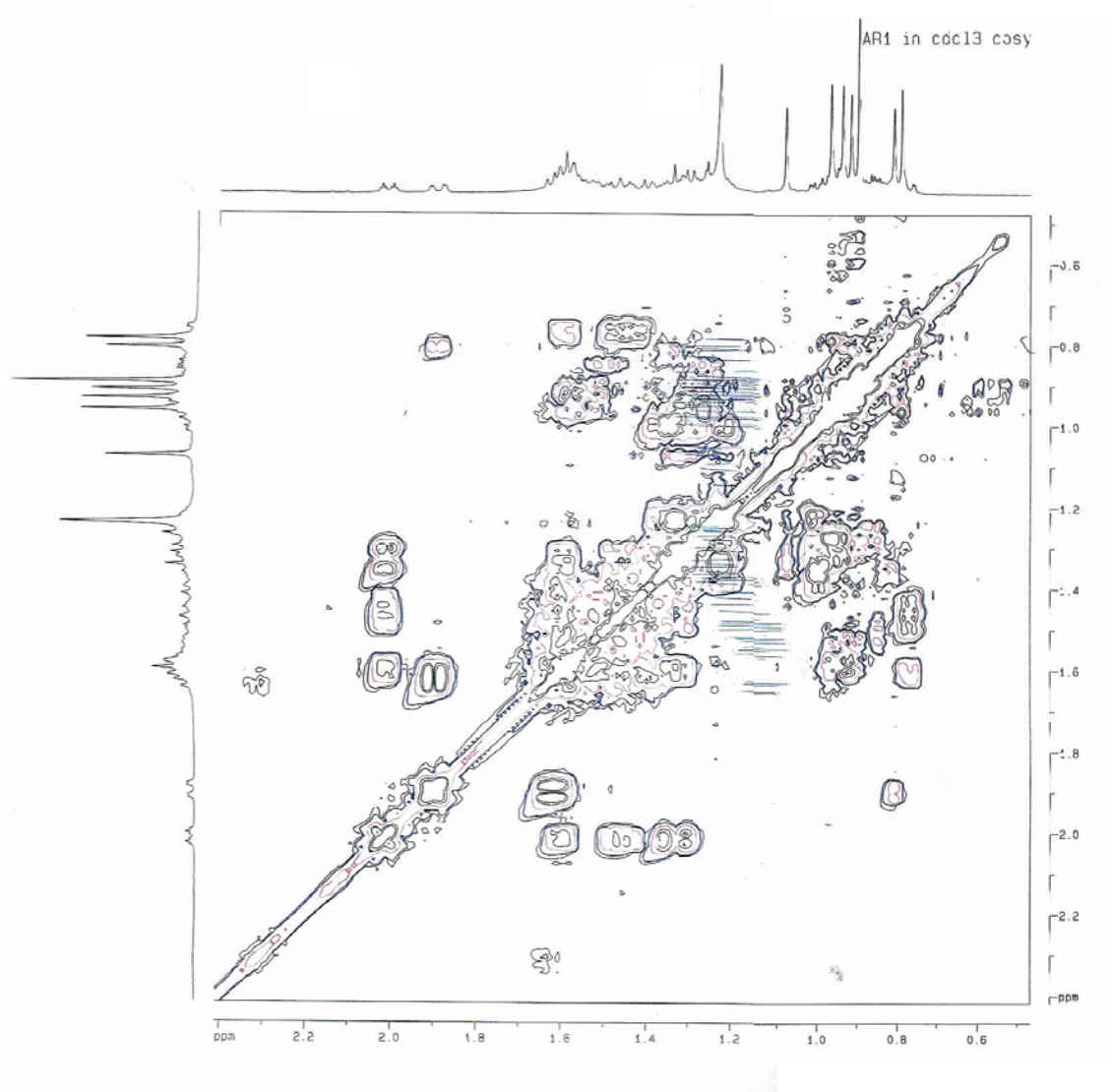


Figure 7.13. The two-dimensional correlated spectroscopy (COSY) NMR spectrum of bota ar1.

When compound bota ar1 dried, it crystallised as colourless needle-like crystals.

Attempts to elucidate the two-dimensional structure of the second compound failed since the material was insufficient (1,8 mg) to obtain the necessary detail.

The 15 other possible pure compounds' structures were not elucidated. They were contaminated by the contents of the deuterated chloroform bottle that was not deuterated chloroform. Efforts to purify these compounds again on Sephadex columns yielded only masses less than 2 mg.

Data from the two-dimensional nuclear magnetic resonance spectra of the first pure compound bota ar1, was compared with similar natural compounds. In Table 7.3, the pure compound isolated (bota ar1) from *P. myrtifolia* leaves, and other similar pentacyclic triterpenes (like taraxerol (2), taraxerone (3), myricadiol (4), and epitaraxerol (5) are listed. It was the same as that of authentic samples of taraxerol, whose details were published in several sources (Sakurai *et al*., (1987), Takasaki *et al*., (1999)).

Table 7.3. ^{13}C NMR values of the pure compound isolated, bota ar1 (1) from *P. myrtifolia* leaves, taraxerol (2), taraxerone (3), myricadiol (4), and epitaraxerol (5). Values for (2) – (5) are from Sakurai *et al*. (1987).

	ppm				
Carbon No.	1 (bota ar1)	2 taraxerol	3 taraxerone	4 myricadol	5 Epitaraxerol
C-I	38.0	38.1	38.4	37.8	37.8
C-2	27.2	27.3	34.1	28	25.2
C-3	79.1	79.2	217.3	78.2	76.3
C-4	39.0	39.1	47.6	41.4	39.1
C-5	55.5	55.7	55.8	56	49.4
C-6	18.8	19.0	20	19.2	18.8
C-7	35.1	35.3	35.2	36.3	33.9
C-8	38.8	38.9	38.9	39.3	38.9

C-9	48.7	48.9	48.7	45.6	48.9
C-10	37.7	37.9	37.6	37.8	37.9
C-11	17.6	17.7	17.5	I7.9	17.5
C-12	35.8	35.9	35.8	31.2	35.2
C-13	37.6	37.9	37.7	38.3	37.9
C-14	158.0	158.1	157.6	158.7	158.1
C-15	116.8	117.0	117.2	116.8	166.6
C-16	36.7	36.9	36.7	33.2	36.8
C-17	38.0	38.1	37.7	38.3	38.1
C-18	49.3	49.4	48.8	49.6	49.1
C-19	41.3	41.4	40.7	41.7	41.4
C-20	29.4	29.0	28.8	28.8	29.0
C-21	33.7	33.9	33.6	33.8	33.2
C-22	33.1	33.2	33.1	28.7	32.4
C-23	28.0	28.1	26.2	28.4	33.1
C-24	15.5	15.6	21.5	16.5	22.0
C.25	14.2	15.6	14.8	15.7	30.0
C-26	31.9	30.1	29.9	30.1	26.5
C-27	25.9	26.0	25.6	26.2	30.0
C-28	29.9	30.1	29.9	64.6	21.2
C-29	33.4	33.5	33.4	33.8	28.1
C-30	21.4	21.5	21.4	22.0	15.1

Bota ar1 gave a molecular ion peak at *m/z* 426.3834 corresponding to the molecular formula $C_{30}H_{50}O$ (calculated 426.3861). The ^{1}H NMR spectrum gave signals for eight tertiary methyl groups: at (δ): 0.98 (H_3-23), 0.80 (H_3-24), 0.93 (H_3-25), 1.09 (H_3-26), 0.95 (H_3-27), 0.82 (H_3-28), 0.91 (H_3-29), 0.91 (H_3-30), 3.19 (dd, *J* = 4.9, 10.9 Hz, H-3α), a typical 3-αH adjacent to 3β-OH and olefinic proton at 5.53 (dd, *J* = 34, 8.0Hz, H-14).

