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Phytochemical analysis and antimicrobial activity of *Piper capense* L.f.

by

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"*So the earth produced all kinds of plants, and God was pleased with what he saw.*" – *Genesis 1:12, GNT*

Abstract

Medicinal plants are the focus of intense study, in particular whether their traditional uses are supported by real pharmacological effects, or merely based on folklore. *Piper capense* L.f. (Piperaceae) is used traditionally for the treatment of infectious diseases, and has the potential to be a source of novel antimicrobial compound(s).

Crude solvent extracts (water, methanol, hexane and acetone) and sequentially extracted subfractions of the root-bark of *P. capense* were prepared, of which the hexane-soluble subfraction $M_SA_SH_S$ was identified as the most promising antimicrobial subfraction. Phytochemical analyses of the various extracts and subfractions using TLC with numerous mobile phases and compound selective visualising reagents revealed the presence of quinones in all of the crude solvent extracts. Alkaloids, lipids/sterols/steroids, phenolic compounds and amino acids/peptides were detected in select subfractions.

Gradient reverse phase HPLC analyses using 0.1% formic acid and methanol indicated three major peaks in $M_SA_SH_S$. IR spectroscopy indicated that carbonyl and hydroxyl functional groups, and aromatic characteristics were present in the major compound present in $M_SA_SH_S$. Further analysis using targeted LC-MS Q-TOF and quadrupole LC-MS/MS analyses indicated an empirical formula of $C_{11}H_8O_3$. This formula was confirmed for the isolated compound by GC-MS (HP5-MS column) that identified the compound as 5-hydroxy-2-methyl-1,4-naphthoquinone ($C_{11}H_8O_3$ MW: 188.18) with 98% certainty using the database. Although 5-hydroxy-2-methyl-1,4-naphthoquinone (also known as plumbagin) is well-known, this is the first time that the presence of this compound is reported in the *Piper* genus.

Antimicrobial activities of *P. capense* root-bark extracts and the subfractions were determined against Gram-negative and Gram-positive bacteria and a yeast strain using the disk diffusion and broth micro-dilution assays. Antimicrobial activity was observed against Gram-positive bacteria, Gram-negative bacteria as well as a yeast strain, indicating broad spectrum activity. The antimicrobial activities of the crude solvent extracts decreased in the order: acetone > methanol > hexane > water. The $M_sA_sH_s$ subfraction demonstrated the highest antimicrobial activity with an MIC of 29 µg/ml against both *Staphylococcus aureus* (ATCC 12600) and *Candida albicans* (ATCC 10231). HPLC eluents of this subfraction that were collected in a drop-wise fashion onto silica TLC plates and assayed by bioautography, indicated that the major compound eluting at 13.6 minutes accounted for most of the antimicrobial activity.

Antioxidant activity was observed for the crude water extract, crude methanol extract, crude acetone extract, $M_sA_sA_s$ subfraction as well as the $M_sA_sH_s$ subfraction.

Cytotoxicity against mammalian cells in culture was observed for the crude methanol extract, crude acetone extract, crude hexane extract and the $M_sA_sH_s$ subfraction when determined using C_2C_{12} cells as well as resting and PHA stimulated lymphocytes.

Stability testing of the $M_sA_sH_s$ subfraction revealed that the antimicrobial compounds found in this subfraction appear to be stable up to 30 days at both 25℃ and 40℃ when assayed against *S. aureus*. However, when assayed against *C. albicans*, there was an increase in antifungal activity from 29 µg/ml to < 7 µg/ml after 30 days at both temperatures tested.

This study provides scientific support for the ethnomedical use of the root-bark of *P. capense* as an antimicrobial. To date, the presence of plumbagin has not been reported in any other plant in the *Piper* genus. Due to the

significant cytotoxic activity against mammalian cells reported in the current study and the mechanism of action of plumbagin, the therapeutic potential of *P. capense* extracts is very limited due to non-selective cytotoxicity, despite its marked antimicrobial activity.

Keywords: Antimicrobial, cytotoxicity, *Piper capense* L.f., phytochemical, plant extracts, plumbagin.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACD	Advanced Chemistry Development
AMB	Amphotericin B
AP	Ampicillin
amu	Atomic mass units
ATCC	American type culture collection
CAE	Crude acetone extract
CFU	Colony forming units
CHE	Crude hexane extract
CME	Crude methanol extract
CWE	Crude water extract
DAD	Diode array detector
DBE	Double bond equivalent
DMEM	Dulbecco's Minimum Eagle's Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenylpicrylhydrazyl
ESI	Electrospray ionisation
FCS	Foetal calf serum
GC	Gas chromatography
GM	Gentamicin
HPLC	High performance liquid chromatography
IC_{50}	50% Inhibitory concentration
INT	p-Iodonitrotetrazolium violet
IR	Infrared
LC	Liquid chromatography
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline
PHA	Phytohemagglutenin
PPM	Parts per million
Q-TOF	Quadrupole time-of-flight mass spectrometer
R*f*	Retardation factor
ROS	Reactive oxygen species
RP	Reverse phase
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
TEAC	Trolox equivalence antioxidant capacity
TIC	Total ion current/ Total Ion chromatogram
TLC	Thin layer chromatography
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TWC	Total wavelength chromatogram
UV	Ultraviolet light
V	Volt
WHO	World Health Organisation
Z	Charge

Chapter 1

Introduction

1 Overview

Infectious diseases are a major concern in Africa (Neuwinger 2000), and have been described as the primary cause of premature death, claiming the lives of almost 50 000 people each day (Ahmad and Beg 2001). Therapy of microbial infection is a problem, due to the emergence of strains resistant to currently available antimicrobials (Koné et al. 2004). One such example is the Methicillin-resistant *Staphylococcus aureus* (MRSA), which is often linked with nosocomial infections (Gibbons et al. 2003). There have been reports where these organisms were resistant even to vancomycin (Gibbons et al. 2003), an antibiotic that is reserved as a treatment of last resort. Resistant strains have emerged because of their remarkable genetic plasticity, as well as the heavy selective pressures of antimicrobial use that compel them to mutate (Kunin 1993). These resistant microorganisms are able to spread easily due to the mobility of the world population (Kunin 1993). An alarmingly narrow range of antimicrobials are still effective against certain pathogenic bacteria (Louw 2002) and this has a negative impact on both human health and the economy.

Different mechanisms are utilized by the microorganisms to protect themselves against antibiotics. These include exclusion of molecules by means of the cell wall, efflux pumps which the bacteria use to pump out unwanted molecules, and enzymatic breakdown of molecules that enter the bacteria (Anomaly 2010). Modulation of bacterial multidrug resistance (MDR) may be one solution to this problem, by targeting the efflux mechanisms of the bacterial cell wall. This could be a practical target to reverse resistance of these organisms as these effect-modulating drugs could be taken with currently available antimicrobials, in order to have a synergistic therapeutic effect (Gibbons et al. 2003). Compounds that modified MDR were isolated in

a study by Gibbons et al. (2003), and were found to be twice as effective when used together with currently available antimicrobials, they at the same therapeutic doses.

A pattern is visible with the discovery, abundant use and sometimes predictable obsolesces that has been repeated after the introduction of each new antimicrobial agent (Kunin 1993). To effectively manage the increasing problem of microbial resistance towards currently effective antibiotics, prevention of the further development of resistance as well as the containment of organisms that have already become resistant is required (Okeke et al. 2005). Most importantly, new antimicrobial compounds/combinations are needed for the treatment of patients.

People have used medicines derived from plants since antiquity (Gilani and Atta-ur-Rahman 2005). The World Health Organisation (WHO) defines herbal medicines as containing plant material in a crude or processed state as active ingredients and which may include excipients, but excludes combinations with chemically defined active substances or isolated constituents (Busse 2000). With the development of the pharmaceutical industry, synthetic drugs dominated the market and were mostly comprised of a single active chemical entity (Gilani and Atta-ur-Rahman 2005). Even from these synthetic drugs, around 25% of currently prescribed medicines are derived from higher plant compounds (Van Wyk et al. 2000; Louw 2002). In a study by Farnsworth et al. (1985), 119 plant metabolites used as drugs were identified, however this was more than twenty years ago. In a more recent review, about a third of the 1010 new chemical entities that became available between 1981 and 2006 were natural products or derivatives thereof (Newman and Cragg 2007). Even though these statistics are inspiring, it should be noted that not many drugs from higher plants have entered the orthodox medicine market in the last couple of decades (Gilani and Atta-ur-Rahman 2005). Some examples of drugs of plant origin include the following: aspirin, atropine, artemesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine,

quinidine, reserpine, taxol, tubocurarine, vincristine, and vinblastine, many of which were discovered in follow-up of leads from traditional usage (Gilani and Atta-ur-Rahman 2005).

South Africa is very rich in plant resources, containing 10% of the world's plant diversity (Eloff 1998a). The biological rationale for the production of secondary metabolites by plants as a defence mechanism makes plants a potential source for antibiotic lead compounds (Gibbons 2003).

Ethnobotany has been defined as "the study of the interrelationship of primitive men and plants" (Jones 1941). It is intriguing that people were aware that plants contained healing properties, before they even knew of the existence of microbes (Gilani and Atta-ur-Rahman 2005). The traditional use of plants serves as an important starting point to obtain new knowledge regarding plant compounds and its bioactivities. This may contribute to modern medicine. The knowledge of traditional medicine practice is regarded as an aid in the search for novel products against various human diseases (Louw 2002). Through careful screening of plant materials, new lead molecules for antimicrobial agents with different mechanisms of action may be discovered (Nostro et al. 2000), and this may be valuable in the treatment of resistant microorganisms (Eloff 1998a).

There are four different approaches for selecting plants with the aim of drug discovery: random selection of plants followed by chemical screening; random selection of plants followed by bioassays; following up on bioactivity reports or follow-up of ethnomedical or traditional uses of plants (Pieters and Vlietinck 2005). Preliminary screening of many plants for antibacterial and anti-inflammatory activity indicates a high correlation between traditional uses and true antimicrobial potential (Shale et al. 1999; Ahmad and Beg 2001; Vlietinck et al. 1995).

In South Africa, 72% of the black African population is estimated to use traditional medicine, which can be translated to an estimate of 26.6 million people (Mander et al. 2007). The WHO estimates that about 75% of the world population depends on traditional medicine to fulfil their healthcare needs (Gilani and Atta-ur-Rahman 2005). It is thus no wonder that medicinal plants have recently become the focus of intense study as to whether their traditional uses are supported by actual pharmacological effects, or merely based on folklore (Rabe and Van Staden 1997).

In an ethnobotanical survey carried out by Arnold and Gulumian (1984), a list of medicinal plants used by traditional healers in Venda was compiled. The authors reported that the bark of *P. capense* was used medicinally to prepare extracts for amongst others the treatment of infections and wounds. A subsequent report by Steenkamp et al. (2007a) tested 32 Venda medicinal plants for their antifungal activity against *C. albicans*. The bark of *P. capense* was found to exert the highest antifungal activity of all the plants tested. Furthermore, *P. capense* was shown to possess antibacterial activity (Steenkamp et al. 2007b).

2 *Piper capense* L.f. (Piperaceae)

The Piperaceae family is composed of about 10 genera of which most species belong to either *Piper* or *Peperomia* (Cronquist 1981). The genus *Piper* is comprised of an estimated 2000 species (Gurib-Fakim 2006). Distributed in the tropical regions of all the major continents, *Piper* species are often shrubs, herbs or lianas commonly found in forest undergrowth (Jaramillo and Manos 2001; Parmar et al. 1997). The greatest diversity of the *Piper* species is found in the American tropics with an estimated 700 species, followed by Southern Asia with an estimated 300 species (Jaramillo and Manos 2001). A wide distribution is also found in the southern half of Africa (Verdcourt 1996). One of the most well-known species from the Piperaceae is *Piper nigrum* L., better known as black pepper, because of its economic importance (Jaramillo and

Manos 2001). The *Piper* species have long been known for their various ethnomedical uses (Parmar et al. 1997). These plants are widely used in the Indian Ayurvedic system of medicine as well as Latin America and the West Indies (Parmar et al. 1997). The widespread ethnomedical use of *Piper* species has led to an increased interest in the search for active compounds from these species, and it has been found that many of these plants contain a number of biological activities (Koroishi et al. 2008).

A characteristic of the *Piper* family is the presence of pungent acidic amides e.g. piperine (Gurib-Fakim 2006). A number of physiologically active compounds have been isolated from the *Piper* species: alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones (Parmar et al. 1997). The first amide isolated from the *Piper* species was piperine, which possesses several biological activities (Parmar et al. 1997). Five phenolic amides isolated from *Piper nigrum* L. have been shown to contain significant antioxidant activity (Nakatani et al. 1986). Piperamides have been reported as containing effective insecticidal activity (Scott et al. 2008). Several compounds with reported antimicrobial activity have been isolated from various *Piper* species (Table 1-1).

Piper capense L.f. (*P. capense*) (Figure 1-1) is a member of the Piperaceae family, and is a pan-tropical shrub or sub-shrub (Verdcourt 1996). It is commonly known as ‘mulilwe’ in Venda, ‘wild pepper’ in English and ‘bospeper’ in Afrikaans (Watt and Breyer-Brandwijk 1962). There are many reports in the literature of the ethnomedical use of *P. capense* extracts (Table 1-2). Experimentally, antifungal (Samie et al. 2010; Steenkamp et al. 2007a, Green and Wiemer 2001; Green et al. 2001) and antibacterial (Steenkamp et al. 2007b) activity has been ascribed to the plant.

Table 1-1: Compounds with known antimicrobial activity isolated from various *Piper* species.

Plant(s)	Compound class	Compound	Reference
Piper aduncum	Amide	Aduncamide	Parmar et al. 1997
Piper aduncum	Benzoic acid derivative	3,5-Bis(3-methyl-2-butenyl)-4-methoxybenzoic acid	Parmar et al. 1997
Piper aduncum	Benzoic acid derivative	4-Hydroxy-3,5-bis(3-methyl-2-butenyl)benzoic acid	Parmar et al. 1997
Piper aduncum			Parmar et al. 1997
Piper aduncum	Benzoic acid derivative	4-Hydroxy-3-(3-methyl-2-butenoyl)-5-(3-methyl-2-butenyl) benzoic acid	Parmar et al. 1997
Piper aduncum	Benzoic acid derivative	Methyl 3-(3,7-dimethyl-2,6-octadienyl)-4-methoxybenzoate	Parmar et al. 1997
Piper aduncum	Benzoic acid derivative	Methyl 4-hydroxy-3-(3-methyl-2-butenyl)benzoate	Parmar et al. 1997
Piper aduncum	Propenylphenol	Pseudodillapiole	Parmar et al. 1997
Piper arboretum; *Piper hispidum;* *Piper tuberculatum*	Amide	Pyrrolidyne	Koroishi et al. 2008
Piper arboretum; *Piper hispidum;* *Piper tuberculatum*	Amide	Piperidine	Koroishi et al. 2008
Piper betle	Propenylphenol	Eugenol	Parmar et al. 1997
Piper betle	Propenylphenol	Allylpyrocatechol diacetate	Parmar et al. 1997

Piper betle	Propenylphenol	Chavibetol	Parmar et al. 1997
Piper betle	Propenylphenol	Chavibetol acetate	Parmar et al. 1997
Piper betle	Propenylphenol	Chavicol	Parmar et al. 1997
Piper betle	Propenylphenol	Hydroxychavicol	Parmar et al. 1997
Piper fadyenii	Propenylphenol	Pseudodillapiole	Parmar et al. 1997
Piper fulvescens	Benzofuran neolignans		Koroishi et al. 2008
Piper hispidum	Propenylphenol	Pseudodillapiole	Parmar et al. 1997
Piper regnellii	Neolignan	Eupomatenoid-3	Koroishi et al. 2008
Piper regnellii	Neolignan	Eupomatenoid-5	Koroishi et al. 2008
Piper sarmentosum	Propenylphenol	1-Allyl-2,6-dimethoxy-3,4-methylenedioxybenzene	Parmar et al. 1997
Piper sarmentosum	Propenylphenol	Asaricin	Parmar et al. 1997
Piper sarmentosum	Propenylphenol	c+Asarone	Parmar et al. 1997
Piper sarmentosum	Propenylphenol	y-Asarone	Parmar et al. 1997

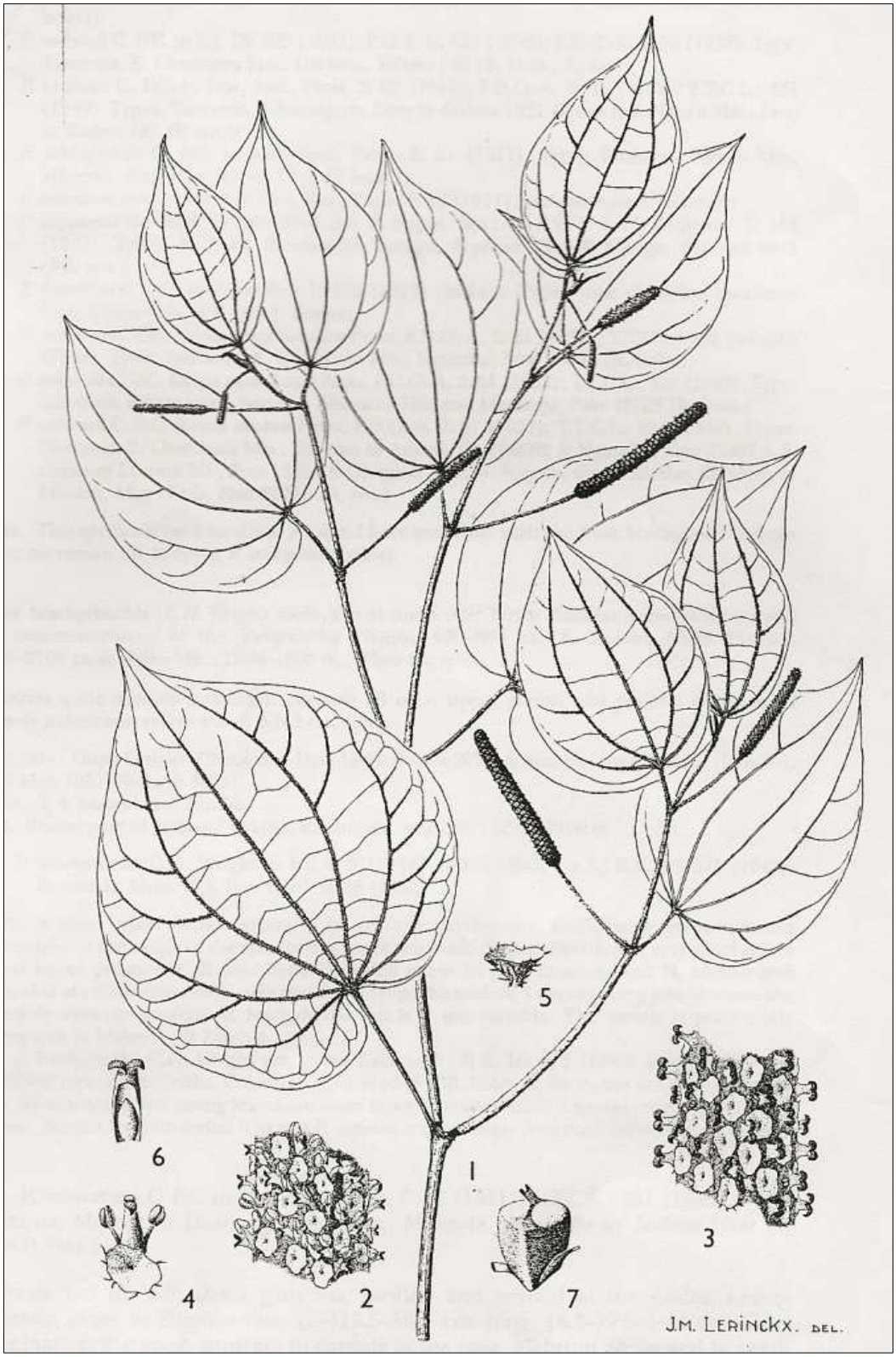


Figure 1-1: *Piper capense* L.f. leaf and fruit structure (Verdcourt 1996).

Table 1-2: Reported therapeutic uses of *P. capense* preparations in traditional medicine.

Therapeutic use	Part used and Preparation	Country	Reference
Sterility	Root - Decoction	NS	Arnold and Gulumian 1984
Wounds and vaginal discharge	Bark – material powdered and applied externally with petroleum jelly	NS	Arnold and Gulumian 1984
Sore throat, chest complaints, tongue ulcers, venereal disease	Bark - maceration is drunk	NS	Arnold and Gulumian 1984
Paralysis caused by cerebral hemorrhage	Root – ointment made and applied to soles of the feet	NS	Watt and Breyer-Brandwijk 1962
Stomach, heart and kidney diseases	Fruit - brandy or water infusion is drunk	Europe, Africa	Watt and Breyer-Brandwijk 1962
Anthelmintic	Root - decoction	Shambala	Watt and Breyer-Brandwijk 1962
Sexual stimulant	Root - eat raw or cooked	Shambala, Pare	Watt and Breyer-Brandwijk 1962
Cough remedy	Root - sweetened decoction	NS	Watt and Breyer-Brandwijk 1962
Cough remedy	Fruit - NS	India, East Africa	Watt and Breyer-Brandwijk 1962
Diuretic and vermifuge	NS - NS	South Africa	Watt and Breyer-Brandwijk 1962
Impetigo	NS - preparations	NS	Neuwinger 2000
Poliomyelitis	Leaves – extracts is drunk	NS	Neuwinger 2000

Abdominal disorders	Leaves – decoction used as enema	NS	Neuwinger 2000
Cough	Leaves – extract is drunk	NS	Neuwinger 2000
Anthelmintic	Seed – decoction is drunk	NS	Neuwinger 2000
Kwashiorkor	Root – decoction is drunk	NS	Neuwinger 2000
Sexual weakness	Leaves – eaten raw of cooked	NS	Neuwinger 2000
Stomachic and carminative in indigestion, flatulence and colic.	NS - NS	S. Tomé e Príncipe	Martins et al. 1998
Diarrhoea	Aerial part or leaves - crunched raw to swallow down the juice	Comoro islands	Kaou et al. 2008
Cough	Aerial part or leaves - NS	Comoro islands	Kaou et al. 2008
External parasitism and acaricide	NS - NS	Rwanda (cows)	Krief et al. 2005
Stomach pains, ulcers, fever, flatulence and kidney disease	NS - NS	Venda	Obi et al. 2002
Malaria	Leaves - boiled	Kenya	Koch et al. 2005
Depression	Roots – mixed with okisusheeit	Kenya	Koch et al. 2005
Sleep inducing remedy	Roots - NS	South Africa	Pedersen et al. 2009

NS: Not specified.

Phytochemical analyses have shown that phenolics are the most frequently isolated compounds found in *P. capense*, and it can be hypothesized that these compounds could be responsible for the antimicrobial activity (Louw 2002). Alkaloids are the second most abundant group of compounds found in these plants and are very common in herbaceous dicotyledonous plants, such as *P. capense* (Louw 2002). An example of an alkaloid that has been extracted from *P. capense* is piperine (Watt and Breyer-Brandwijk 1962; Pedersen et al. 2009). Chavicine, an isomere of piperine, has been reported to be the pungent compound present in *P. capense* (Watt and Breyer-Brandwijk 1962). Another amide, 4,5-dihydropiperine, has been isolated from the roots (Pedersen et al. 2009). The first true alkaloid group that was extracted from the *Piper* species is oxaporphines (Neuwinger 1996). *Piper* species are not generally known for their abundance in terpenes, but monoterpenes have been isolated as the main component of *P. capense* essential oils (Martins et al. 1998). A novel sesquiterpenoid named capentin has also been isolated (Chen et al. 1992). Studies done by the University of Iowa, found 7 new aromatic neolignan compounds in *P. capense* (Green and Wiemer 1991; Agrios 1997), these are phytoestrogens with antioxidant activity and may also possess antimicrobial activity. Despite the promising antimicrobial activity of this plant, an extensive search of the literature depicts that there have been no studies to determine which compound(s) could be responsible for the antimicrobial activity in *P. capense* extracts. This lack of information with respect to the antimicrobial activity prompted the investigations in the current study.

3 Study Aim

The aim of this study was to identify and characterise the active compound(s) responsible for the antimicrobial activity in *P. capense* L.f. (Piperaceae) root-bark extracts.

4 Study Objectives

The objectives of the study were to:

- Determine the antimicrobial activity of crude solvent extracts of *P. capense* against American type culture collection (ATCC) strains of *C. albicans*, *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* using the disk diffusion assay;
- Quantify the antimicrobial activity of crude plant extracts using the broth micro-dilution assay;
- Identify the most promising subfraction using differential solubility, liquid-liquid extraction, thin layer chromatography and high performance liquid chromatography;
- Re-assess the subfractions for biological activity;
- Determine the presence of various phytochemical groups in the crude solvent extracts and subfractions primarily by qualitative analysis employing thin layer chromatography (TLC) and various chemical class selective spray reagents and ultraviolet light visualisation;
- Determine the presence of antioxidant activity in the crude solvent extracts as well as the subfractions by means of a TLC based method as well as the 2,2-diphenylpicrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assays;

- Attempt to isolate and chemically characterise and identify the most active compound through the utilisation of chromatographic and spectroscopic techniques;
- Determine *in vitro* cytotoxicity of the crude solvent extracts and most promising subfraction against mammalian cells;
- Assess the stability of the most promising subfraction.

Chapter 2

Materials and Methods

1 Extraction and phytochemical characterisation

1.1 Plant material

Root-bark of *Piper capense* L.f. (Piperaceae) was collected in Venda and a voucher specimen (LT16) is lodged at the herbarium in the Department of Toxicology, Onderstepoort Veterinary Institute (Pretoria). Identity of the plant was confirmed by the South African National Biodiversity Institute (Tshwane).

Plant material was inspected for any contamination, air-dried at room temperature, ground to a fine powder (Ika Analytical Mill) and stored in sealed brown bottles in a dark area, until extracts were prepared.

1.2 Extraction

A flow-diagram showing the step-wise procedure for extraction and fractionation is provided in Figure 2-1.

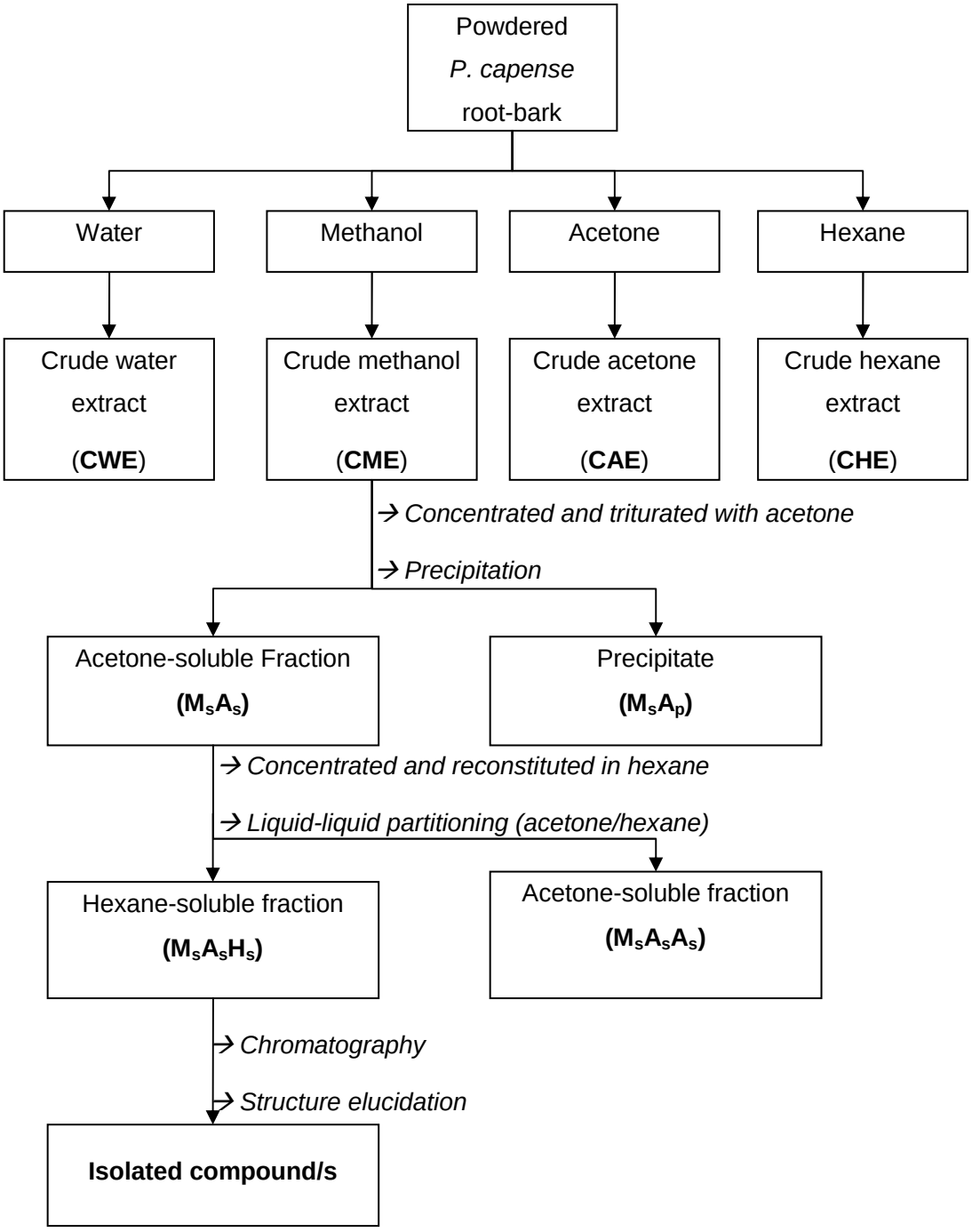


Figure 2-1: Flow diagram representing the extraction process of *P. capense*.

1.2.1 Crude extracts

A mass of 1 g of powdered plant material was added to 10 ml solvent (distilled water, methanol, acetone or hexane). Tubes were lightly shaken and then sonicated in a ultrasonic bath for 30 min. Preparations were allowed to stand for 24 h in the refrigerator (4℃) after which the extracts were centrifuged at 400 *g* for 15 min. The supernatants were removed and filtered sequentially through 0.45 µm and 0.22 µm syringe filters (Millipore). Extracts were stored sealed at -18℃ until used in order to limit chemical decomposition.

The crude methanol extract (CME) was subjected to further fractionation. This extract was concentrated down to 10% of the initial volume under a constant flow of nitrogen gas where after which it was triturated with acetone, resulting in a precipitate (M_sA_p) and supernatant (M_sA_s). The supernatant (M_sA_s) was concentrated under a flow of nitrogen gas, after which liquid-liquid extraction (acetone/hexane) was employed. The different solutes distributed themselves between the two layers, the top layer ($M_sA_sH_s$) and the bottom layer ($M_sA_sA_s$). Yields were determined gravimetrically throughout.

1.3 Phytochemical screening

1.3.1 Thin Layer Chromatography (TLC)

To assess complexity of the crude extracts and the subfractions aliquots (5 µl) were spotted onto normal phase silica gel plates (Si_{60} F_{254}; Macherey-Nagel Alugram) and developed using a mobile phase that consisted of methanol:water (50:50, v/v). Separated compounds were visualised under ultraviolet as well as visible short and long wave as light (UV; 254 nm and 360 nm; Camag Universal UV lamp, TL-600). Retardation factors (R*f* values) for the specific compounds visualised were calculated as follows:

$$Rf = \frac{\text{Distance run by solute}}{\text{Distance run by solvent front}}$$

The presence of specific phytochemical groups was determined using the methods as described by Stahl (1969) (Table 2-1).

1.3.2 High performance liquid chromatography (HPLC) fingerprinting

HPLC was carried out using an Agilent 1100 instrument with a diode array detector (DAD) and C18 column (150 mm × 4.6 mm × 5µm; Alltech). All mobile phases were made up using HPLC grade solvents. The binary mobile phase consisted of A: water (0.1% formic acid) and B: methanol (0.1% formic acid). The gradient program was as follows: 20% B for 2.5 min, 20% B to 80% B in 16.5 min, 80% B for 2 min, return to initial conditions in 2 min and re-equilibrate for 3 min. The total run time was 26 min at a flow-rate of 1.0 ml/min. The injection volume was 20 µl of a 20 mg/ml solution.

Table 2-1: Spray reagents used for the detection of various phytochemical classes (Stahl 1969).

Spray reagent	Preparation
Antimony (III) chloride	10% Solution of antimony (III) chloride in chloroform
Chromic acid-sulphuric acid	5 g Potassium dichromate dissolved in 110 ml 40% sulphuric acid
Diphenylpicryl-hydrazyl (DPPH)	0.06 g DPPH (Sigma) was dissolved in 100 ml chloroform
Dragendorff's reagent	Solution A: 1.7 g basic bismuth nitrate dissolved in 20 ml acetic acid and 80 ml water. Solution B: 40 g potassium iodide in 100 ml of water. 5 ml of solution A was added to 5 ml solution B and 10 ml acetic acid and 70 ml water.
Folin-Ciocalteu	Spray I: 20% aqueous sodium carbonate. Spray II: Folin-Ciocalteu reagent (Sigma) was diluted with three times its volume water before spraying. Spraying was carried out using Spray I first, briefly drying the TLC plate and then Spray II was applied
Molybdo-phosphoric acid	5% Solution of molybdophosphoric acid in ethanol
Ninhydrin	0.3 g Ninhydrin (Sigma) dissolved in 100 ml n-butanol and 3 ml acetic acid added
Potassium hydroxide	5% Solution of potassium hydroxide in methanol
Sodium hydroxide	5% Solution of sodium hydroxide in ethanol
Sulphuric acid	50% concentrated sulphuric acid in methanol
Vanillin-sulphuric acid	1 g Vanillin dissolved in 100 ml 40% concentrated sulphuric acid in methanol

1.4 Structure elucidation

1.4.1 Infrared spectroscopy

Infrared (IR) spectra were obtained using KBr windows prepared by applying the hexane solution of the $M_SA_SH_S$ subfraction onto standard KBr windows and allowing the solvent to evaporate. The KBr disks were scanned a minimum of 32 scans between 4000 and 400 wave numbers with a 2 cm^{-1} resolution on a Bruker Tensor 27 spectrophotometer and the data collected and analysed with OPUS software version 5.5 (Bruker).

1.4.2 Liquid chromatography-mass spectrometry (LC-MS/MS)

Analysis was performed on an Agilent 6530 series quadrupole time-of-flight mass spectrometer (Q-TOF) with an electrospray ionisation (ESI) source operated in both the negative and positive ionization modes. The purified $M_SA_SH_S$ subfraction was diluted to a concentration of 0.1 mg/ml in methanol and 5 µl injected via a 1369A autosampler. This was separated on a Zorbax SB-C3 column (100 mm × 2.1 mm × 3.5 µm particle size) at 300 µl/min using an isocratic mobile phase of 0.1% formic acid in water/acetonitrile (50:50) for the first 5 min followed by a gradient increasing the acetonitrile to 80% by 10 min before returning to the starting conditions at 10.5 min. A re-equilibration time of 3 min was allowed before the next injection. The column eluent was introduced directly into an Agilent 6530 series Q-TOF mass spectrometer.

Mass spectrometer conditions were as follows: ESI at 4000 V (or -4000 V) and 300ºC with drying gas flow rate of 10 l/min N_2. Scans were monitored from 50 – 1000 amu in positive mode and from 50 – 1100 amu in negative mode. The MassHunter software was programmed to scan automatically for a precursor at 1 scan/sec and MS/MS at 3 scans/sec when a compound was detected. Collision energy of 4 V/100 amu with a +15 V offset was used. A targeted scan was performed on all compounds detected at the compound

mass using a 20 V collision energy and 1 scan/sec. The data was collected for 12 min and analysed by MassHunter workstation software.

1.4.3 Gas chromatography-mass spectrometry (GC-MS)

The purified $M_SA_SH_S$ extract was prepared by diluting 5 µl of the hexane solution in 100 µl of methanol which resulted in a sample concentration of 0.1 mg/ml. A volume of 1 µl of this sample was injected using an autosampler into a split mode injector with a 10:1 split ratio. The temperature program increased at 10ºC/min and was run from 60ºC to 300ºC using an HP5-MS column (Agilent) (30 m in length, 250 µm internal diameter and 0.25 µm thickness). The MS parameters were set to scan for compounds 36 – 600 amu in size. EI was at -70 V. The data was analysed with the Agilent ChemStation with a National Institute of Standards and Technology (NIST) natural product GC-MS library.