The chemical shifts in the ^{1}H and the ^{13}C NMR spectra of bota ar1 closely resembled those of ^{14}-taraxerene derivatives, particularly the chemical shift and multiplicities of the olefinic proton and the chemical shifts of ring C, D and E carbon atoms. The relative stereochemistry of 3-C, was confirmed by NOE difference measurements. Irradiation at δ3.71 (6- βH) resulted in a 9.12% NOE at δ1.10 (26-H_3), a 7.6% NOE at δ0.97 (25-H_3) and a 10.99% NOE at δ0.90 (24-

H_3). Irradiation at δ0.90 (24-H_3) resulted in a 12.10% NOE at δ3.71 (6-βH), a 15.6% NOE at δ0.97 (25-H_3) and a 6.6% NOE at δ1.4 (2-H_2). Irradiation at δ0.97 (25-H_3) resulted in an 8.25% NOE at δ3.71 (6-βH), a 17.2% NOE al δ1.10 (26-H_3), a 14.1% NOE at δ0.90 (24-H_3) and a 5.2% NOE at δ1.4 (2-H_2). Finally irradiation al δ1.10 (26-H_3) resulted in an 8.9% NOE at δ3.71 (6-βH), a 16.3% NOE at δ0.97 (25-H_3) and a 7.7%NOE at δ1.37 (12-H_2).

The ^{1}H and the ^{13}C NMR data of bota ar1 is the same as data published previously for taraxerol, and is therefore taraxerol. Taraxerol had a melting point of 281 - 282°C (Takasaki *et al*., 1999).

7.3.5 Mass spectrometry

The mass spectrum (Figure 7.14) was characteristic of the ^{14}-taraxerene series of triterpenes. In these molecules a retro-Diels-Alder decomposition would be expected to operate and the collapse of ring D should occur (Indicated in Figure 7.15). The mass spectrum graph of bota ar1 was the same as that of taraxerol.

The mass spectra of members of the □^{14}-Taraxerenes (taraxerone, taraxerol, and myricadiol diacetate) have been measured, thus offering the necessary labels for assigning structures to the major fragments (Budzikiewicz *et al*., 1963). Mass spectrometry can be of great value in the structure elucidation of pentacyclic triterpenes. In general, the presence and position of a nuclear double bond control the fragmentation behaviour, which frequently allow assignment of membership of a given triterpene in one of the major classes (saturated and unsaturated members of the α- and β-amyrin group as well as members of the taraxerol, bauerene, friedelane and lupane series). In addition, the location of functional groups can often be narrowed down by consideration of the fragment pattern (Budzikiewicz *et al*., 1963).

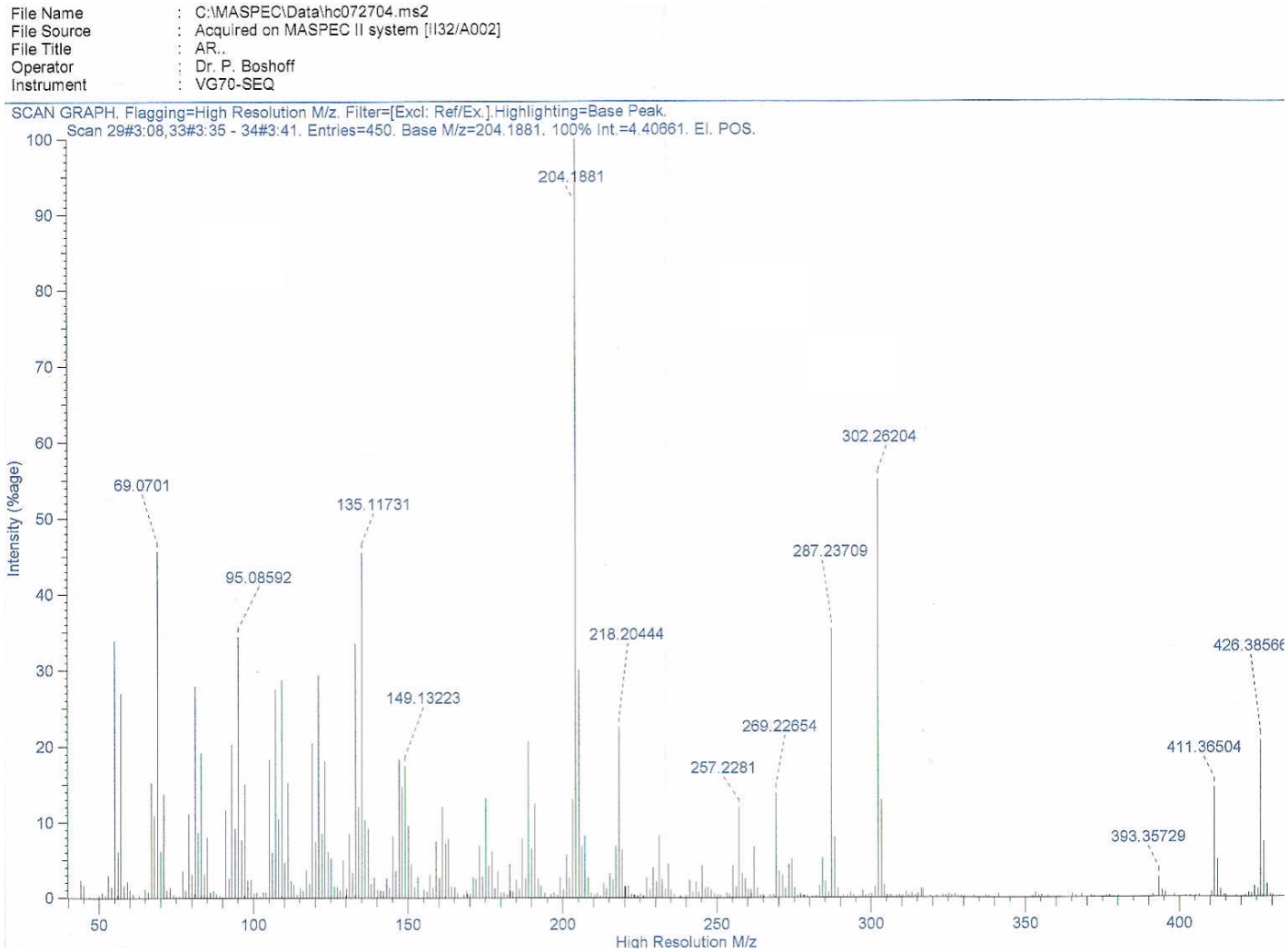


Figure 7.14. The mass spectrum of bota ar 1.

A two-dimensional representation of the structure of taraxerol, with a likely retro-Diels-Alder decomposition which would be expected to operate with collapse of ring D, is presented by Figure 7.15.

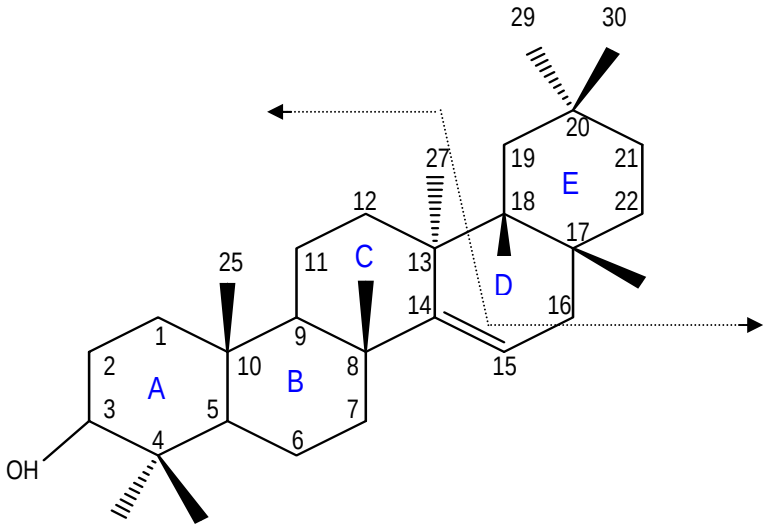


Figure 7.15. A two-dimensional representation of taraxerol. (A retro-Diels-Alder decomposition would be expected to operate with the collapse of ring D).