2 Biological activity

Biological activity was determined at the indicated stages (Figure 2-2) using the methods described below.

2.1 Antimicrobial activity

2.1.1 Microorganisms

Antimicrobial activity was determined against two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 12600) and *Staphylococcus epidermidis* (clinical isolate, Department of Microbiology, NHLS, Pretoria), two Gram-negative bacteria: *Escherichia coli* (ATCC 1175) and *Pseudomonas aeruginosa* (ATCC 9027) as well as the yeast; *Candida albicans* (ATCC 10231). Stock cultures of *S. aureus* and *S. epidermidis* were maintained on MacConkey Agar with salt, whereas *C. albicans*, *E. coli* and *P. aeruginosa* cultures were maintained on Mueller-Hinton Agar (Davies Diagnostics) at 4℃.

2.1.2 Preparation of Inocula

Inocula were freshly prepared from 24 h subcultures in sterile saline (0.85%) which was colorimetrically adjusted (Sherwood) until a turbidity of 0.5 McFarland standard was reached at a wavelength of 560 nm.

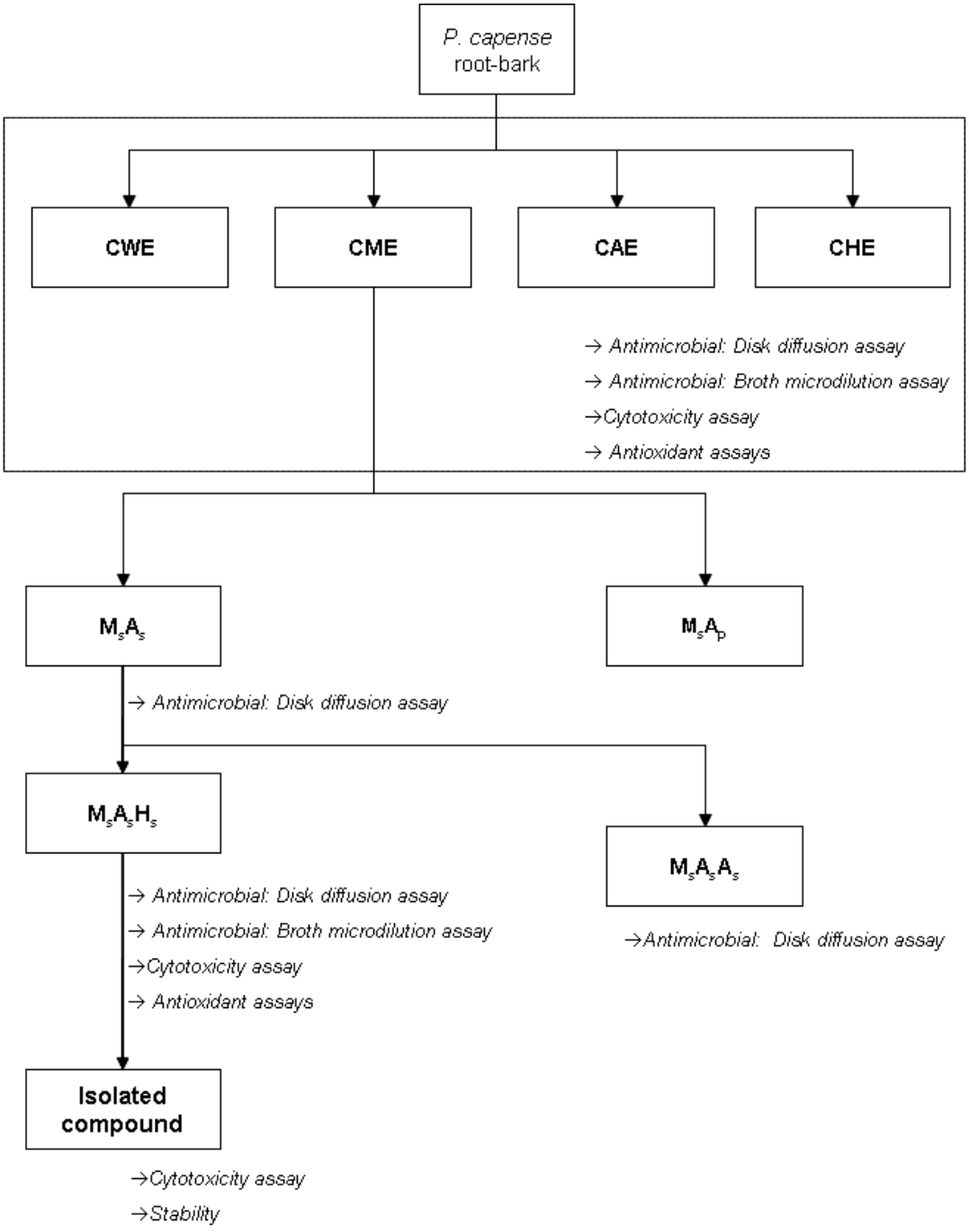


Figure 2-2: Flow-diagram representing the bioassays performed on the crude extracts/subfractions.

2.1.3 Disk Diffusion Assay

The method as described by Bauer et al. (1966) was used to determine antimicrobial activity. Sterile filter paper discs (Whatmann, 10 mm) were impregnated with 200 µl of the respective plant extract. The discs were allowed to dry as to ensure that all solvent was driven off. A Petri dish was filled with 23 ml of Mueller-Hinton Agar and allowed to gel. A volume of 100 µl of the specific bacterial/fungal culture inocula was spread on the surface of each plate and the test sample impregnated filter paper discs were placed on the Agar. Plates were incubated at 37℃ for 24 h. Antimicrobial activity was expressed as the mean diameter of the zone of inhibition (mm) around the discs as measured with a calliper. For positive controls antibiotic discs (Ampicillin (AP), 10 µg – Gram-positive bacteria; Gentamicin (GM), 10 µg – Gram-negative bacteria; Amphotericin B (AMB), 20 µg - yeast) (Mast diagnostics) were placed on similarly prepared plates with the appropriate bacterial/fungal culture. A negative control was prepared using the respective test sample solvent only.

2.1.4 Broth micro-dilution Assay

The broth micro-dilution assay as described by Eloff (1998b) was used. Serial two-fold dilutions (1000 – 31.125 µg/ml) of the plant extracts were made using Mueller-Hinton broth. A volume of 150 µl of each dilution was then transferred into the wells of a 96-well microtitre plate. A volume of 50 µl inocula (1×10^5 CFU/ml) was added to the wells to give a final volume of 200 µl. Plates were incubated at 37˚C for 24 h, after which 30 µl of a 0.2 mg/ml aqueous solution of p-iodonitrotetrazolium chloride (INT, Sigma) was added to the wells. The plates were allowed to incubate further until maximum colour intensity had developed (± 30 min). The extracts/subfractions were prepared as follows: non-aqueous extracts were evaporated to dryness in vaccuo at 40˚C, after which the dry residue was re-dissolved in ± 2-3 ml dimethyl sulfoxide (DMSO) (Merck). These

preparations were further diluted with distilled water to obtain the relevant concentrations of test compounds. The final concentration of DMSO in the extract was < 2.5% (v/v). Aqueous extracts were freeze-dried, and re-dissolved in distilled water to obtain the relevant concentrations. Antibiotics were included as positive control (0.062 – 2 µg/ml) and 2.5% DMSO as solvent control. Wells containing 150 µl of Mueller-Hinton Broth without plant extract or antibiotics were used as untreated growth controls. Inhibition of microbial growth was indicated by the failure of the well to change colour, whereas bacterial/fungal growth resulted in a pink colour. The minimum inhibitory concentration ($MIC_{p\text{-}INT}$) was defined as the lowest concentration of plant extract that inhibited the reduction of INT, indicating a reduction of microbial viability.

2.1.5 Bioautography

Bioautography was carried out according to the method of Begue and Kline (1972) which was modified by Hamburger and Cordell (1987). TLC chromatograms were developed in methanol:water (50:50) (Chapter 2, Section 1.3.1) and air dried overnight at ambient temperature to allow for the evaporation of solvents from the plate. Plates were sprayed with a saturated suspension of either *S. aureus* or *C. albicans* in growth media until fairly wet but not enough to run. The plates were incubated overnight at 37℃ at 100% relative humidity. After incubation the plates were sprayed with a 2 mg/ml solution of INT in water (Sigma). Inhibition of microbial growth was visible as white zones against a pink background on the chromatographic plate. Bioautography was also carried out in the same way on subfractions collected from HPLC analysis where the eluents of the HPLC analyses were collected manually in a drop-wise fashion onto large silica TLC plates where after they were left to air dry at ambient temperature for 48 h to ensure that all formic acid had evaporated.

2.2 Antioxidant activity

2.2.1 DPPH radical scavenging activity

The effects of the crude extracts as well as the $M_sA_sH_s$ subfraction on DPPH radical were determined using the method of Liyana-Pathirana and Shahidi (2005), with minor modifications. A solution of 0.135 mM DPPH (Sigma, SA) in methanol was prepared and 185 µl of this solution was mixed with 15 µl of varying concentrations of the extract (0.125, 0.25, 0.5 and 1 mg/ml) in a 96-well plate. The reaction mixture was vortex mixed and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 570 nm using a microplate reader (Bio-Tek Instruments, Inc.). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Fluka, SA) was used as the reference antioxidant compound. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{contol} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of DPPH radical + methanol and A_{sample} is the absorbance of DPPH radical + sample extract/standard.

2.2.2 ABTS radical scavenging activity

The method of Re et al. (1999) was adopted for the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay. ABTS radical stock solution was freshly prepared 12 - 16 h before use, and stored in a dark cupboard. The resultant ABTS radical solution was diluted with methanol to obtain an absorbance of 0.700 ± 0.001 at 734 nm. Varying concentrations (0.125, 0.25, 0.5 and 1 mg/ml) of the extract (20 µl) were allowed to react with 2 ml of the ABTS radical solution and the absorbance (734 nm) was recorded spectrophotometrically. The ABTS radical scavenging capacity of

the crude extracts as well as the $M_sA_sH_s$ subfraction was compared to that of Trolox and the percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of ABTS radical + methanol and A_{sample} is the absorbance of ABTS radical + sample/standard. The ABTS radical scavenging activity (%) was plotted as a function of concentration of extracts and Trolox. The gradient obtained from the graph of a particular sample was divided by the gradient of the Trolox graph to yield a Trolox equivalence antioxidant capacity (TEAC) value.

2.3 Cytotoxicity

2.3.1 Human lymphocytes

Venous blood was collected in heparin vacutainers (Becton Dickinson) from healthy volunteers (blanket ethics approval obtained for Department of Pharmacology from the Research Ethics Committee of the University of Pretoria). The lymphocytes were isolated according to the method of Anderson (1993). Heparinized blood (35 ml) was loaded onto 15 ml Histopaque 1077 (Sigma-Aldrich). The lymphocyte monolayer was harvested and transferred to sterile 50 ml centrifuge tubes. The tubes were filled with sterile Roswell Park Memorial Institute (RPMI) 1640 medium, without bovine foetal calf serum (FCS), and then centrifuged at 200 *g* (room temperature) for 15 min. After discarding the supernatant, the pellet was gently mixed and the tube filled with 10 % RPMI medium. The suspension was centrifuged for a further 10 min at 200 *g* after which the supernatant was discarded, the pellet manually mixed and the tube filled with ice-cold ammonium chloride (NH_4Cl) for red blood cell lysis induction. The cell suspension was left on ice for approximately 10 min, to allow any remaining

red blood cells to be lysed. The suspension was again centrifuged at 200 *g* (room temperature) for 10 min, and the supernatant discarded. Tubes were filled with RPMI 1640 medium containing 10% bovine FCS. The suspension was centrifuged for a further 10 min at 200 *g* (room temperature). The supernatant was discarded and the pellet resuspended in 1 ml of 10% RPMI medium containing 10% bovine FCS. The concentration of lymphocytes was determined by adding 50 µl of the cell suspension to 450 µl counting fluid. This suspension was loaded onto a haemocytometer and cells were counted using a Reichert-Jung Microstar 110 microscope at a 40 times magnification. The cells were re-suspended to obtain a concentration of 2 × 10^6 cells/ml.

Into 96-well plates (AEC-Amersham P/L) was placed 60 µl RPMI 1640 medium containing 10% bovine FCS and 100 µl cell suspension (2 × 10^6 cells/ml). Plates were incubated at 37℃ in a 5% CO_2 atmosphere for 60 min after which 20 µl of the experimental extracts/subfractions at varying concentrations was added. Both resting and phytohemagglutenin (PHA) – stimulated lymphocytes were used to determine cytotoxicity. Stimulated lymphocytes received 20 µl PHA whereas the untreated (resting) lymphocytes received 20 µl of RPMI 1640 medium containing 10% bovine FCS. The final volume in each of the wells was 200 µl. Plates were incubated for 3 days where after cell enumeration was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay (Mossman 1983).

2.3.2 C_2C_{12} cells

C_2C_{12} mouse myoblast cells (CRL-1772) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS. These cells were passaged every three days. The culture medium was discarded and 5 ml Trypsin Versene (obtained from the National Institute for communicable Diseases, Johannesburg, SA) was added in order to rinse

the cells. The Trypsin Versene was then discarded and a further 15 ml of Trypsin Versene was added to the cells. The flask was placed in the incubator where the cells were incubated for 20 min at 37℃ in a 5% CO_2 atmosphere until the cells were detached from the flask. Five millilitres of medium supplemented with FCS were added to the cells in order to neutralize the action of the Trypsin Versene. The latter suspension was then aspirated to a 15 ml centrifuge tube and centrifuged at 200 *g* for 5 min. The supernatant was discarded after which 1 ml medium supplemented with FCS were added to the cell pellet and aspirated to form a suspension.

Into 96-well plates (AEC-Amersham P/L) was placed 80 µl of DMEM medium and 100 µl cell suspension (50 000 cells/ml). Plates were incubated at 37℃ in a 5% CO_2 atmosphere for 60 min after which 20 µl of the experimental extracts/subfractions at varying concentrations was added. The final volume in each of the wells was 200 µl. Plates were incubated for 3 days where after cell enumeration was determined using the MTT assay (Mossman 1983).

2.3.3 MTT assay

The method of Mossman (1983) was employed. After the incubation period, 20 µl of a MTT solution (5 mg/ml phosphate buffered saline (PBS) was added to each well. Plates were incubated for another 4 h at 37℃ in a CO_2 incubator where after they were centrifuged at 800 *g* for 10 min. The supernatant was removed and the pellet washed by adding 150 µl PBS. The cells were left to dry whereafter 100 µl DMSO was added to solubilise the formazan crystals by shaking for 1-2 h. These crystals are formed when enzymes, that are present in the mitochondria of viable cells, reduce the yellow coloured MTT to a purple coloured formazan. The absorbance of the DMSO/formazan solution was determined spectrophotometrically at a wavelength of 570 nm and a reference wavelength of 630 nm. For the cytotoxicity assays, the extracts/subfractions were prepared as follows: non-

aqueous extracts were evaporated to dryness in vacuo at 40˚C, after which the dry residue was re-dissolved in ± 2-3 ml DMSO (Merck). These preparations were further diluted with distilled water to obtain the relevant concentrations. The final concentration of DMSO in the sample was < 0.5% (v/v). Aqueous extracts were freeze-dried, and re-dissolved in distilled water to obtain the relevant concentrations.

2.4 Stability

The open plate and accelerated stability tests were performed according to The United States Pharmacopoeia (1999) principles. The $M_sA_sH_s$ subfraction was exposed to 60% relative humidity at 25℃ and 40℃ respectively for 30 days. The subfractions were assayed for antimicrobial activity at day 0 and day 30 as described in Section 2.1.4 (Chapter 2). HPLC analysis (Section 1.3.1, Chapter 1) was also carried out to assess whether there were any changes in peak areas and that a similar chromatographic fingerprint for the compounds was obtained on days 0 and 30.

3 Statistical analysis

Tests were carried out where possible at least in triplicate and on three different occasions. Results for the disk diffusion assay are expressed as mean ± SD. For the DPPH radical scavenging assay the 50% Inhibitory concentration (IC_{50}) values were calculated from linear regression plots using GraphPad Prism® 4 software. In the ABTS radical scavenging assay the gradient of each graph was also determined using GraphPad Prism® 4 software. Cytotoxicity results are expressed as the percentage cell survival compared to the untreated control using a non-linear dose response curve (curve fit), and by choosing a bottom constraint of 0% for the sigmoid dose-response (variable slope), which extrapolated to the concentration at which 50% of cells survived (IC_{50}). The curve was created using GraphPad Prism

4 Software©. LC-MS/MS results were analysed using the MassHunter workstation software. The compounds analysed using GC-MS were identified with the Agilent ChemStation software which has a NIST library of mass fragmentations.

Chapter 3

Results and Discussion

1 Phytochemical characterisation

A herbal extract can be described as compounds/mixtures of compounds that have been retrieved from either fresh or dry plant parts by different extraction procedures (Assis et al. 2006). The ideal extraction method should retain the compounds with the biological activity of interest and eliminate most of the unwanted compounds. In this study, crude extracts were prepared with water, methanol, acetone and hexane in order to extract compounds across the polarity spectrum. Although water is the solvent most commonly used by traditional healers, it is not necessarily the solvent that will produce optimal extraction of plant compounds (Louw 2002).

It is known that the compounds and activity in an extract may differ depending on various factors including time/date of collection and the plant part (Houghton and Raman 1998). To ascertain which of the root-bark, inner root or whole root material were more active, it was decided to first compare the different parts of root material prior to any other experiments. The yields of these extracts were determined in relation to the original dry mass of the material used (Figure 3-1). By determining yields throughout the study ensures that a degree of efficacy and safety is measured (Houghton and Raman 1998). In order to compare the complexity of the extracts visually, TLC analysis was carried out (Figure 3-2). Although TLC provides rapid qualitative information, it is limited in that it has poor detection in comparison to other techniques such as HPLC (Gurib-Fakim 2006). CME and CWE of the whole root, and CWE of the root-bark each contained four major compounds (*Rf* values of 0.42, 0.76, 0.89 and 0.95) whereas CME of the inner root contained only two major compounds (*Rf* values of 0.42 and 0.95).

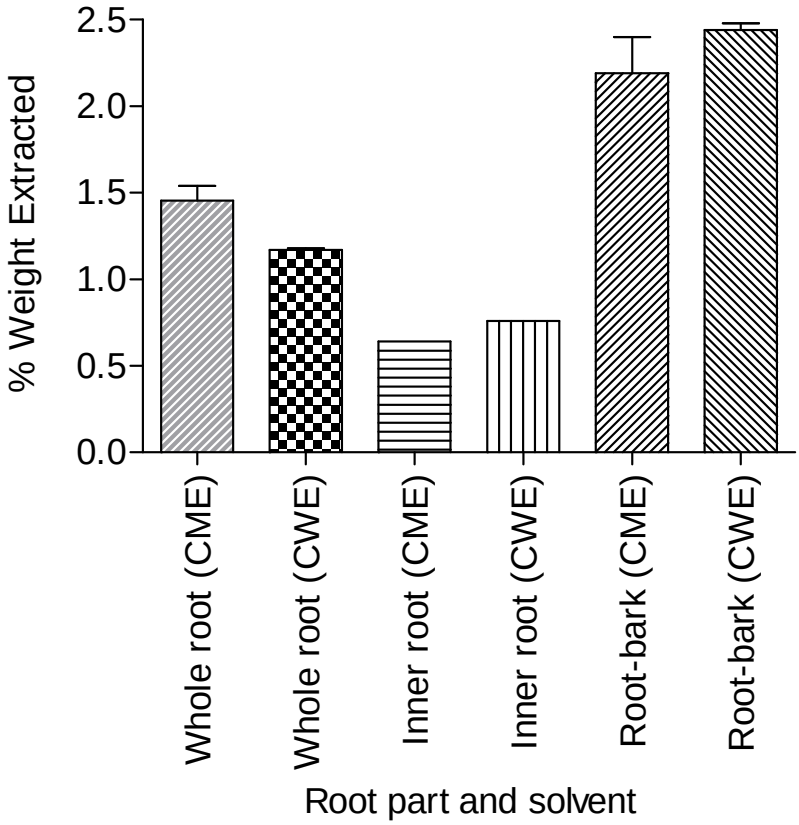


Figure 3-1: Extraction efficiency of extracts prepared from the whole root, inner root and root-bark material of *P. capense* respectively, based on original dry mass. The extraction efficiency is presented as average % weight extracted (n=2).

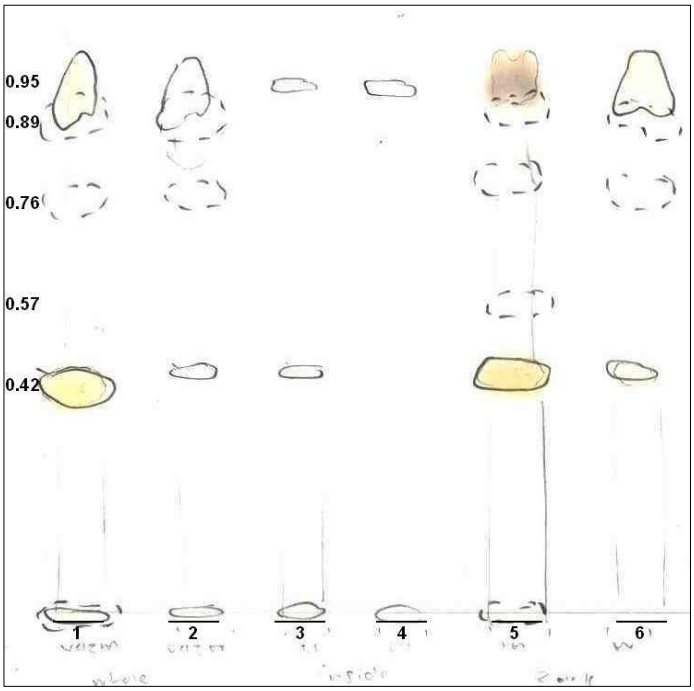


Figure 3-2: TLC chromatogram on a normal phase silica plate, developed in methanol:water (50:50). 5 µl of extracts were spotted from left to right: 1: CME of the whole root material; 2: CWE of the whole root material; 3: CME of the inner root material; 4: CWE of the inner root material; 5: CME of the root-bark material; 6: CWE of the root-bark material. UV visualisation: dotted lines represent compounds detected at 360 nm and solid lines represent compounds detected at 254 nm.

CWE of the inner root contained only one compound (*Rf* value of 0.95) and CME of the root-bark contained five compounds (*Rf* values of 0.42, 0.57, 0.76, 0.89 and 0.95). The inner root material did not contain as many compounds when compared to the whole root or root-bark material. The compound with an *Rf* value of 0.42 was found to be present in all extracts except the CWE of the inner root material. From the colour intensity of the spots it would appear as if the root-bark material contained higher concentrations of compounds when compared to the whole root material. This finding together with the antimicrobial results which were run in parallel, led to the conclusion that the root-bark would be used for all further analyses.

The water and methanol solvents of the root-bark provided the highest yields (Figure 3-3). It would appear as if there are higher concentrations of polar compounds present in the root-bark material of *P. capense*, than non-polar compounds. TLC analysis of the different solvent extracts indicated that hexane extracted the least compounds from the root-bark (Figure 3-4). Three apparently similar compounds were visualised for each of CWE, CME and CAE (*Rf* values of 0.45, 0.77 and 0.93) and only one compound in CHE (*Rf* value of 0.45*)*. All the extracts contained the compound with an *Rf* value of 0.45. The high *Rf* values indicated that the compounds were of a less polar nature.

Yields of the subfractions were also determined in relation to the original CME yield (Figure 3-5). The yields indicated that most of the compounds remained in M_sA_s, as the percentage weight extracted was high (67.24%). After liquid-liquid partitioning, the majority of compounds remained in $M_sA_sA_s$. TLC analysis indicated the presence of three major compounds in M_sA_s (*Rf* values: 0.45, 0.77 and 0.93) and four compounds in $M_sA_sA_s$ (*Rf* values: 0.45, 0.75, 0.91 and 0.93) as can be seen in Figure 3-6. $M_sA_sH_s$ appeared to be the least complex and only two compounds could be visualised (*Rf* values: 0.45 and 0.93).

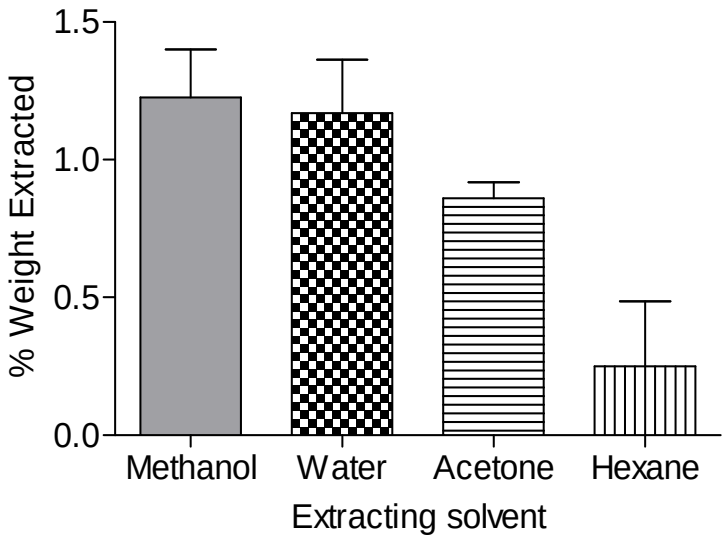


Figure 3-3: Extraction efficiency of different solvents for the crude extracts prepared from *P. capense* based on original dry mass. This is presented as % weight extracted ± SD (n=4).

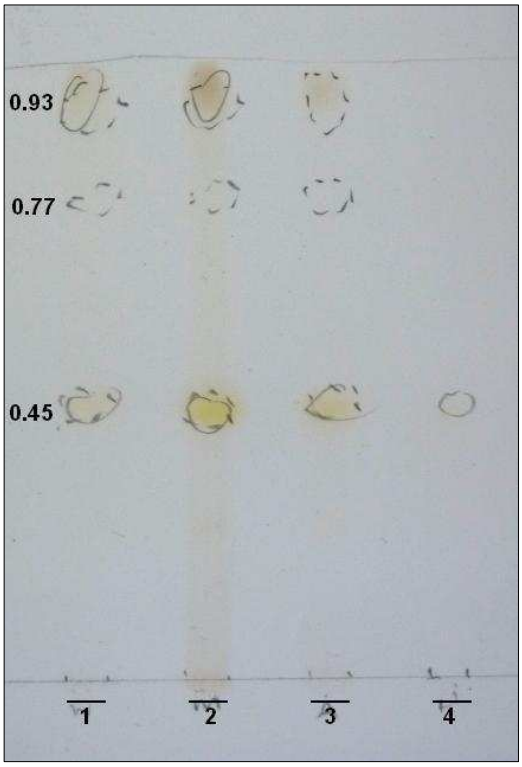


Figure 3-4: TLC chromatogram of the crude extracts of *P. capense* after development in methanol:water (50:50). From left to right: 1: CWE; 2: CME; 3: CAE; 4: CHE. UV visualisation: dotted lines represent compounds detected at 360 nm and solid lines represent compounds detected at 254 nm. R*f* values are indicated on the left side of the plate.

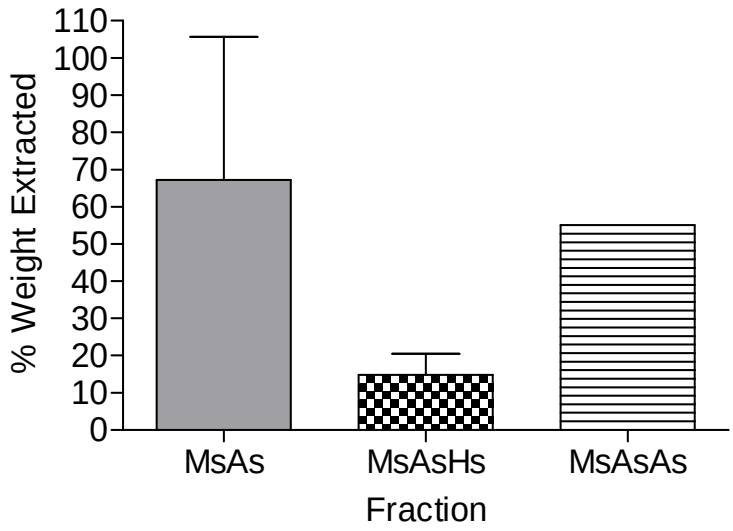


Figure 3-5: Weight distribution for the M_sA_s, $M_sA_sH_s$ and $M_sA_sA_s$ subfractions prepared from *P. capense* CME, as a percentage of CME. The distribution is presented as % weight extracted ± SD (n=4, except $M_sA_sA_s$ where n=1).

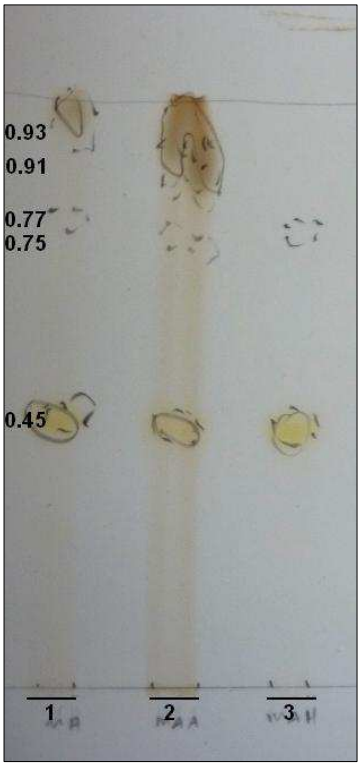


Figure 3-6: TLC chromatogram of the resultant subfractions prepared from CME after development in methanol:water (50:50). From left to right: 1: M_sA_s; 2: $M_sA_sH_s$; 3: $M_sA_sA_s$. UV visualisation: dotted lines represent compounds detected at 360 nm and solid lines represent compounds detected at 254 nm. R*f* values are indicated on the left side of the plate.

TLC has been described as the chromatographic system with the widest application in phytochemistry (Harborne 1998). The reason is that it can be applied to nearly all classes of compounds with the exception of very volatile compounds. It is therefore a useful tool in the preliminary detection of classes of compounds in extracts/subfractions, which may assist in the consequential elucidation of a structure of a compound with biological activity. The findings of the phytochemical assays is summarised in Table 3-1.

Dragendorff's spray reagent had a positive colour reaction for $M_SA_SA_S$, indicating the presence of alkaloid/s (Stahl 1969). Alkaloids are the second most abundant group of compounds found in plants and are very common in herbaceous dicotyledonous plants, such as *P. capense* (Louw 2002). Several alkaloids have previously been isolated from the *Piper* species, with the most prominent being piperine (Parmar et al. 1997). Aduncamide has also been isolated and this compound has been reported to possess antimicrobial activity (Parmar et al. 1997). Kaousine, an amide alkaloid, has been extracted from *P. capense* (Kaou et al. 2010).

Many terpenes/terpenoids have been isolated from the *Piper* species to date (Parmar et al. 1997). In *P. capense* the sesquiterpenoid, capentin, has been isolated from the roots and monoterpene hydrocarbons from the aerial parts (Chen et al 1992; Martins et al. 1998). However, no terpenes/terpenoids were visualised in any of the crude extracts/subfractions in the current study.

Table 3-1: Phytochemical classes detected in *P. capense* crude extracts/subfractions.

Phyto-chemical group	Extract/Subfraction						
	CWE	**CME**	**CAE**	**CHE**	**M_sA_s**	**$M_sA_sA_s$**	**$M_sA_sH_s$**
Alkaloids	–	–	–	–	–	+	–
Terpenes/ terpenoids	–	–	–	–	–	–	–
Flavonoids	–	–	–	–	–	–	–
Phenolic compounds	+	+	+	-	+	+	+
Quinones	+	+	+	+	+	+	+
Primary amines	+	+	–	–	+	+	–
Lipids/ sterols/ steroids	–	–	–	–	–	+	–

+: present.

–: absent.

With the exception of CHE, all the crude extracts and subfractions tested positive for the presence of phenolic compounds. A number of propenylphenols have previously been isolated from *Piper* species, and many of these have been shown to contain antimicrobial properties (Parmar et al. 1997). Phenolic compounds are known to act as antimicrobials either by inhibition of enzymes by the oxidized compounds or by reacting with sulfhydryl groups but also by nonspecific interactions with proteins (Cowan 1999). Coumarins are phenolic compounds which may, amongst others, have antimicrobial activity (Cowan 1999). However, the presence of coumarins was not confirmed in any of the extracts/subfractions.

No flavonoids could be visualised in any of the extracts/subfractions tested upon spraying with antimony (III) chloride. To date there are no reports on the identification of flavonoids in *P. capense*, and very few flavones and flavanones have been isolated from the *Piper* species (Parmar et al. 1997).

All of the extracts/subfractions showed a positive reaction upon spraying with sulphuric acid which, although not exclusively, may be indicative of the presence of quinones. Quinones also fall under the phenolic group of compounds as they contain an aromatic ring with two ketone substitutions (Cowan 1999). These compounds are known to irreversibly bind with nucleophilic amino acids in proteins which in return may effect a loss of function to the protein – one of the ways in which it exerts its antimicrobial effects (Cowan 1999).

The presence of primary amines was confirmed in CWE, CME, M_SA_S and $M_SA_SA_S$ with the use of the ninhydrin spray reagent. Peptides may be inhibitory to microorganisms through either forming ion channels in the microbial membrane or by inhibiting the adhesion of microbial proteins to host receptors (Cowan 1999).

The presence of lipids, long chain alcohols, phenols, polyphenols and terpenes could be visualised in $M_SA_SA_S$ with the use of molybdophosphoric acid. The presence of phenols in the $M_SA_SA_S$ subfraction was revealed earlier, but this is the first indication of terpenes in any of the extracts/subfractions. This presence of the lipids is an indication that this subfraction is not pure, as lipids are not known to have antimicrobial activity and may interfere with downstream assays (Verpoorte 1998). The presence of these compounds would also have been expected in M_S and M_SA_S, but this was not observed.

HPLC fingerprinting was done for $M_SA_SH_S$ (Figure 3-7). Three major peaks were evident on the chromatogram eluting at 11.38, 11.81 and 13.60 min.

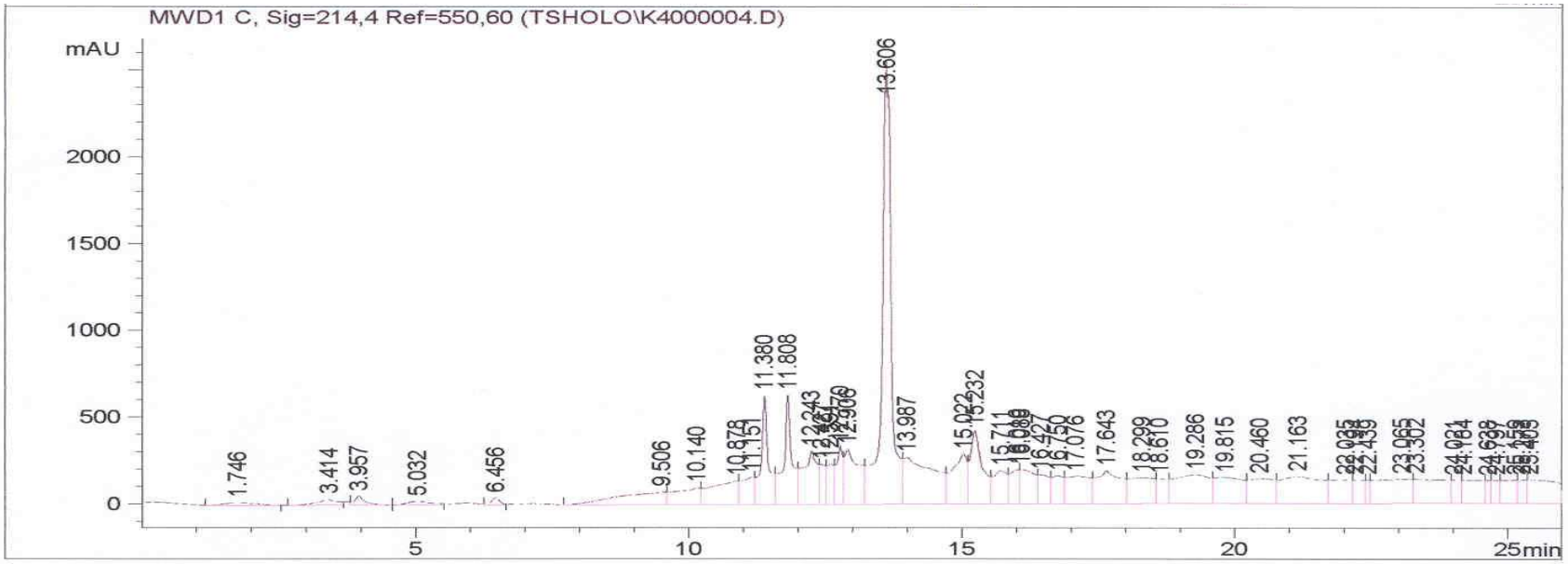


Figure 3-7: HPLC chromatogram of $M_sA_sH_s$ recorded at a wavelength of 214 nm. The mobile phase was run as a gradient consisting of water (0.1% formic acid) and methanol (0.1% formic acid).