According to literature sources, the alcohol isolated here, taraxerol, $C_{30}H_{50}O$ was first isolated from *Taraxacum* root, by Burrows & Simpson (1938) who prepared the acetate and benzoate form, but had insufficient material for further study. In 1942, it was isolated from the Japanese *Skimmia* species and largely characterized by Takeda. Although taraxerane derivatives occur in nature in abundance, little study has been done on the parent hydrocarbon in the years before 1962. Confusion arose from literature because Takeda prepared 14α-Taraxerane which he called dihydroskimmiene (Rahman, 1986). In the same year Winterstein assumed Takeda's dihydroskim-miene was oleanane (β-amyrane) and it is so described in Elsevier's Encyclopedia of 1952 (Corbett and Cumming, 1955). Taraxer-14-en-3α-ol was isolated in a previous study in 1962 from the bark of *Suttonia australis.* At that time, it had not been reported to occur naturally, but has since been isolated from *Euphorbia* from Hong Kong and several other plants.

The configuration of rings A and B of 14α-taraxerane is the same as that of ursane, lupane, oleanane and 18α-oleanane (Rahman, 1986).

More recent investigations of taraxerol, indicated that it is identical to skimmiol (Rahman, 1986). 14-Taraxeren-3-ol has a molecular formula of $C_{30}H_{50}O$ and a molecular weight of 426.724. Its synonym is D-Friedoolean-14-en-3-ol. Its appearance is colourless needle-like crystals. The 3α-form is also called Epitaraxerol and the 3β-form Taraxerol or Skimmiol. The 3α-form has been found in *Euphorbia royleana*, *Suttonia australis*, *Macranga denticulate*, *Skimmia wallichii* and other plants. The 3β-form has been found in *Taraxacum officinale* (dandelion), *Alnuss* spp, *Skimmia japonica*, *Rhododendron* spp, *Euphorbia* spp and other plants. It is probably identical with Pertusarin from the lichen *Pertusaria communis*, described by Hesse in 1898 (Takasaki *et al*., 1999).

7.4 Conclusions

Pure compounds were isolated from a bacterially active fraction from *P. myrtifolia* leaves. One pure compound's structure was elucidated as a pentacyclic triterpenoid, taraxerol ($C_{30}H_{50}O$) (synonyms: 14-taraxeren-3-ol, D-friedoolean-14-en-3-ol, or skimmiol). No reports on taraxerol's MIC values for bacteria or tests against human cancer cell lines could be found.

7.5 Literature references

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Chapter 8

Antibacterial, antioxidant and cytotoxic activity of taraxerol, a pentacyclic triterpenoid, isolated from *Pteleopsis myrtifolia* leaves

Abstract

A known pure pentacyclic triterpenoid, taraxerol (14-taraxeren-3-ol), that was isolated from *Pteleopsis myrtifolia* leaves by bioassay-guided fractionation in a previous investigation (Chapter 7), was investigated for its bioactivity. It had antibacterial activity and MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli,* respectively. It did not have significant antioxidant activity. It had cytotoxic activity and showed significant growth inhibition of the human cancer cell line $WHCO_3$ (oesophagus).

8.1 Introduction

8.1.1 Triterpenoids

Probably no other group of metabolites throughout the Plant and Animal kingdom has such diversity, so many functions and is produced by so many organisms than terpenoids (Harrewijn *et al*., 2001). Plant hormones are often derivatives of terpenoids, such as cytokines, gibberellins and abscissic acid. The steroid hormones of mammals are terpenoids with an advanced but not very complex structure (Harrewijn *et al*., 2001).

Terpenes have a unique structure: they consist of an integral number of five-carbon (5C or isoprene) units. Two such units can form a monoterpene (C-1O), and sesquiterpenes (C-15), diterpenes (C-20), triterpenes (C-30), tetraterpenes (C-40) and polyterpenoids (>40C) are also

possible. Many terpenoids are produced via the mevalonic acid pathway (MAD) that probably had its origin during the early development of life on this planet. Other terpenoids are biosynthesised via a recently discovered pathway, a mevalonate independent route (MAI) ((Harrewijn *et al*., 2001). The mechanisms that regulate the biosynthesis of mevalonate are finely tuned. In many organisms, end products in which isoprenoids are incorporated can reduce the activity of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase(s) via a feedback and regulatory system, in this way achieving for example; cholesterol homeostasis. Figure 8.1 shows the several places in steroid biosynthesis where feedback for regulation takes place. Terpenoids can have a simple aliphatic or a cyclic structure. The cyclic structure(s) can exist in mono-, bi-, tri- and polycyclic formations and many of them can be polymerised. Polymerisation can be artificially induced by strong acids, such as nitric acid, UV light, temperature, oxygen and co-polymers that result in complicated structures.

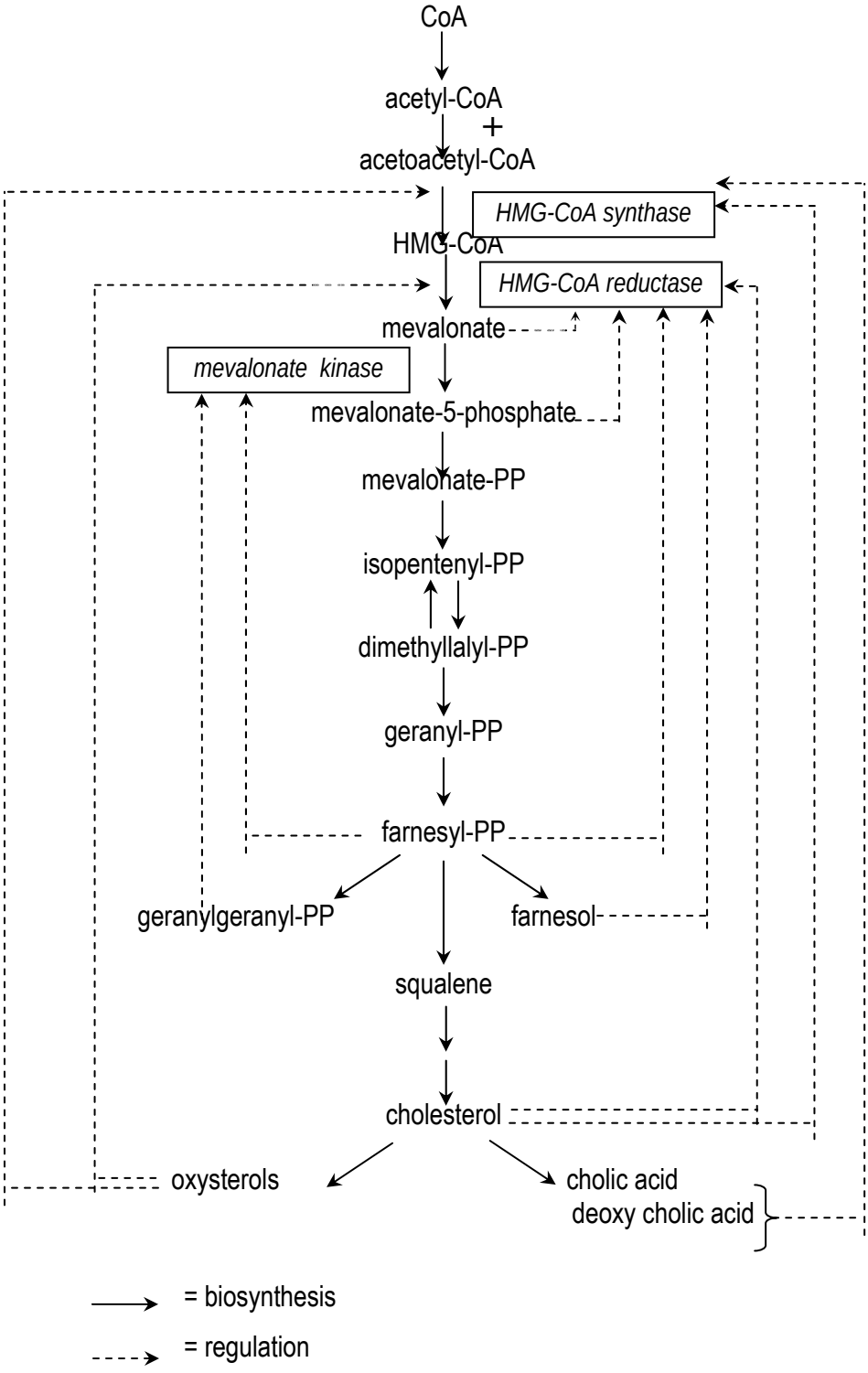

Figure 8.1. Multivalent regulation systems of steroid biosynthesis of the mevalonic acid pathway (MAD) metabolism in vertebrates (Harrewijn *et al*., (2001). (CoA = coenzyme A, HMG = β-hydroxy- β-methylglutaryl, PP = pyrophosphatase).

8.1.2 Specific functions of terpenoids

Terpenoids have a role in the regulation of isoprenoid metabolism and signal transduction and as such can exert a profound effect on cell growth, differentiation, apoptosis and multiplication. According to Penuelas *et al*. (1995), no other biochemical group of secondary metabolites has such a potential to interfere with processes ranging from cell level to ecological interactions. Moreover, the lower terpenes are rather volatile, an essential physical property for air-borne long distance effects. Nature uses terpenes in a "chemical language" between plants, insects, vertebrates and even humans. Terpenes and other isoprenoids have also important functions as messengers: they can act as defensive substances in plants (allomones) and animals; they can be used by plants to deter herbivores or to inform conspecifics, or to attract natural enemies of herbivores; within organs and within the cell body, in particular between the cell surface and the cell nucleus. They can have free radical scavenger capacities as antioxidants, keep homeostasis of cell numbers, have effects on Ras proteins, effect cancer cells in different ways, impair mevalonic acid synthesis in tumours and cause unexpected effects. Terpenoids can be toxic to micro-organisms, insects and other animals.

Often their effects are additive or even synergistic with other mevalonate metabolites, or they are inhibitors of parts of the mevalonate pathway. Thus, their mode of action should be viewed with respect to the role of other mevalonate metabolites in growth, development and behaviour of the organism studied.

8.1.3 Importance of knowledge about terpenoids

From ancient times, humans have utilized the messenger functions (volatile properties) of natural terpenoids for several purposes without knowledge of their structure. Terpenoids' structure elucidation had to wait until the second half of the 20th century for their eventual revelation. Since its identification in 1956, mevalonic acid has been recognized as a key

substance in the biosynthesis of a wide range of isoprenoids, including terpenoids. Mevalonic acid is produced in many organisms from acetate via a generally occurring enzyme, acetyl co-enzyme A. End products of the mevalonic pathway include sterols such as cholesterol, involved in membrane structure; haem A and ubiquinone, active in electron transport; dolichol, required for glycoprotein synthesis; carotenoids with many functions; steroid hormones in animals; hormones in insects and isopentenyl adenine and isoprenoid proteins, both involved in DNA synthesis. A study of a basic function of a messenger molecule in a particular organism increases our understanding of regulatory systems in distant taxa. The more these systems are involved in basic processes (e.g. gene expression), the greater scientific disciplines will benefit from such a study (Harrewijn *et al*., 2001).

Specific targets of messenger molecules in fully developed organisms are usually studied by specialists. It is highly likely that they are unaware of the same compound having profound effects in organisms belonging to other groups, although this knowledge is somewhere amidst a wealth of accessible information. Terpenoids are such compounds. Insects are needed to pollinate our crops, but they can also destroy them and defoliate our forests, or cause epidemic diseases both for livestock and humans. Terpenoids have become of widespread importance in perfumery, detergents, foods and beverages, chemical manufacturing industries, pharmacy and biotechnology, yet knowledge of their potential utilization is only just beginning to be revealed (Harrewijn *et al*., 2001).

8.1.4 Natural terpenoids to the benefit of human health

Examples of benefits of terpenoids to human health form literature are: antimicrobials, analgesics, cholesterolemia and vascular problems, tracheal and bronchial disorders, arthritis, rheumatism, inflammatory disorders, intestinal disorders, stress-related problems and sedatives, cancer therapies, cosmetics, sex attractants, dermatological preparations, terpenoid

analogues and derivatives applied in agriculture and in medicine (Harrewijn *et al*., 2001). Antimicrobial properties and anticancer action of taraxerol are listed in Tables 8.1 and 8.2 respectively.

8.1.4.1 Antimicrobial activity

Only a few examples of terpenoids acting as antimicrobials are given in Table 8.1.

Table 8.1. Effect of volatile terpenoids on bacteria (Harrewijn *et al*., 2001).

Species	Gram-	Gram+	Inhibiting terpenoids
Bacillus subtilis			acorenone, rimulene
Citrobacter freundii (sym-bionts in gut of termites)			thujone
Enterobacter spp. (sym-bionts in gut of termites)			linalool
Escherichia coli			carvacrol (ph.t.), citronellal, citronellyl acetate, geraniol, linalool, neral, pulegone, terpinen-4-ol, α-pinene, α-terpineol, δ-3-carene
Flavobacterium suaveolens			germacrene, sabinene
Klebsiella oxytoca			cineole, thujone
Klebsiella pneumoniae			thujone, 1,8-cineole
Mycobacterium smegmatis			alantolactone, isoalantolactone
Proteus vulgaris			limonene (toxic to cat flea), 1,8-cineole
Pseudomonas aeruginosa			linalool, pulegone
Salmonella spp. *Shigella shiga*			β-caryophyllene, β-caryophyllene oxide β-caryophyllene, β-caryophyllene oxide
Staphylococcus aureus			carvacrol (ph.t.), citronellal, citronellol, manool, pulegone, β-caryophyllene
Streptococcus faecalis			carvacrol (ph.t.), citronellal, citronellol, thymol
Vibrio cholerae			carvacrol, thymol (ph.t.), β-caryohyl-lene, β-caryophyllene oxide

((ph.t) stands for phenolic terpenoids)

8.1.4.2 Cancer therapies

The triterpenoids taraxasterol and taraxerol exhibited potent antitumour-promoting activity in

cacinogenesis tests of mouse skin (induced by a chemical initiator and promoter). In addition, they had an inhibitory effect on mouse spontaneous mammary tumours (Takasaki *et al.,* 1999). The anticancer activity of some terpenoids are listed in Table 8.2.

Table 8.2. Terpenoids' modes of action against tumour cells AG = angiogenesis; cytos. = cytoskeleton; CA = carcinogenes; HMGR = HMG-CoA reductase; diff. = (re)differentiation; ? = unknown (Harrewijn *et al*., 2001).