2 Structure elucidation

IR spectroscopy yields results by spectral absorbance bands or peaks in the IR spectrum above 1200 cm^{-1} that is created by the vibrations of bonds or functional groups in the molecule (Harborne 1998). The region below 1200 cm^{-1} is known as the "fingerprint" region and is very complex because bands that appear here are due to vibrations of the whole molecule (Harborne 1998). For $M_sA_sH_s$ (Figure 3-8), the fingerprint region shows very little detail below 1200 cm^{-1} and this may be indicative of either the lack of substitution patterns or due to traces of impurity often present in natural samples (Harborne 1998).

The fact that $M_sA_sH_s$ was hexane-soluble indicated that the compound of interest was lipophilic. The broad very strong absorbance band around 1700 cm^{-1} is suggestive of a carbonyl group being present in the molecule. The band in the region of 3000 cm^{-1} may indicate that the compound is aromatic in nature and may contain an alkyl group. There also appears to be a hydroxyl present (3600 to 3200 cm^{-1}), which is consistent with the fact that we know the compound is not an acid as there was no salt formation on addition of a base. IR spectrum is a relatively simple and reliable method to assign a compound to a compound class because of the ease of identification of many functional groups (Harborne 1998).

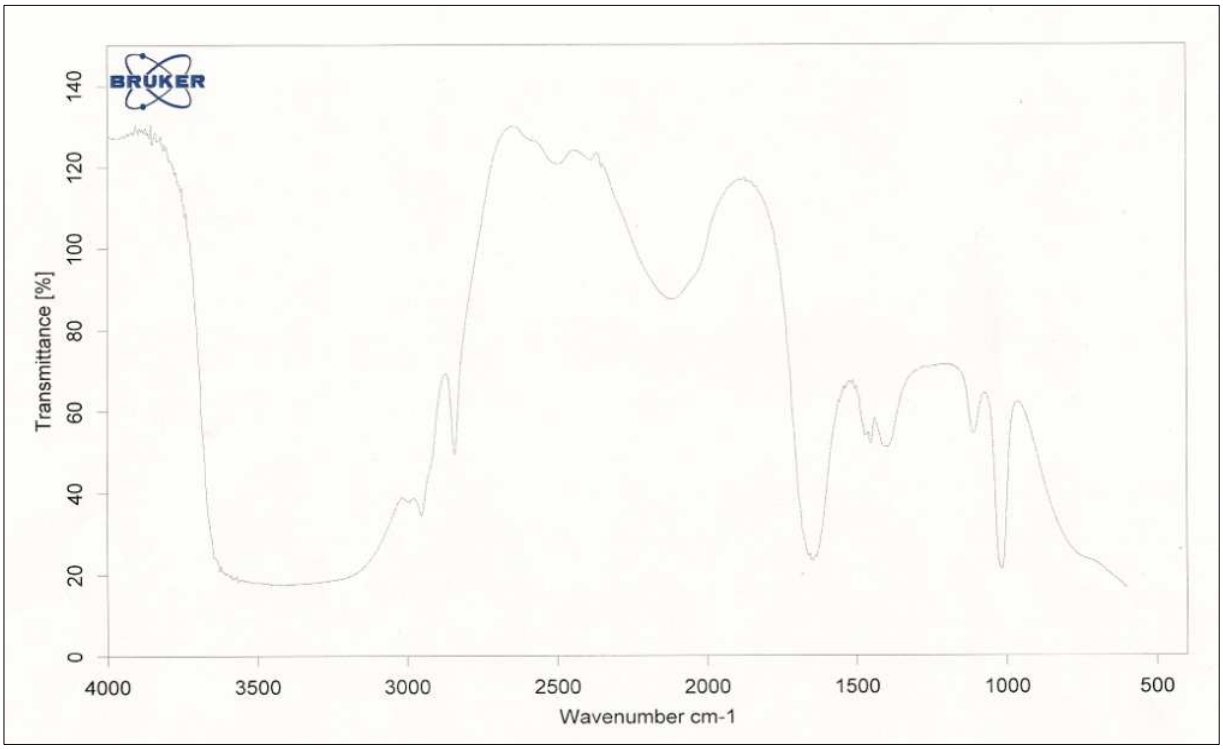


Figure 3-8: Infrared spectra of $M_sA_sH_s$ for the measurement range 4000 to 600 cm^{-1}.

LC-MS/MS was run on a Q-TOF instrument, an accurate mass instrument which provides improved data and can predict empirical mass of precursor ions. LC-MS analysis was first done in the positive mode. A DAD was also used to measure the absorbance of the column eluent over a wavelength of 220 nm to 400 nm to reveal a total wavelength chromatogram (TWC). The mass scans from 50 – 1000 amu revealed a total ion current/total ion chromatogram (TIC) from where the mass of 188+1 was targeted selectively (Figure 3-9). An advantage of high resolution MS such as LC-MS QTOF is that one can accurately measure the isotopic distribution which can be used to confirm the empirical formula with a high degree of confidence (Figure 3-10 and Table 3-2).

The accurate mass data obtained reveals the empirical formula of the major compound as found in the purified $M_sA_sH_s$ sample to be $C_{11}H_8O_3$. The actual isotope abundances as found in the analyses correlated well with the calculated abundances which confirmed this empirical formula.

The targeted mass of 188+1 in positive mode was further fragmented and analysed to give the product ions at a collision voltage of 20 V. The mass of the MS and the MS/MS scans is shown in Figure 3-11 (A) and Figure 3-11 (B) respectively. It should be noted that the MS/MS shows discrete peaks without the isotope distribution due to only the most abundant precursor ion being fragmented. The mass of the product ions are summarised in Table 3-3 with the empirical formula for each fragment, the abundance and the neutral loss.

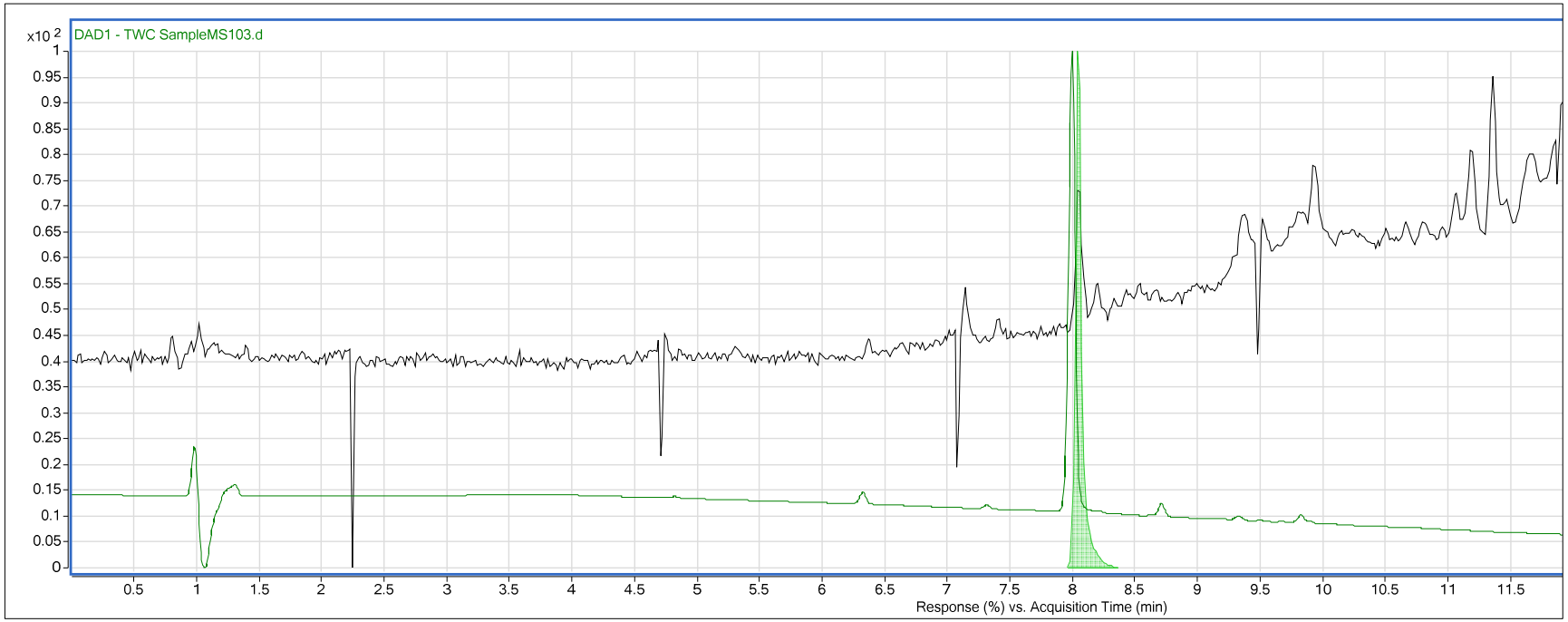


Figure 3-9: LC-MS/MS TWC chromatograms for $M_sA_sH_s$ in the positive mode. The green coloured peak eluting from 7.9 – 8.4 min is where the mass of 188+1 eluted. The solid green line is the DAD signal measuring the total UV absorbance signals between 220 and 400 nm. The black line represents the TIC, between 50 and 100 amu/z. The dropdown spikes are due to calibrant being introduced into the TOF system at regular intervals during the chromatographic run.

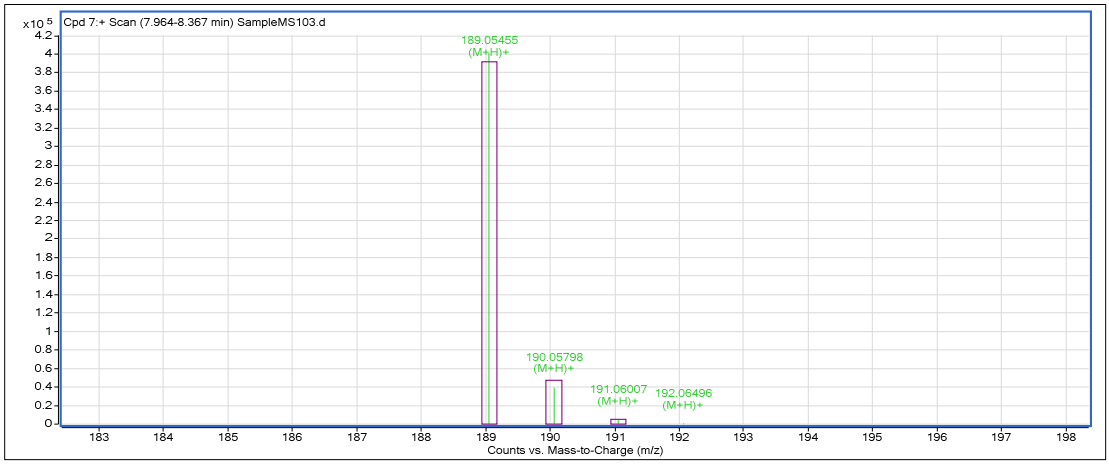


Figure 3-10: Isotopic distribution of the selected mass in the TIC scan from 7.9-8.4 min in the positive mode showing the counts versus the mass-to-charge ratio. The green lines represent the actual abundance of the masses found in the analysis, and the purple blocks represent the calculated abundance of the isotopic distribution of the compound with a mass of 188+1 and an empirical formula of $C_{11}H_9O_3$.

Table 3-2: Isotopic distribution abundance calculations of the selected mass in the TIC scan from 7.9 - 8.4 min in the positive mode. Actual isotope abundance versus calculated abundance is revealed. The double bond equivalent (DBE) is also given for the compound.

m/z	Ion	Formula	Abundance
189.05455	(M+H)+	$C_{11}H_9O_3$	400500.3

Best	Formula (M)	Ion Formula	Score	Cross Score	Mass	Calculated Mass	Difference (ppm)	Absolute Difference (ppm)	DBE
TRUE	$C_{11}H_8O_3$	$C_{11}H_9O_3$	100	100	188.04728	188.04734	0.36	0.36	8

Isotope	Abundance %	Calculated Abundance %	m/z	Calculated m/z	Difference (ppm)
1	100	100	189.05455	189.05462	0.35
2	9.78	12.12	190.05798	190.05801	0.13
3	1.09	1.29	191.06007	191.06019	0.59
4	0.1	0.1	192.06496	192.06284	-11.04

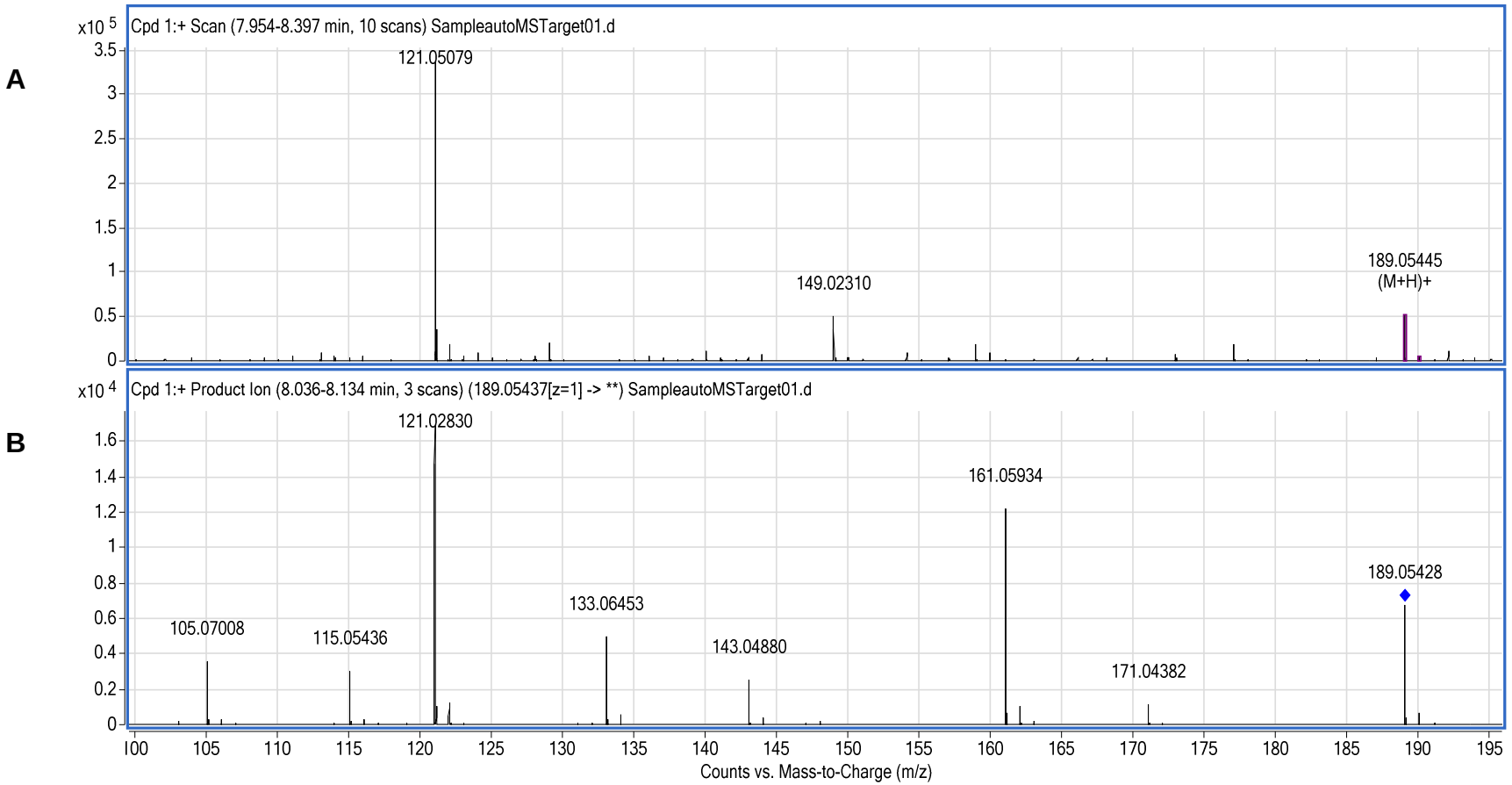


Figure 3-11: Results for the targeted MS/MS in the positive mode when looking for the compound with a mass of 188+1, expressed as counts versus mass-to-charge ratio. Mass spectra of fragment patterns obtained for (A) the peak eluting at 7.9 – 8.4 min in MS mode, (B) the peak eluting at 8.0 – 8.1 min in MS/MS mode. The red block in (A) indicates the precursor that was selected for further fragmentation in automated MS/MS mode and the fragments of that precursor is shown in (B).

Table 3-3: Product ions obtained after LC-MS/MS analysis in the positive mode. Mass, empirical formula, abundance and neutral loss is shown.

m/z	Formula	Abundance %	Difference (ppm)	Loss Mass	Loss Formula
105.07008	C_8H_9	7.34	-1.95	83.98474	C_3O_3
115.05436	C_9H_7	6.14	-1.2	74.00039	$C_2H_2O_3$
121.0283	$C_7H_5O_2$	37.07	0.87	68.02621	C_4H_4O
121.06662	C_8H_9O	2.27	-15.08	67.98983	C_3O_2
133.06453	C_9H_9O	10.28	1.93	55.98983	C_2O_2
143.0488	$C_{10}H_7O$	5.1	2.41	46.00548	CH_2O_2
161.05934	$C_{10}H_9O_2$	27.34	2.25	27.99491	CO
171.04382	$C_{11}H_7O_2$	2.34	1.37	18.01056	H_2O

LC-MS/MS analysis was also carried out in the negative mode. The scan was run from 50 – 1100 amu from where the mass of 189 was targeted selectively (mass of 189 (M^*-)) due to formation of the more stable tauteromeric form in the negative mode ionisation. The negative mode is less sensitive but can be more selective especially with hydroxy and oxygen substituted aromatic ring compounds. Isotopic distributions from the negative mode ionisation are illustrated in Figure 3-12.