Terpenoid	AG	DNA	cytos.	G1 arrest	CA	HMGR	diff.	?
aphidicolin		✓					✓	
asprellic acids								✓
betulinic acid derivatives								✓
carotenes							✓	
corosolic acid	✓							✓
curcumins							✓	
dehydrothyrsiferol								✓
farnesol		✓	✓	✓		✓		
geranylgeraniol		✓	✓	✓		✓		
geranylstilbenes								✓
ginsenoides	✓							
gossypol		✓						
β-ionone				✓				
kansuiphorin								✓
limonene				✓	✓	✓		
limonoids								✓
menthol						✓		
perillyl alcohol				✓	✓	✓		
protolichesterinic acid		✓						
oleanolic acid	✓							
pinene						✓		
remangilones								✓
retinoids							✓	
taraxasterol					✓			
taraxerane					✓			
taraxerol					✓			
taxamairins			✓					
taxol			✓					
tingenone								✓
tocotrienols							✓	
ursolic acid	✓							
vitamin K2					✓		✓	

Parallel studies on the different aspects of terpene and steroid chemistry gradually revealed that squalene, a rare C_{30} hydrocarbon, was a conceivable progenitor of the higher terpenoids. Squalene was first isolated from shark liver, *Squalus* spp., which was later found to be

ubiquitously distributed. By folding this compound, one can construct the basic triterpenoid skeleton with the angular methyls and side chain in correct positions, to incorporate into, for example cholesterol.

One previous report of taraxerol's isolation in the Combretaceae was from leaves of *Terminalia glabrescens* in Brazil (Garcez *et al*., 2003). No reports of taraxerol's antibacterial activity or effect on human cell lines could be found. In a previous investigation in Angola and the Cape Basin, taraxerol and *Rhizophora* (a mangrove tree, dominant in equatorial and subequatorial west Africa) pollen, found in mid-Pleistocene sediments, was indicative of past mangrove ecosystems (Versteegh *et al*., 2004). *Rhizophora mangle* and *Rhizophora racemosa* leaves are extraordinary rich in taraxerol.

Pteleopsis myrtifolia leaf extracts have antibacterial activity (Chapters 3 and 4). Taraxerol, a pentacyclic triterpenoid, was isolated from *Pteleopsis*' leaves by bioassay-guided fractionation. The aim of this research was to determine taraxerol's bioactivity: - biological activity against various bacteria, against various human cancer cell lines, as well as its free radical scavenging or antioxidant capacity.

8.2 Materials and Methods

8.2.1 Plant material

Plant material were collected and prepared as described in 2.2.1 of Chapter 2.

8.2.2 The isolation of taraxerol

Taraxerol was isolated as described in Chapter 5.

8.2.3 Antibacterial activity of taraxerol

8.2.3.1 Minimum inhibitory concentration

MIC values were determined as described in 3.2.4.1 of Chapter 3.

8.2.3.2 Bioautography

For bioautography on the thin layer chromatograms, 20 µl of a10 mg/ml solution of taraxerol in acetone was applied to Merck Silica gel F_{254} plates) and developed with a solution of *n*-hexane and chloroform, (3:7). The bioautography method is described in 3.2.4.3 of Chapter 3.

8.2.4 Investigation of activity of taraxerol against human cell lines

A dried form of taraxerol was redissolved in dimethylsulfoxide (DMSO) to a final concentration of 1000 mg/ml (which served as a stock from which dilutions were made) and stored in a tightly sealed dark glass container at 5 ºC. The human cell lines used were MCF-12 (non-cancerous mammary gland), MCF-7 (cancerous breast), H157 (cancerous lung), $WHCO_3$ (cancerous oesophagus) and HeLa (cancerous cervix) (detail about cell lines in 6.2.2 of Chapter 6).

8.2.4.1 Human cell line cultures

The human cell lines were seeded in multiwell plates as described in 6.2.5 of Chapter 6.

Initially each cell line was tested at 10 and 100 µg/ml of taraxerol. The experimental layout of the MWP is shown in Figure 8.2. The wells bordering the MWP contained only Triton X-100.

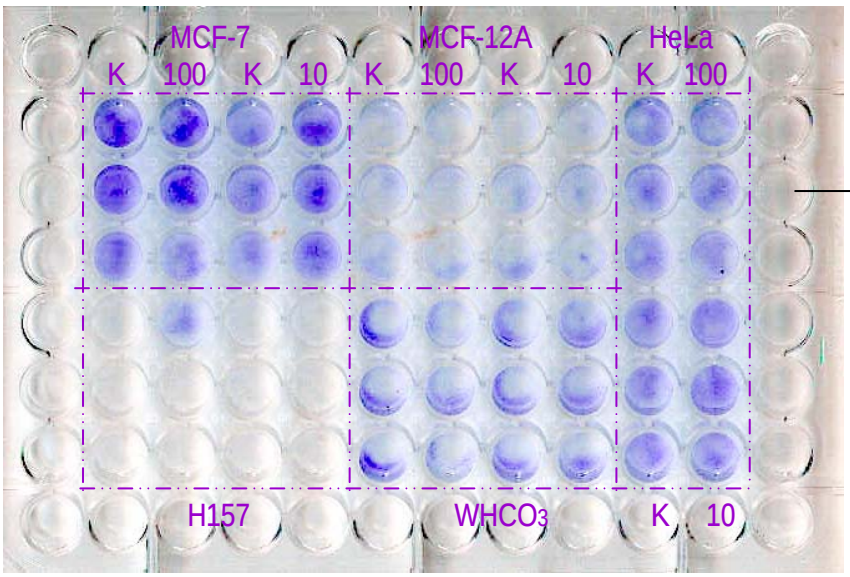

Figure 8.2. Scan of a 96-multiwell plate with the human cell lines MCF-7 (top left), MCF-12A (top right), H157 (bottom left) and $WHCO_3$ (bottom right). Each cell line's reaction to taraxerol was tested at control (K), 10 and 100 µg/ml values in triplicate against taraxerol. • The wells bordering the MWP contained only Triton X-100.

Three days after the plant extracts were added, the medium was discarded from the wells. Fixation, staining and spectrophotometer readings were done as described in 6.2.5 of Chapter 6.

Only the H157 (lung) cell line was tested at more concentrations against taraxerol.

8.2.5 Antioxidant activity

To investigate the free radical scavenger activity of taraxerol, a dried pure form thereof was redissolved in acetone to a 10 mg/ml final concentration.

8.2.5.1 The dot-blot DPPH staining procedure

The method, as described in 7.2.4 of Chapter 7, was followed.

8.3 Results and Discussion

8.3.1 Antibacterial activity of pure compound

8.3.1.1 Minimum inhibitory concentration (MIC)

Taraxerol's MIC values against the bacteria *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli* are listed in Table 8.3. The activity for the Gram-positive bacteria, especially *E. faecalis* is very good. In Table 8.4 of section 8.4, its MIC values are compared to that of other compounds from Combretaceae.

Table 8.3. Minimum inhibitory concentration values of taraxerol against the bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

	Minimum inhibitory concentration (MIC) in mg/ml			
	Staphylococcus aureus	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Escherichia coli*
Taraxerol	0.04	0.16	0.63	0.31

8.3.1.2 Bioautography

Taraxerol had visible antibacterial activity against the bacteria *S. aureus*, *E. faecalis* and *E. coli* (Figure 8.3).