The accurate mass and isotope distribution data obtained again confirmed the empirical formula to be $C_{11}H_8O_3$ for the major compound as found in the $M_sA_sH_s$ subfraction (Table 3-4). The actual isotope abundance found in the analyses correlated well with the calculated abundance.

Advantages of GC-MS include the electron impact fragmentation and the availability of libraries of fragment patterns such as the NIST library. This library of natural products for GC-MS contains a mass spectral fragmentation database of thousands of organic compounds coupled to GC data. It is the worlds most widely used mass spectral reference library.

A requirement for a compound to be analysed by GC-MS is that the compound must be volatile. This was expected for the $M_sA_sH_s$ sample because of its lipophilic character due to its solubility in hexane and as it eluted late on a reverse phase (RP) column. An empirical formula obtained from the LC-MS/MS using TOF also gave the chance of the compound being volatile a high probability. The only question was whether the compound would be stable at high temperature. The use of gas phase electron impact at high voltage ensured reproducible fragmentation of the compound which allowed identification using the NIST library.

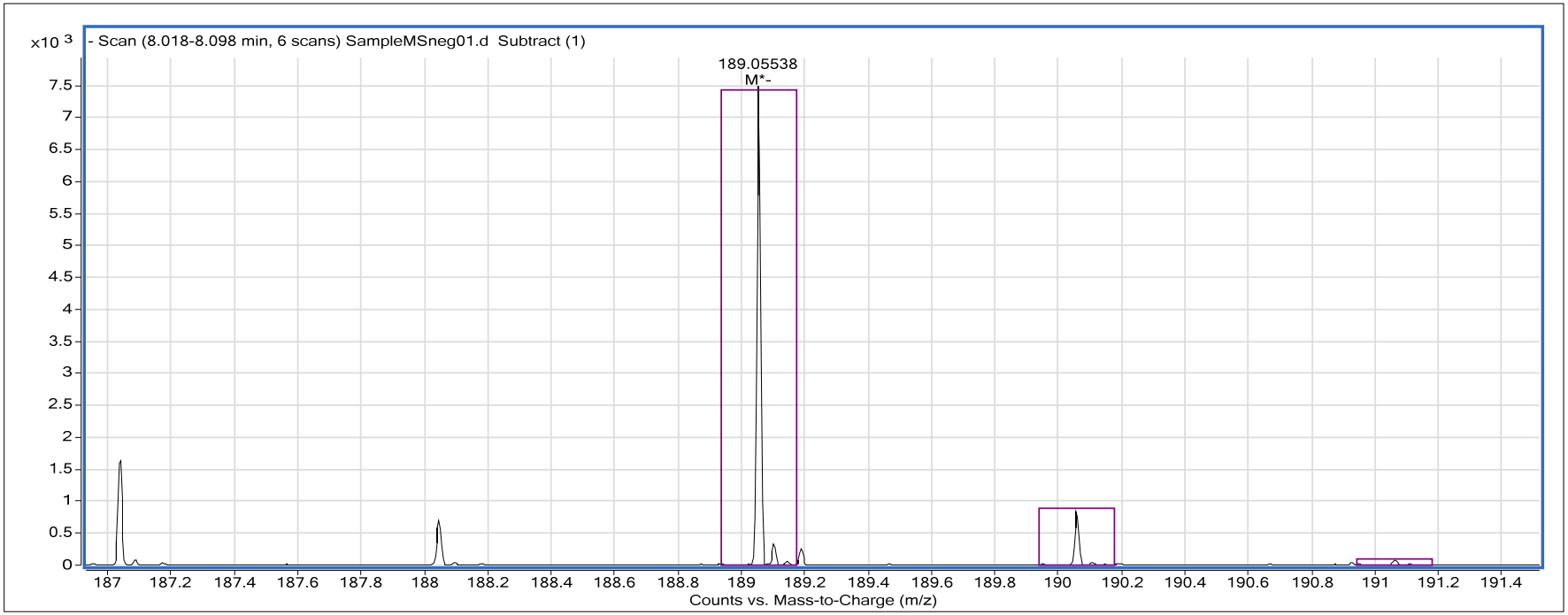


Figure 3-12: Isotopic distribution of the selected mass in the scan from 8.0 - 8.1 min in the negative mode (LC-MS/MS) showing the counts versus the mass-to-charge ratio. The purple blocks represent the calculated abundance of the isotopes. Note that the ion M^{*}- has a mass of 189.05538.

Table 3-4: Isotopic distribution abundance calculations of the selected mass in the scan from 8.0 - 8.1 min from LC-MS/MS in the negative mode. Actual isotope abundance versus calculated abundance is revealed. The DBE is also given for the compound.

m/z	Ion	Formula	Abundance
189.05538	M*-	$C_{11}H_9O_3$	7507.6

Best	Formula (M)	Ion Formula	Score	Cross Score	Mass	Calculated Mass	Difference (ppm)	Absolute Difference (ppm)	DBE
TRUE	$C_{11}H_9O_3$	$C_{11}H_9O_3$	100		189.05483	189.05517	1.78	1.78	7.5

Isotope	Abundance %	Calculated Abundance %	m/z	Calculated m/z	Difference (ppm)
1	100	100	189.05538	189.05572	1.78
2	11.17	12.12	190.05893	190.05911	0.93
3	1.06	1.29	191.06268	191.06128	-7.32

The GC-MS TIC (Figure 3-13) revealed that $M_sA_sH_s$ was a fairly clean sample. The predominant peak eluted at 11.6 min and the fragmentation pattern of the mass spectra of this peak was then compared to mass spectra fragmentation patterns in the NIST library (Figure 3-14). There was a 98% certainty match to the fragmentation pattern of 5-hydroxy-2-methyl-1,4-naphtalenedione. This compound has a molecular formula of $C_{11}H_8O_3$ and molecular weight of 188.05. When this compound (CAS number 481-42-5) was searched in the Merck Index (Merck 1997) the compound was identified as "plumbagin" (Figure 3-15). The GC-MS spectrum (Figure 3-16) and spectral assignment (Figure 3-17) for 5-hydroxy-2-methyl-1,4-naphtalenedione from Advanced Chemistry Development (ACD) labs is also shown.

Plumbagin is a naturally occurring yellow pigment which forms yellow needles during dilution with alcohol (Merck 1997). These yellow needles were observed with $M_sA_sH_s$ upon evaporation of the solvents. Quinones, such as plumbagin, are often found in the bark, heartwood or roots, or sometimes in tissues of plants, where their pigment colours are easily masked by other pigments (Harborne 1998). The presence of quinones was confirmed by the phytochemical analysis done (Section 1, Chapter 3) and the infrared spectra matched the high intensity of a dicarbonyl.

Many biological activities have been ascribed to plumbagin which include: antioxidant (Sugie et al. 1998), anti-inflammatory (Sugie et al. 1998), chemopreventative (Sugie et al. 1998), cytotoxic (Lin et al. 2003; Montoya et al. 2004; Ichihara et al. 1980; Nguyen et al. 2004; Hsieh et al. 2005), antimicrobial (Ichihara et al. 1980; De Paiva et al. 2003; Abdul and Ramchender 1995; Hsieh et al. 2005), contraceptive (Hsieh et al. 2005) and anti-leishmanial (Hsieh et al. 2005).

Plumbagin was first isolated in 1885 from the roots of *Plumbago europoea* (Roy and Dutt 1928). Both the genus *Drosera* and the genus *Plumbago* is known to

contain plumbagin (Durand and Zenk 1971). To our knowledge this is the first report of the presence of plumbagin in any of the *Piper* species.

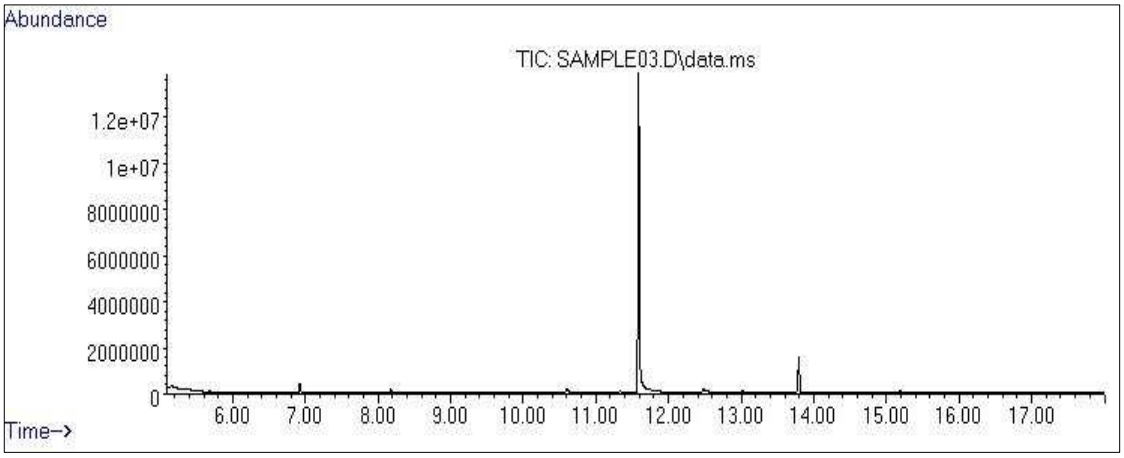


Figure 3-13: GC-MS TIC of $M_sA_sH_s$, demonstrating the high abundance of a single compound eluting at 11.6 min.

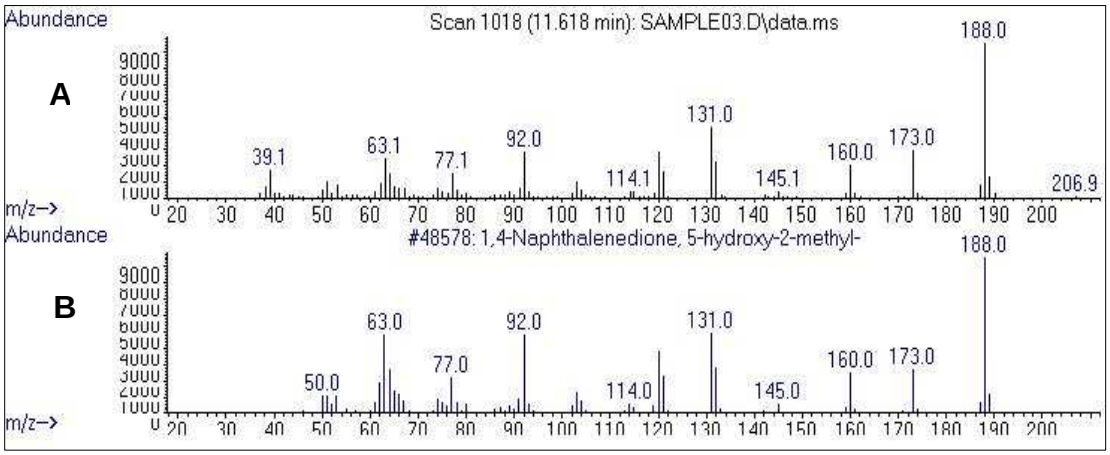


Figure 3-14: (A) Mass spectra from the GC-MS scan revealing the fragmentation pattern of the peak that eluted at 11.6 min for $M_sA_sH_s$ with the mass to charge ratio of all the fragments versus abundance. (B) Spectrum of the fragmentation pattern of 5-hydroxy-2-methyl-1,4-naphtalenedione showing the mass to charge ratio of all the fragments versus abundance which provided a 98% certainty match for (A).

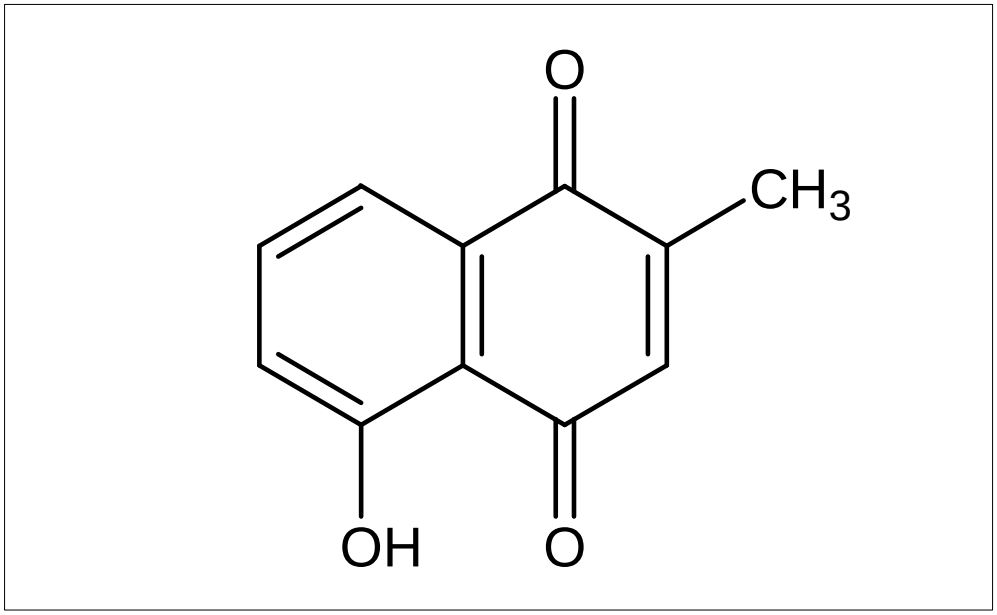


Figure 3-15: Chemical structure of plumbagin.

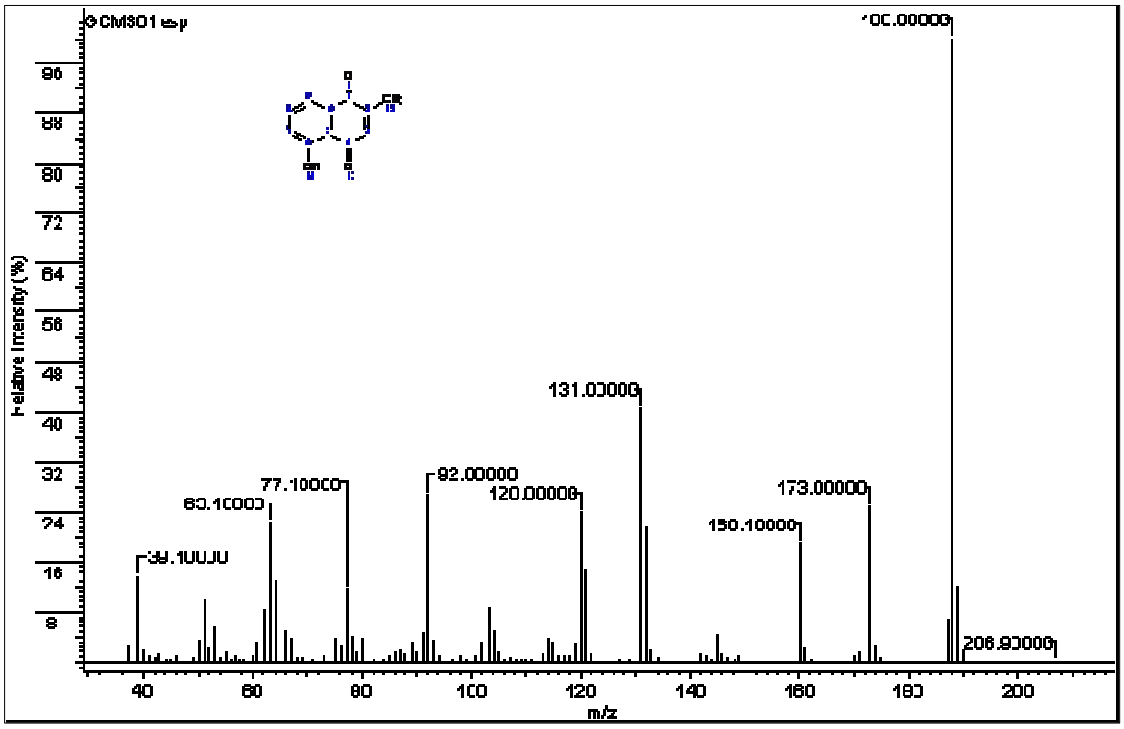
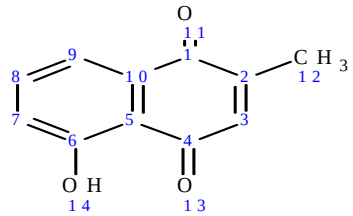


Figure 3-16: The predicted GC-MS spectrum of 5-hydroxy-2-methyl-1,4-naphtalenedione (ACD labs).



No.	Fragment	Formula	m z-Calc.	Difference-Da
1	M	C11H8O3	188.047	-0.047
2	1-11,13-14	C10H5O3	173.023	-0.023
3	1-3,5-12,14	C10H8O2	160.052	0.048
4	2-10,12-14	C10H8O2	160.052	0.048
5	1-3,5-11,14	C9H5O2	145.028	-0.028
6	2-10,13-14	C9H5O2	145.028	-0.028
7	1,4-11,14	C8H4O2	132.021	0.079
8	1,4-11,14+H	C8H5O2	133.013	-0.013
9	1,4-10,13-14+H	C8H5O2	133.013	-0.013
10	1,4-11,13	C8H5O2	133.013	-0.013
11	1-5,8-11,13	C8H5O2	133.013	-0.013
12	1,5-11,14	C7H4O2	120.021	-0.021
13	1-5,10-13	C7H4O2	120.021	-0.021
14	4-10,13-14	C7H4O2	120.021	-0.021
15	1-3,5-7,10-11,14	C7H3O2	119.013	0.087
16	2-5,8-10,12-13	C8H6O	118.041	-0.041
17	1-3,5-7,10-12	C8H5O	117.033	-0.033
18	2-3,5-10,12	C9H7	115.054	0.046
19	2-5,8-10,13	C7H3O	103.013	0.087
20	1,5-11	C7H3O	103.013	0.087
21	1-3,5,8-11	C7H3O	103.013	0.087
22	1-3,5-7,10-11	C7H2O	102.010	0.090
23	5-10,14	C6H4O	92.026	-0.026
24	3-5,8-10,13+H	C6H4O	92.026	-0.026
25	4-5,8-10,13	C5H2O	78.010	0.090
26	1,5,8-11	C5H2O	78.010	0.090
27	1-4,12-13+2H	C5H2O	78.010	0.090
28	1-4,11-12+2H	C5H2O	78.010	0.090
29	1,5-7,10-11	C5HO	77.002	0.098
30	3-5,8-10+H	C6H4	76.031	0.069
31	3-5,8-10	C6H3	75.023	0.077
32	5-10+H	C6H2	74.015	-0.015
33	1-4,11	C4HO	65.002	0.098

Figure 3-17: The predicted spectral assignment of 5-hydroxy-2-methyl-1,4-naphtalenedione fragments during GC-MS using the same fragmentation conditions used experimentally during the analysis (ACD labs).