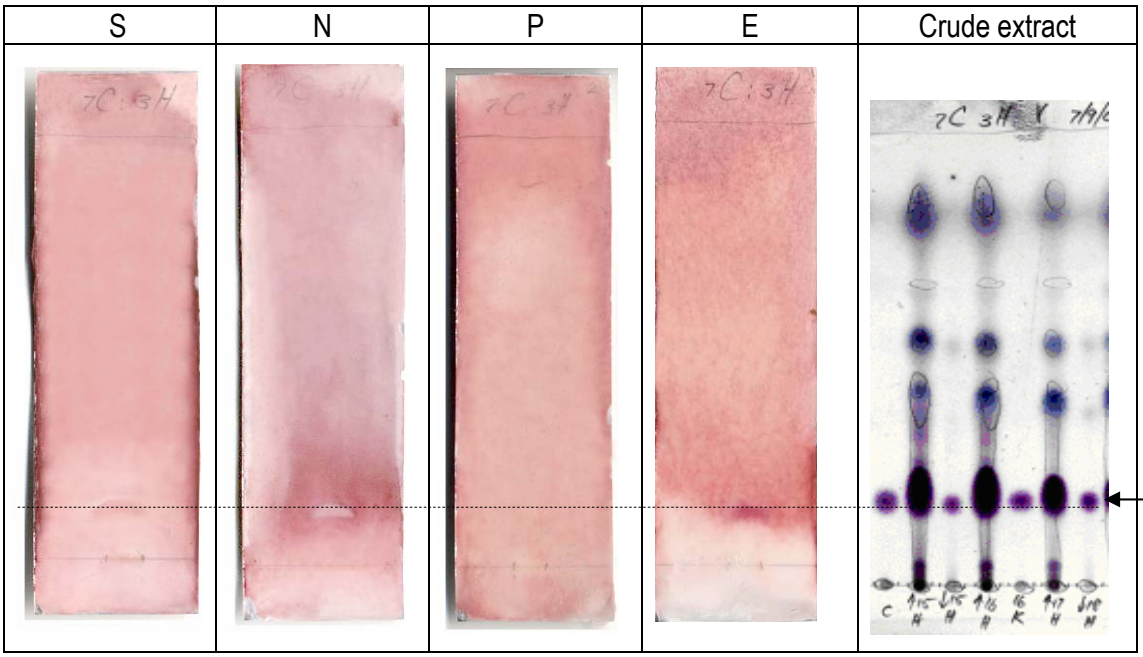

Figure 8.3. Bioautograms showing the effect of taraxerol on *Staphylococcus aureus* (S), *Enterococcus faecalis* (N), *Pseudomonas aeruginosa* (P) and *Escherichia coli* (E), after spraying with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet solution, and (extreme right) a thin layer chromatogram (which contained taraxerol – position of arrow) developed in same eluent (chloroform : *n*-hexane; 7:3) and sprayed with vanillin.

An important observation here is that the R_f value of the inhibition zones were the same as that of the pure compound (taraxerol) isolated (chromatogram at the right).

8.3.2 Cancer cell growth inhibition by taraxerol

The 10 μg/ml concentrations of taraxerol did not inhibit growth of cell lines MCF-7, $WHCO_3$ and HeLa significantly different than the 100 μg/ml concentrations (Figure 8.4).

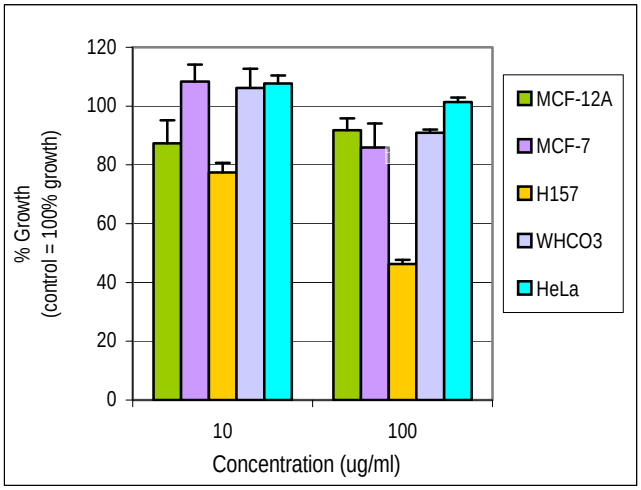

Figure 8.4. Effect of taraxerol, isolated from *Pteleopsis myrtifolia* leaves, on different human cell lines MCF-12A (non-cancerous breast), MCF–7 (breast adenocarcinosma), H157 (cancerous lung), $WHCO_3$ (cancerous oesophagus) and HeLa (cancerous cervix) at 10 μg/ml (left) and 100 μg/ml (right).

Taraxerol was inhibitory to cancer cell line H157 and this significant difference is indicated with a star at the 100 μg/ml in Figure 8.4. Figures 8.5 and 8.6 also show the inhibitory effect of taraxerol to the cancerous cell line H157 (lung).

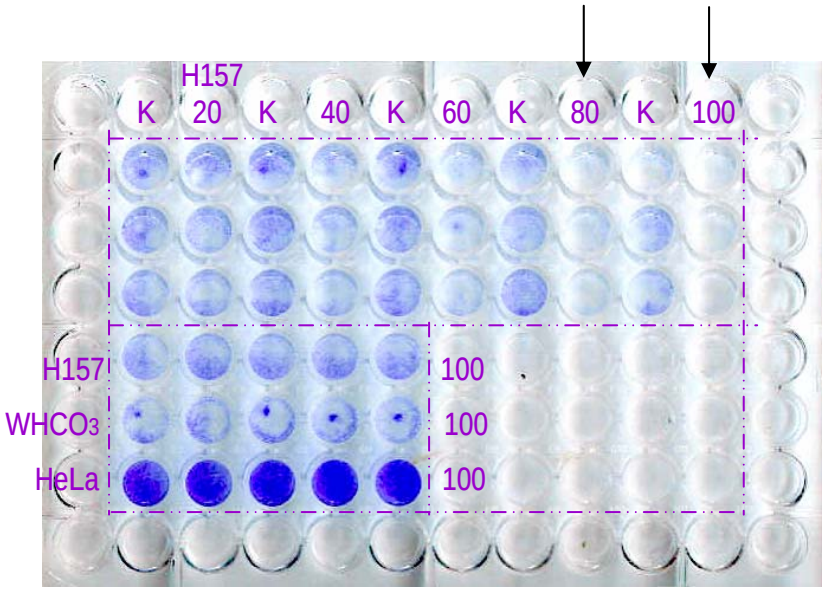

Figure 8.5. Scan of multiwell plates where taraxerol was tested at different concentrations (20, 40, 60, 80 and 100 μg/ml) against the H157 (lung) cancer cell line.

Lighter purple areas in Figure 8.5, (indicated with arrows) indicate less cancer cell growth.

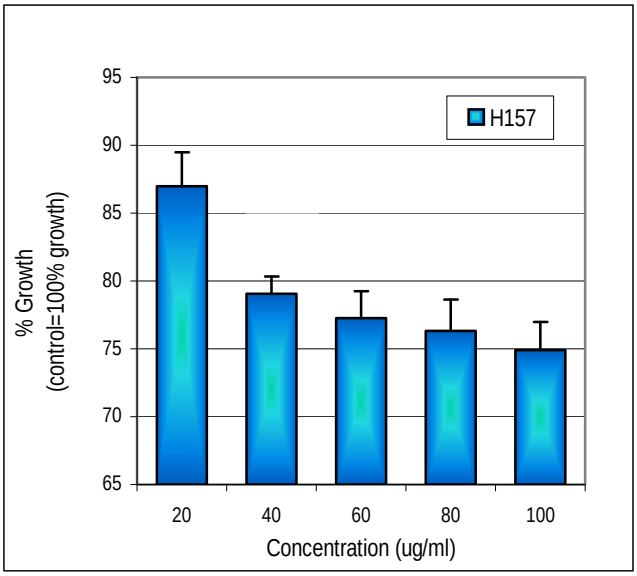

Figure 8.6. Graph of the effect of different concentrations of taraxerol (20, 40, 60, 80 and 100 μg/ml), isolated from *Pteleopsis myrtifolia* leaves, on the human cancer cell line H157 (lung).

Taraxerol's effect on the H157 cell line at 20 μg/ml concentration's were significantly less than the 40, 60, 80 μg/ml concentration's effects and this is indicated by a above the 20 μg/ml. In addition, its effect at 60 μg/ml was significantly less than at 100 μg/ml and this is indicated by a above the 60 μg/ml.

Taraxerol did not reach GI50 or LC values for the concentrations examined. No other reports of taraxerol's activity for the human cell lines tested could be found.

8.3.3 Antioxidant activity of taraxerol

8.3.3.1 Dot-blot DPPH staining procedure

The dot-blot assay indicates coloured (yellow) spots where aliquots of compounds with free radical scavenger activity were placed on the TLC plate. A more intense yellow colour is indicative of increased antioxidant activity. The purple area on the plate indicates no free radical scavenging (antioxidant) activity. Although extracts of *P. myrtifolia* leaves gave

antioxidant (free radical scavenger) activity (the extract 14-taraxen-3-ol was isolated from as well) in a previous study (Chapter 7), taraxerol did not have any antioxidant activity (lane at the right in Figure 8.7, indicated with an arrow).

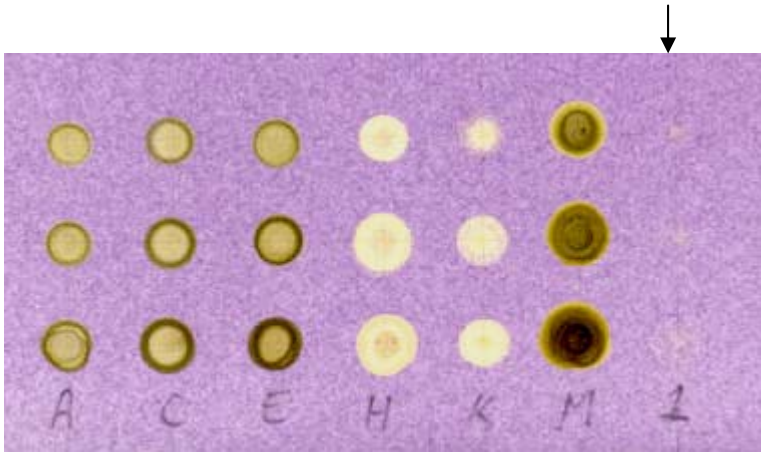

Figure 8.7. Scan of a dot-blot test of a thin layer chromatography plate sprayed with a 0.4 mM solution of 1,2-diphenyl-2-picrylhydrazyl in methanol after extracts A, C, E, H, K, M and 1 were applied (A = acetone extract, C = chloroform extract, E = ethanol extract, H = hot water extract, K = cold water extract, M = methanol extract and 1 = taraxerol). The dot blots applied were 20 μg (bottom row), 10 μg (middle row) and 5 μg (top row).

Cisplatin is a standard anticancer drug that does not have antioxidant activity (like taraxerol). It is extremely toxic and it acts as an alkylating agent (Yasuda *et al.,* 2000).

8.4 Summary and comparison of taraxerol's activity

Taraxerol had MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli* respectively. A *Terminalia sericea* extract that contained terminoic acid and had a MIC value of 0.33 mg/ml for *S. aureus* (lower than taraxerol's MIC for *S. aureus*), was developed into a topical ointment for use (Kruger, 2004). In an experiment with mice, this ointment was found to be more effective than commercial Gentamycin cream. This ointment may find a veterinary application. If Taraxerol could be commercialised as an ointment it may be even more effective than the ointment prepared from *Terminalia* extracts because of

its lower MIC. This might however, not be a viable option due to limited distribution and numbers of *P. myrtifolia* trees, insufficient leaves and considering conservational aspects. The Pharmaceutical developer would have to find ways to synthesize taraxerol. Alternatively, other plants that contain this terpenoid and that occur more abundantly, might be used to isolate it from. In this study 7.7 mg was isolated from 1 kg of dried leaf material, indicating that a big amount of dry leaves will be needed. To prepare 30 ml of a 10 mg/ml cream, provided the yield of taraxerol is similar, 39 kg of dried leaves would have to be extracted. A leaf extract from *Pteleopsis* trees may find an application by rural people who live in the vicinity of trees and who can gather leaves to apply a crude leaf extract to skin infections.

MIC values found from pure compounds isolated from members of Combretaceae in previous investigations, are listed in Table 8.4.

Taraxerol's MIC values indicated that it was very active against *E. faecalis* and *S. aureus*. Its' MIC values to the Gram-negative bacteria were not as low.

Taraxerol did not have significant antioxidant activity (although the fraction it was isolated from, had).

Taraxerol significantly inhibited growth of the human lung cancer cell line H157. It was previously reported to have a less defined mode of action against tumour cells (Harrewijn *et al*., (2001). It, together with 10 other triterpenoids from the roots of *Taraxacum japonicum* (Compositae) were examined for its inhibitory effects on Epstein-Barr virus early antigen (EBV EA) at the University of Kyoto (Takasaki *et al*., 1999). This antigen was induced by the tumour promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), in Raji cells as a primary screening test for anti-tumour promoters (cancer chemopreventive agents). Of these triterpenoids, taraxasterol and taraxerol exhibited significant inhibitory effects on EBV-EA induction. Further-

Table 8.4. Minimum inhibitory concentration values from pure compounds isolated from members of Combretaceae.

Compounds from Combretaceae	MIC values in μg/ml			
	S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*
Taraxerol	40	16	630	310
Combretastatin B5[1]	16	>250	125	125
5 hydroxy-7,4'-dimethoxyflavone [2]	>100	50	100	50-100
Rhamnazin [2]	>100	25	100	100
Rhamnocitrin [2]	50-100	25-50	100	100
Genkwanin [2]	50-100	50-100	100	100
Terminoic acid [3]	330	-	-	-
Alpinentin [4]	40	40	130	250
Pinocembrin [4]	80	40	300	130
Flavokwavaine [4]	40	400	300	600
Ampicillin[c]	80	160	125	160
Chloramphenicol[c]	160	40	125	160

[1] = Famakin (2003), [2] = Martini (2002), [3] = Kruger (2004), [4] = Serage (2003), [c] = control

more, these two compounds exhibited potent anti-tumour promoting activity in the two-stage carcinogenesis tests of mouse skin using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter. (Takasaki *et al*., 1999). The inhibition of TPA co-carcinogenesis took place because signal-regulated cyclic AMP-dependant protein kinases were inhibited. Taraxerol can also inhibit proteases by targeting trypsin and being anti-inflammatory to phorbol ester-induced inflammation (Polya, 2003).

8.5 Conclusions

Results found in this study contributed to knowledge of the phytochemistry of Combretaceae – that taraxerol occur in the leaves of *P. myrtifolia.* This is the first time it was isolated from *P. myrtifolia* leaves.

No literature reporting on the MIC values of taraxerol could be found, and this might be the first report of taraxerol's MIC values against the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli.* It had good antibacterial activity, a MIC of 0.016 mg/ml against the Gram-positive *E. faecalis.*

Taraxerol significantly inhibited growth of the human lung cancer cell line H157. Growth inhibition of the H157 cell line was significantly less at 20 μg/ml than at 60 μg/ml and 100 μg/ml. No other reports of taraxerol's effect on human cell lines could be found.

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Chapter 9

General discussion, conclusions and recommendations

9.1 Less known genera of the Combretaceae

Previous work on the African Combretaceae has concentrated mainly on the genus *Combretum* and to a lesser extent on *Terminalia*. This is probably because they occur in larger numbers over a wide geographical area and consequently are easier to collect (Katerere, 2001). This study extended phytochemical and pharmacological investigations to other related genera of Combretaceae, *Pteleopsis* and *Quisqualis.* Initially, investigations involved extracts of *Pteleopsis myrtifolia* leaves and fruit as well as of *Quisqualis littorea* leaves, but later investigations involved only *P. myrtifolia* leaves. This was due to the fact that fruit were only available during a part of the season and not in large enough quantities. The material available from *Quisqualis,* was from a relatively young climber, and leaves were not available in large enough quantities.

9.2 Antibacterial activity of extracts from *P. myrtifolia* and *Q. littorea*

A primary objective of this investigation was to investigate the antibacterial activity of extracts of *P. myrtifolia* leaves and fruit and *Q. littorea* leaves. MIC values, thin layer chromatography and bioautography indicated that extracts of *P. myrtifolia* leaves and fruit and *Q. littorea* leaves contain several antibacterial compounds. *Q. littorea* leaf extracts exhibited good activity against the Gram-negative bacterium, *E coli*, in contrast to previous reports where the majority of plants did not have activity against Gram-negative bacteria (Vlietinck *et al*., 1995). Isolation of compounds from an extract of *Q. littorea* holds the potential to lead to an antibiotic for Gram-negative bacteria, but was not investigated due to lack of enough material.