3 Biological activity

3.1 Antimicrobial activity

The disk diffusion assay was employed as an indicator of antimicrobial activity. The zones of inhibition for the whole root, inner root and root-bark extracts are presented in Table 3-5. In all preparations CME exerted the highest antimicrobial activity. Of the three root parts tested, the root-bark produced the greatest zone of inhibition. As mentioned earlier (Section 1, Chapter 2), only the root-bark was used to prepare extracts for further analysis.

All the crude solvent extracts and subfractions contained antimicrobial activity (Table 3-6). CME exhibited the highest activity against *S. aureus*, whereas CAE had the highest activity against *C. albicans*. CHE had the lowest antimicrobial activity, which could possibly be attributed to the fact that hexane extracts are expected to contain more non-polar compounds, and the disk diffusion assay is not an appropriate assay for non-polar compounds, as these compounds are not likely to diffuse into the agar (Cos et al. 2006). It has been reported that in some instances the crude extract will retain the higher activity as either the compounds could have decomposed during the fractionation procedure, or the biological activity of the extract may be due to a synergistic effect of compounds in the crude extract (Houghton et al. 2005). The $M_sA_sH_s$ subfraction which contained the lowest yield (Figure 3-5), had the highest antifungal activity of all the subfractions. Because of its retention of antimicrobial activity this subfraction was subjected to further investigation.

The broth micro-dilution assay was used to quantitate the antimicrobial activity. An advantage of this method is that it allows for testing of both polar and non-polar compounds (Cos et al. 2006). MIC $_{p\text{-INT}}$ values for the crude extracts ranged from 0.125 mg/ml to > 2 mg/ml (Table 3-7). The highest concentration tested for CWE, CME, and CHE was 2 mg/ml and for CAE 1 mg/ml due to the lower yield of the extract.

Table 3-5: Antimicrobial activity as measured by the agar disk diffusion method of CWE from the whole root, inner root and the root-bark material, respectively. Zones of inhibition are measured in mm (average), n=2.

Plant part	Zone of Inhibition (mm ± SD)		
	Extract	***S. aureus***	***C. albicans***
Whole root	CME	40.00	44.20
	CWE	15.12	17.56
Inner root	CME	14.97	15.48
	CWE	–	–
Root-bark	CME	44.16	42.36
	CWE	22.93	19.85
Positive control	AMB	N/A	19.24
	AP	34.38	N/A

–: No activity.

N/A: Not applicable.

AMB: Amphotericin B, 20 µg (positive control).

AP: Ampicillin, 10 µg (positive control).

Table 3-6: Antimicrobial activity as measured by the agar disk diffusion assay of the CWE, CME, CAE, CHE, M_sA_s, $M_sA_sH_s$ and $M_sA_sA_s$ as well as the positive controls against *S. aureus* and *C. albicans.* Zones of inhibition are measured in mm ± SD*.

Extract/ subfraction	Zone of Inhibition (mm ± SD*)	
	S. aureus (*n=6)	***C. albicans*** (*n=6)
CWE	34.45 ± 1.58	34.38 ± 2.47
CME	44.79 ± 2.45	42.40 ± 5.00
CHE	18.00 ± 1.63	19.16 ± 1.63
CAE	42.98 ± 0.92	46.45 ± 1.84
M_sA_s	28.8 ± 0.76	36.19 ± 1.99
$M_sA_sH_s$	17.36 ± 0.16	36.21 ± 6.18
$M_sA_sA_s$	25.03 ± 0.84	30.34 ± 0.97
AMB	N/A	22.01 ± 0.42
AP	38.45 ± 1.21	N/A

ND: Not done.

N/A: Not applicable.

AMB: Amphotericin B, 20 µg (positive control).

AP: Ampicillin, 10 µg (positive control).

Table 3-7: Antimicrobial activity using the broth micro-dilution assay of the crude extracts against *S. aureus, C. albicans, S. epidermidis, E. coli* and *P. aeruginosa*. $MIC_{p\text{-}INT}$ values are measured in mg/ml, n=3.

Extract/ Sub-fraction	**$MIC_{p\text{-}INT}$ (mg/mL)**				
	S. aureus	***C. albicans***	***S. epidermidis***	***E. coli***	***P. aeruginosa***
CWE	1	0.5	1	2	> 2
CME	0.5	0.33	0.5	> 2	1
CHE	0.5	1	0.5	2	2
CAE	0.125	0.25	0.125	> 1	0.25
$M_SA_SH_S$	0.029	0.029	-	-	-

-: Insufficient quantities available for testing.

$MIC_{p\text{-}INT}$ values greater than 1 mg/ml are considered to be clinically insignificant (Gibbons 2004). The $M_SA_SH_S$ subfraction exhibited the best antimicrobial activity when compared to all crude extracts against both *S. aureus* and *C. albicans*. Antimicrobial activity against *S. aureus* is of importance since *S. aureus* is one of the most persistent infectious microorganisms (Kang and Moon, 1990) and the most common Gram-positive bacterium found in nosocomial infections (Hugo and Russell 1995). Furthermore, it is also the leading cause of wound infections (Nester et al. 2001). The Gram-positive bacterium, *S. epidermidis*, is also relevant in wound infections. *P. aeruginosa* is also a major cause of nosocomial infections (Nester et al. 2001). *C. albicans* is often part of the normal epidermal flora, but can infect wounds (Nester et al. 2001). Gram-negative bacteria possess a lipopolysaccharide layer which acts as a barrier, and makes them less susceptible to many antimicrobial compounds (Nester et al. 2001). Furthermore, due to the increase in incidence of fungal infections coupled with the toxicity induced by prolonged treatment with antifungal drugs (Giordani et al. 2001), it is important to determine activity against *C. albicans*. Infections due to the above microorganisms may delay wound healing, and therefore it was relevant to determine activity against these microorganisms in the current study. The broad spectrum activity noted for *P. capense* extracts makes it a good antimicrobial candidate. As the $M_SA_SH_S$ subfraction was identified as the most promising preparation it was used for further analyses.

There have been many reports on antimicrobial activity of plants from the *Piper* species (Orjala et al. 1994; Parmar et al. 1997; Pessini et al. 2005; Koroishi et al. 2008; Scott et al. 2008; Naz et al. 2009). Antimicrobial activity against *C. albicans* has previously been reported for hexane and acetone root extracts of *P. capense* (Samie et al. 2010) as well as aqueous and methanol extracts of the bark of this plant (Steenkamp et al. 2007a). However, in both cases the MIC values reported by the authors were higher than those obtained in the

present study. Obi et al. (2002) found methanol root extracts of *P. capense* to have antimicrobial activity against *S. aureus*, *Bacillus cereus* and *Streptococcus pyogenes*. These findings are supportive of the results obtained in the present study.

Bioautography has been described as "the most important detection method for new or unidentified antimicrobial compounds", as the antimicrobial activity can be localised on a chromatogram when using this method (Rios et al. 1988). Bioautography was performed on the eluents of the HPLC analyses for the $M_SA_SH_S$ subfraction. Droplets of HPLC eluent collected at fractions 90 - 94 directly onto the TLC plate lacked colour change after exposure to *S. aureus*, whereas all the other droplets were pink in colour, which indicated inhibition of *S. aureus* growth for the compounds eluting in droplets 90 - 94 only (Figure 3-18). These droplets could be correlated back to the time in the HPLC run at 13.6 min which was the dominant peak (Figure 3-7). As this was the only area where inhibition of bacterial growth was visualised, it could be deduced that the antimicrobial activity of $M_SA_SH_S$ was restricted to the peak eluting at 13.6 min during the HPLC analyses.

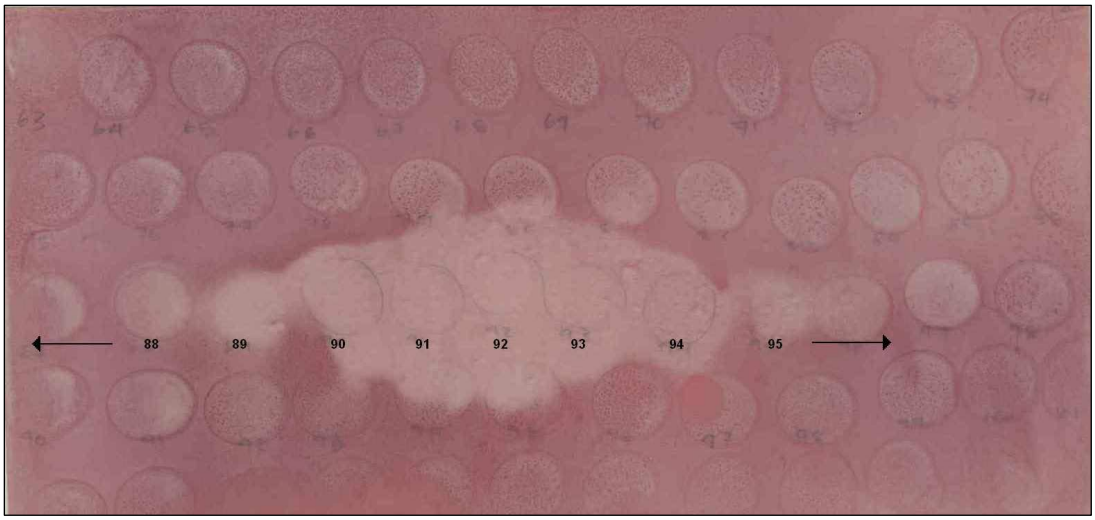


Figure 3-18: Bioautography plate where the collected eluents of the HPLC analyses were collected in a drop-wise fashion onto a silica TLC plate, and coated with a thin layer of *S. aureus*. After incubation, plates were sprayed with INT to visualise bacterial viability. Inhibition of growth is visible as white zones against a pink background.

3.2 Antioxidant activity

Antioxidants have many health benefits, and may contribute to wound healing in that it reduces the amount of free radicals in the wound (Mensah et al. 2001). As plants naturally produce antioxidants to protect themselves from reactive oxygen species (ROS), they may be a rich source of antioxidant compounds (Huda-Faujan 2009). Phenolic compounds are a major group of secondary metabolites that may contribute to the antioxidant activity of plants (Mamyrbékova-Békro et al. 2008). Due to the presence of phenolic groups in *P. capense* extracts/subfractions (Section 1, Chapter 3), it was expected that antioxidant activity would also be present in these extracts/subfractions.

DPPH spray reagent on TLC plates was used as an indicator of the presence of antioxidant compounds in the extracts/subfractions. The presence of antioxidant activity is visualised as the formation of yellow zones against a purple background (Figure 3-19). $M_SA_SA_S$ exhibited the most prominent reaction revealing large yellow zones and streaking of this yellow zone from the origin to the solvent front of the TLC plate. No antioxidant compounds were detectable for CHE and $M_SA_SH_S$ using this method.

Antioxidants transfer a hydrogen/electron atom to a purple DPPH free-radical when they come into contact, which neutralises the DPPH free-radical which results in the change of colour from purple to yellow (Dasgupta and De 2004). Advantages of this method include its simplicity, sensitivity, rapidness and its independence of sample polarity (Koleva et al. 2002). All the crude extracts as well as the $M_SA_SH_S$ subfraction quenched the DPPH radical in a dose-dependent manner. CME (1 mg/ml) had the highest DPPH radical scavenging activity (68%) (Figure 3-20). None of the extracts tested had activity as effective as the positive control, Trolox (1 mg/ml).

A

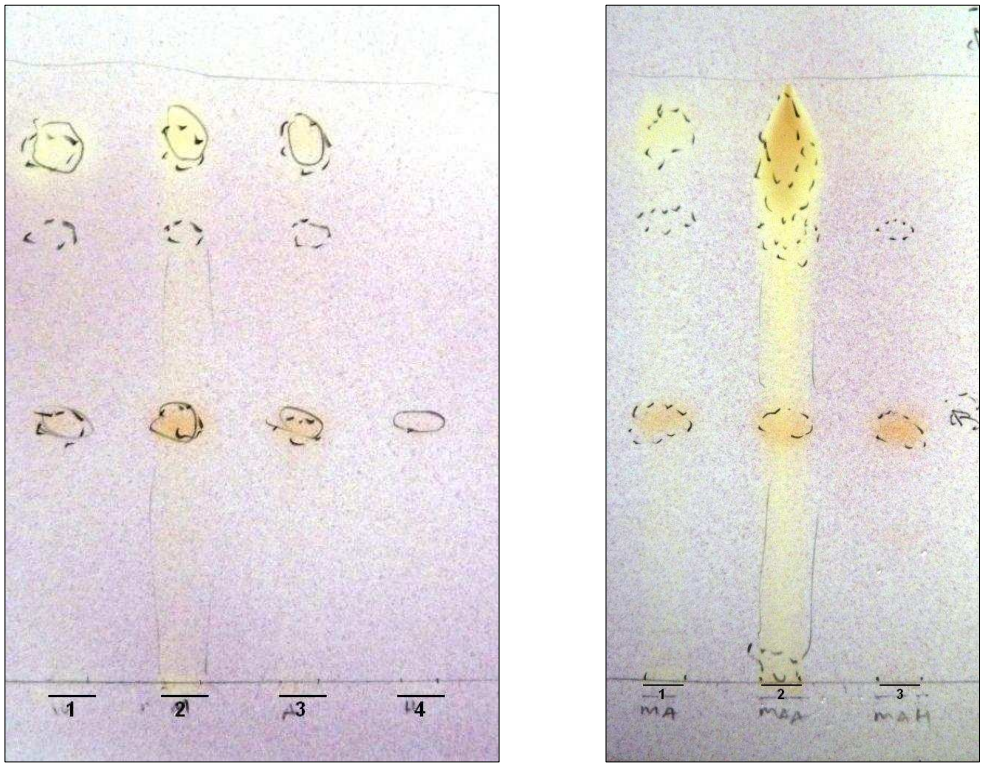


Figure 3-19: TLC chromatogram on a normal phase TLC plate, developed in methanol:water (50:50). (A) Crude *P. capense* extracts; (B) subfractions of *P. capense*. 5 µl of extract was spotted on the plate from left to right: 1: CWE; 2: CME; 3: CAE; 4: CHE; 5: M_sA_s; 6: $M_sA_sA_s$; 7: $M_sA_sH_s$. UV visualisation: dotted lines represent compounds detected at 360 nm and solid lines represent compounds detected at 254 nm. Plates were then sprayed with DPPH spray reagent.

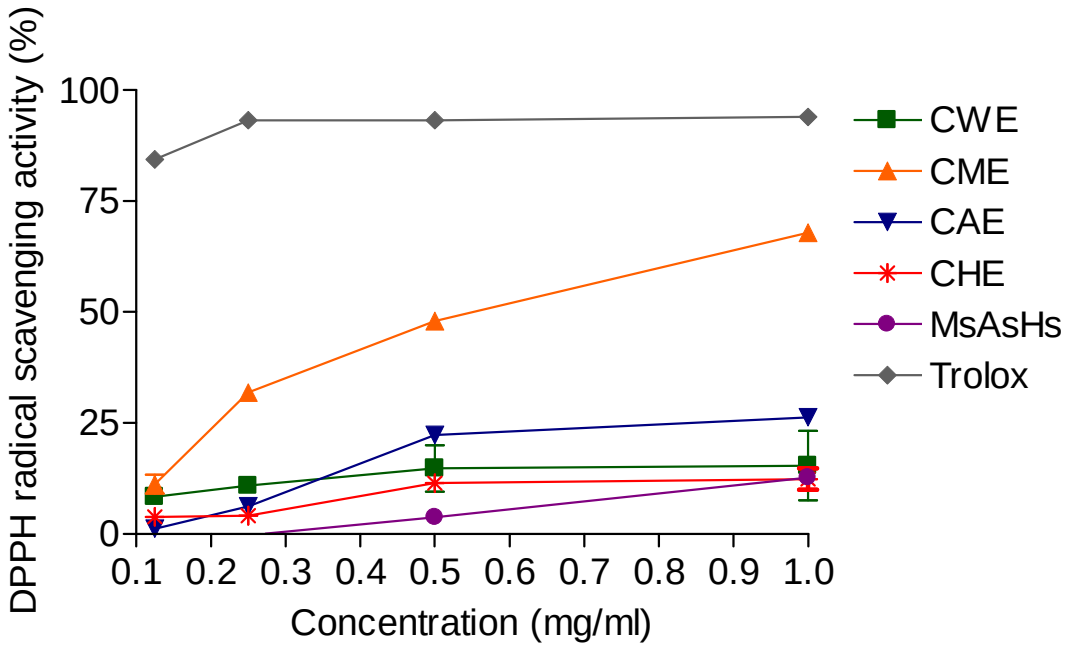


Figure 3-20: Graph indicating the DPPH radical scavenging activity (%) for different concentrations of the crude methanol extract, crude water extract, crude hexane extract, crude acetone extract, $M_sA_sH_s$ subfraction as well as Trolox (positive control), n=3.

In the ABTS radical scavenging assay, CHE showed no antioxidant activity (Fig 3-21). CME yielded the highest ABTS radical scavenging activity of all the samples tested at a concentration of 1 mg/ml (97.21%). At the same concentration CAE, CWE and $M_SA_SH_S$ exhibited ABTS radical scavenging activities of 61.31%, 56.20% and 18.17%, respectively. All of the crude extracts as well as the $M_SA_SH_S$ subfraction tested in this assay showed rather low or even no antioxidant activity according to their Trolox equivalence. CME showed the highest activity with a TEAC value of 0.45, whereas the CWE and CAE extracts had TEAC values of 0.21 and 0.05, respectively. No TEAC values could be determined for CHE and $M_SA_SH_S$ as a correlation coefficient of $r^2 > 0.95$ could not be achieved. The strength of this assay is that it can be used on both aqueous and lipophilic systems.