9.3 Growth inhibition effect of *P. myrtifolia* leaf extracts on human cell lines

Another objective of this investigation was to investigate the cytotoxic activity of extracts of *P. myrtifolia* leaves. The growth of the cancerous cell line $WHCO_3$ (which was the most differentiated from the cell lines used) was inhibited by all plant extracts - 20 μg/ml had significant less growth inhibition than 60 μg/ml. The growth of cell lines MCF-7 and H157 were inhibited by all plant extracts except the methanol without tannin extract - the 20 μg/ml had significant less growth inhibition than the 100 μg/ml concentration. The HeLa cell line's growth was inhibited by all plant extracts except the hot water and chloroform extracts, with significant less growth inhibition at 20 μg/ml than 100 μg/ml.

Extracts with tannin inhibited the growth of the cancer cell lines MCF-7 and HeLa more than the same extracts without tannins. For the H157 cell line, extracts without tannin inhibited the growth more than extracts with tannins. A possible explanation could be that the H157 cells were more sensitive to tannins present than the other cell lines. After the tannins were removed, the active compound(s) had more pronounced activity. This was not the case for the other cell lines, as they were less sensitive to tannin or responded to other compounds in the extract.

GI_{50} values were reached for all extracts ≥ 100 μg/ml for the MCF-7 and $WHCO_3$ cell lines. All, except the chloroform extract attained GI_{50} values for the HeLa cell line. No extracts reached GI_{50} values for the MCF-12A cell line, and only the hot water, methanol and chloroform extracts exhibited their GI_{50} values for the H157 cell line. LC values were attained for the cold water, hot water, methanol and ethanol extracts for the MCF-7, $WHCO_3$ and HeLa cell lines. No extracts reached constant lethal concentration values for the cell lines H157 and MCF-12. Some extracts exhibited their GI_{50} values but not their LC values at a specific concentration for a specific cell line, especially with $WHCO_3$ cell line. This was the desired effect, since the ideal

would be to find plant extracts or compounds that inhibit cell growth, but that are not toxic (lethal). The cell lines differed in their sensitivity to the plant extracts and were most sensitive to the hot water and methanol extracts.

9.4 Antioxidant activity of *P. myrtifolia* leaf extracts

The antioxidant activity of extracts of *P. myrtifolia* leaves was also investigated. All *P. myrtifolia* leaf extracts gave positive scavenging capacity (antioxidant activity) with DPPH, but the quantity differed for all different extracts. Compared to black tea, where one gram of dry mass, had a vitamin C equivalent of 0.126 mg, the cold water extract of *P. myrtifolia* leaves, had a vitamin C equivalent of 0.34 mg, more than twice that of black tea. Further, the vitamin C equivalent of the methanol and hot water extracts were both more than that of black tea, 0.20 and 0.147 mg/g respectively.

9.5 Solvent-solvent separation to find best fraction for purification

Bioassay-guided fractionation by solvent-solvent separation identified that all fractions: *n*-hexane, carbon tetrachloride, chloroform, 35% water in methanol, *n*-butanol and water had antibacterial activity for at least two of the bacteria tested. The chloroform fraction was antibacterial to all bacteria tested, and seven areas of inhibition were observed. This fraction was chosen for further purification and isolation of compounds. The *n*-hexane fraction also had high antibacterial activity and contained mostly waxes and fat-like substances. It was not considered for further development, because waxes cannot be utilised in medicine due to the non-specificity for host or bacterial cells as well as their poor pharmacokinetic profile (Martini, 2002).

9.6 Pure compounds isolated from *P. myrtifolia* leaves

In total, 17 pure compounds were isolated from the chloroform fraction of *P. myrtifolia* leaves.

One compound, a triterpenoid taraxerol, was present in sufficient quantities for structure elucidation. It is not a novel structure, but is reported for the first time in this species. Taraxerol is usually bound in the leaf matrix of some plant's leaves, probably ester-bonded to cutin (Killops and Frewin, 1994). One might expect this rather non-polar compound to be associated with the *n*-hexane fraction, not the chloroform fraction which it was isolated from in this investigation. The presence of saponins offers an explanation to non-polar substances being soluble in the chloroform fraction.

Results found in this study contributed to knowledge of the phytochemistry of Combretaceae – that taraxerol occur in the leaves of *P. myrtifolia.* Taraxerol was isolated from another genus of Combretaceae, namely from *Terminalia glabrescens* leaves by Garcez *et al*., in 2003.

9.7 Bioactivity of taraxerol

When the bioactivity of taraxerol was investigated (the final objective for this study), no other reports about its antibacterial activity could be found. It had MIC values of 0.04, 0.016, 0.63 and 0.31 mg/ml for the bacteria *S. aureus*, *E. faecalis, P. aeruginosa* and *E. coli* respectively. In *Terminalia sericea* an extract which contained terminoic acid and had a MIC value of 0.033 mg/ml for *S. aureus*, was developed into a topical ointment for veterinary use (Kruger, 2004). In an experiment with mice, this ointment was found to be more effective than commercial Gentamycin cream. Since *T. sericea*'s activity is less than taraxerol's activity for *S. aureus*, taraxerol's commercialisation as an ointment may be even more effective than the ointment prepared from *Terminalia* extracts. This is however, not a viable option due to (as mentioned earlier), the low yield of taraxerol from leaves, limited distribution and numbers of *P. myrtifolia* trees and thus insufficient leaves. Before the spectrum of antimicrobial activity is further researched, a Pharmaceutical developer may not be convinced that it can be specific enough for identified bacteria. A leaf extract from *Pteleopsis* trees may find an application by rural

people who live in the vicinity of trees and who can gather leaves to apply a crude leaf extract (as an antibiotic) for topical infections.

Taraxerol did not in itself have free radical scavenging (antioxidant) activity, but the extract it was isolated from, had.

Taraxerol significantly inhibited growth of the human lung cancer cell line H157. With the examined concentrations of up to 100 µg/ml, the H157 cell line did not reach GI_{50} values or lethal concentrations. The activity of the extracts could have been due to synergy of more than one compound.

Results from this study indicated that extracts from *P. myrtifolia* and *Q. littorea* had antibacterial activity, that extracts from *P. myrtifolia* leaves had cytotoxic and antioxidant activity, and that a pure compound from *P. myrtifolia* leaves had antibacterial and cytotoxic activity.

9.8 Recommendations for future

Future research on antibacterial extracts will benefit by using apparatus that can handle large quantities of dried material throughout the extraction and chromatography process. According to a company representative of Shimoda in the Eastern Cape, they have developed an apparatus for investigating bulk material. When using samples of up to five kilograms of dried material to isolate pure compounds, it is highly probable that compounds will not be in sufficient quantities to determine their chemical structures. The complexity of plant material from Combretaceae as well as too small quantities of possible pure compounds have also been reported by Famakin (2002), Serage (2003) and Kruger (2004).

Where a large enough quantity of plant material from *Q. littorea* can be found, isolation of compounds from an extract with activity for Gram-negative bacteria should be persued, since

it holds the potential to lead to an antibiotic for Gram-negative bacteria.

The use of optical density of bacterial cultures should be explored to see of results of different laboratories or researchers might be more uniform.

An investigation of the antimicrobial activity of taraxerol against many bacteria and fungi is recommended. As more knowledge about its specific activity becomes available, it might be that, although it is a non-polar triterpenoid, its activity against specific bacterial cells can be very well defined and be commercialised. It is recommended that it be tested in *in vivo* experimental conditions in future.

9.9 Literature references

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