No literature could be found with information on antioxidant activity for *P. capense* extracts. However, there are reports on antioxidant activity of other *Piper* species. Not only has *Piper betle* L. been shown to contain significant antioxidant activity *in vitro* (Dasgupta and De 2004), but it has also been reported to elevate antioxidant status in animals after oral administration of the extracts (Choudhary and Kale 2002). *Piper samentosum* were also reported to possess antioxidant properties (Hafizah et al. 2010). Five phenolic amides have been isolated from *Piper nigrum* L., all of which contain antioxidant activity, which has been shown to be higher than alpha-tocopherol, a naturally occurring antioxidant (Nakatani et al. 1986).

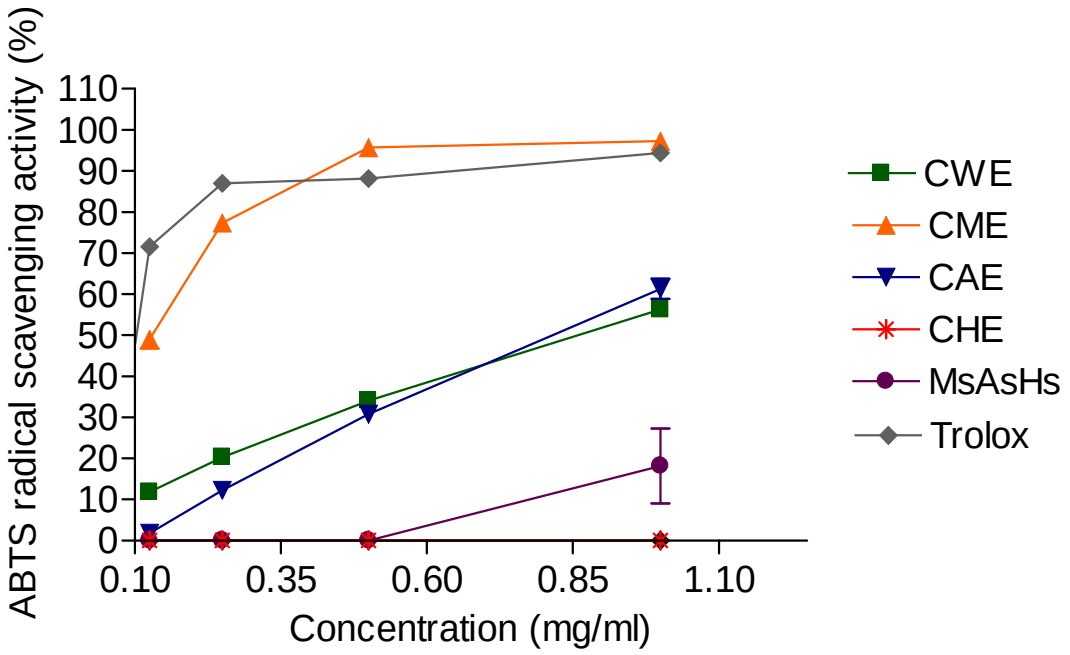


Figure 3-21: Graph indicating the ABTS radical scavenging activity (%) for different concentrations of the CME, CWE, CHE, CAE, $M_sA_sH_s$ as well as Trolox (positive control), n=3.

3.3 Cytotoxicity

Although *in vitro* assays are limited in that they do not mimic a full biological system, they are used to assist in predicting certain aspects of *in vivo* activity. Considerable interest has been focused on identifying antimicrobial compounds that are pharmacologically potent with low or no side effect/s or toxicity, and therefore cytotoxicity was determined to give an indication of the toxicity of the extracts/subfractions. Plumbagin was included in the cytotoxicity assays as it was earlier identified as the major compound present in the extracts (Section 2, Chapter 3). Furthermore, a previous study which reported antimicrobial activity of *P. capense* extracts, suggested that toxicological studies should be performed in order to prove safety for potential use in humans (Samie et al. 2010).

The results, recorded as percentage of cell growth compared to the untreated control for C_2C_{12} cells are presented graphically in Figure 3-22 A-F, for resting lymphocytes in Figure 3-23 A-F and for PHA stimulated lymphocytes in Figure 3-24 A-F. IC_{50} values were determined by extrapolating the data to the concentration at which 50% of cells survived, and is depicted in Table 3-8.

CHE exhibited the highest cytotoxic activity against all cells tested with IC_{50} values of 1.64, 0.62 and 3.01 µg/ml in the resting human lymphocytes and PHA stimulated lymphocytes and C_2C_{12} cells, respectively. The concentrations of plumbagin tested in this study were at the same concentration as found in the extracts in an attempt to obtain comparative data, but these concentrations proved to be excessively toxic to the lymphocytes. It can be noted that all the cell survival results for the resting and PHA stimulated lymphocytes are below 50% of relevant untreated control lymphocytes. For all the concentrations tested, $M_sA_sH_s$ inhibited cell growth by more than 50% in the resting human lymphocytes. The C_2C_{12} cells were much more resistant to the cytotoxic effects of plumbagin. When C_2C_{12} cells were treated with CAE, all the concentrations tested were too low to give a dependable IC_{50} value.

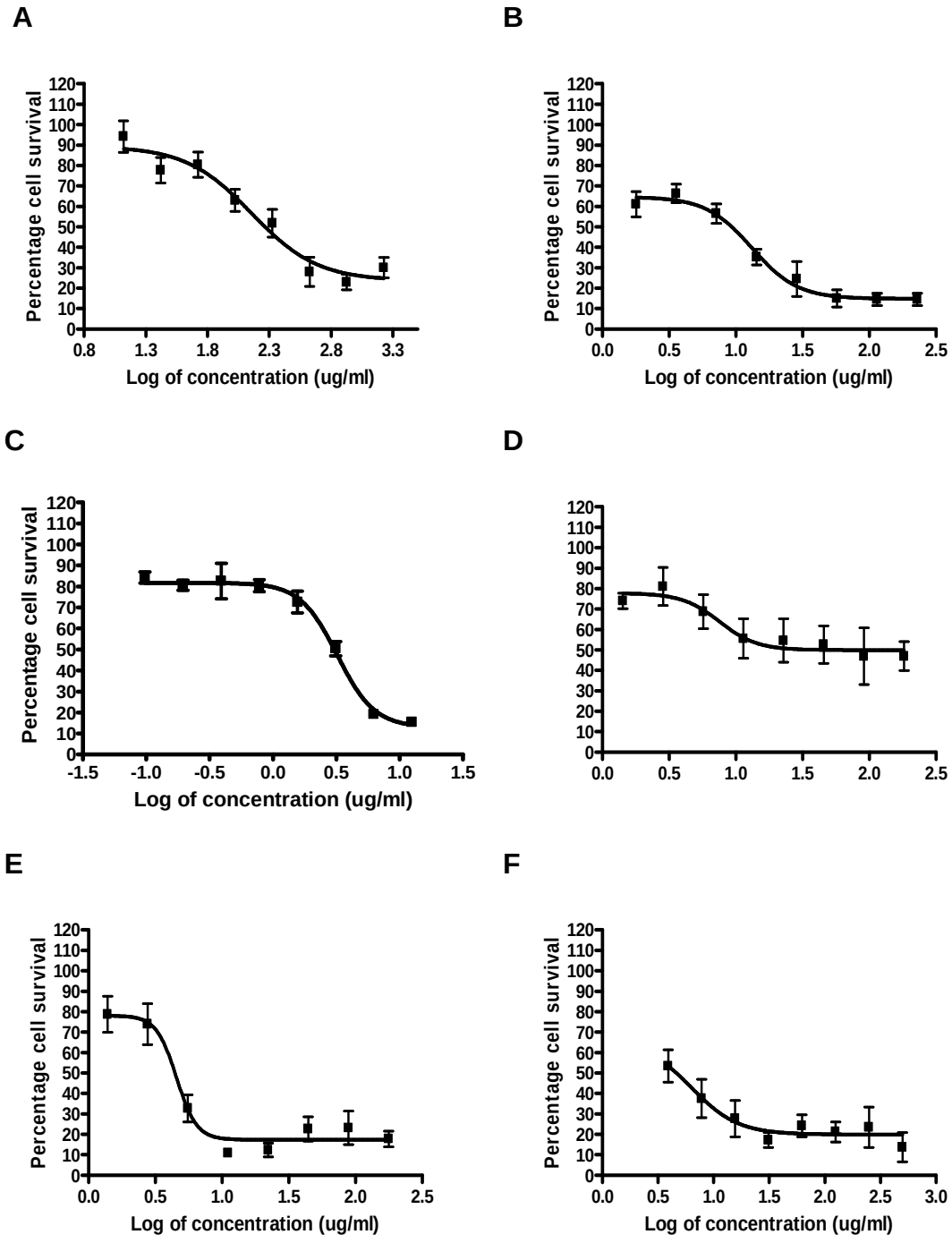


Figure 3-22: Effects of crude extracts/subfractions on the growth of C_2C_{12} cells. (A) CWE; (B) CME; (C) CHE; (D) CAE; (E) $M_sA_sH_s$; (F) plumbagin. Each endpoint represents the mean of three different experiments ± standard deviation (SD).

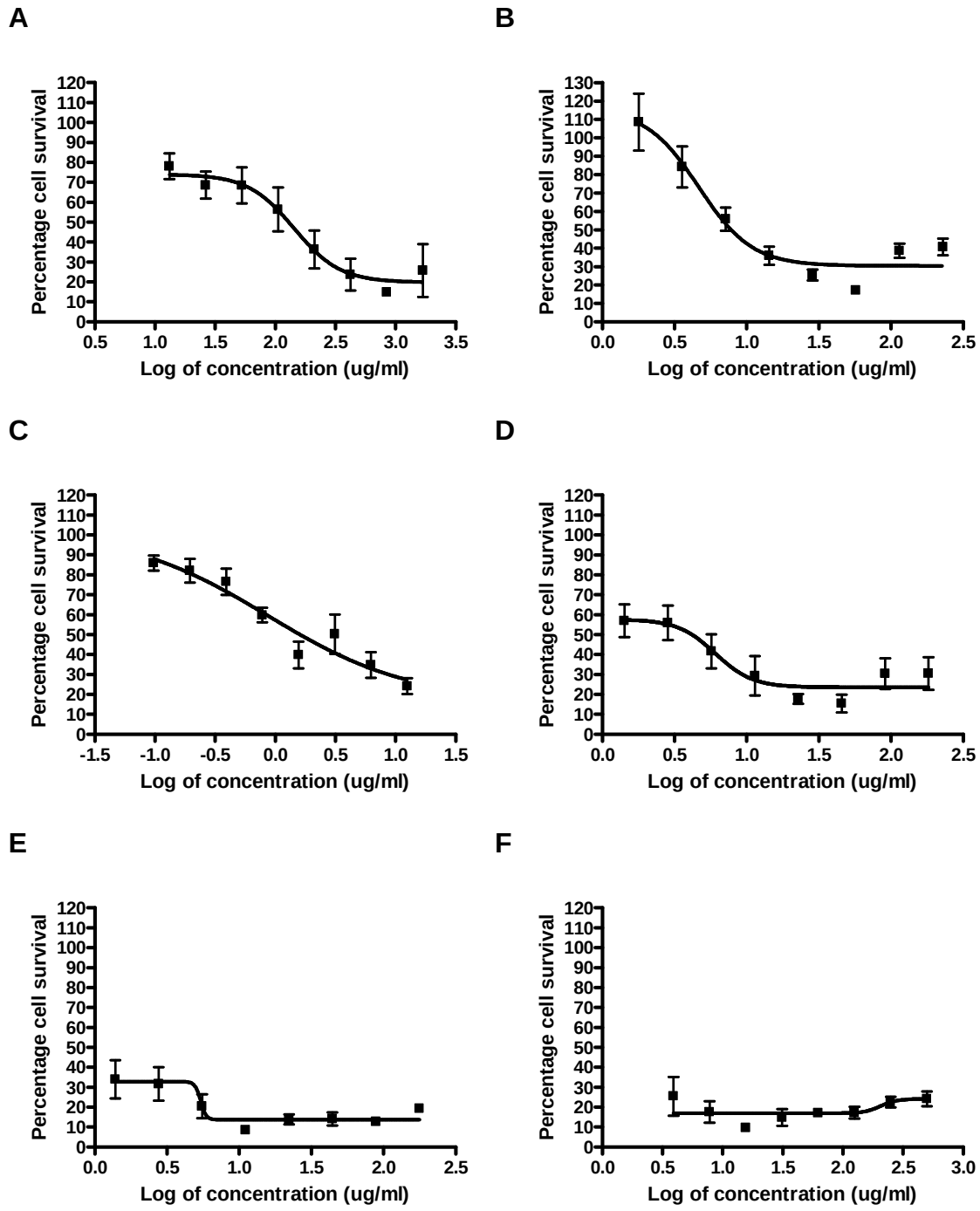


Figure 3-23: Effects of crude extracts/subfractions on the growth of resting lymphocytes. (A) CWE; (B) CME; (C) CHE; (D) CAE; (E) $M_SA_SH_S$; (F) plumbagin. Each endpoint represents the mean of three different experiments ± standard deviation (SD).

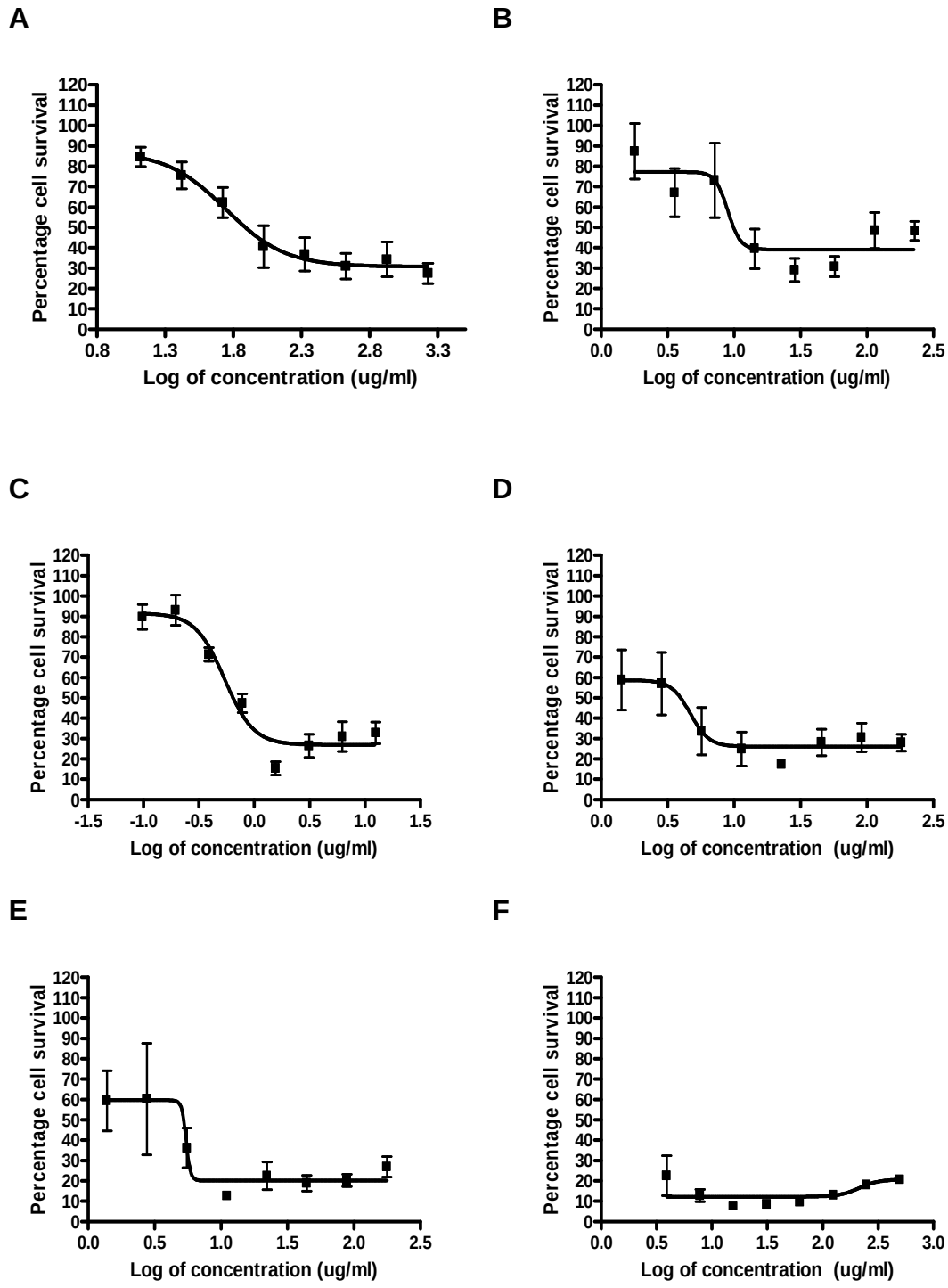


Figure 3-24: Effects of crude extracts/subfractions on the growth of PHA stimulated lymphocytes. (A) CWE; (B) CME; (C) CHE; (D) CAE; (E) $M_sA_sH_s$; (F) plumbagin. Each endpoint represents the mean of three different experiments ± standard deviation (SD).

Table 3-8: Extract/subfraction/plumbagin concentration (µg/ml) causing 50% cell death (IC_{50}) in human lymphocytes (resting and PHA stimulated) and C_2C_{12} cells.

Extract/ Subfraction	IC_{50} (µg/ml) ± SD (n=3)		
	Human Lymphocytes (resting)	Human lymphocytes (PHA stimulated)	C_2C_{12} cells
CWE	49.74 ± 14.70	52.30 ± 2.74	144.44 ± 57.96
CME	6.23 ± 5.25	28.42 ± 28.43	11.94 ± 3.49
CHE	1.64 ± 1.84	0.62 ± 0.27	3.01 ± 0.61
CAE	16.82 ± 13.51	28.13 ± 25.34	43.89 ± 67.45
$M_sA_sH_s$	38.98 ± 30.85	23.07± 22.49	4.23 ± 1.30
Plumbagin	24.95 ± 24.93	79.68 ± 64.14	10.20 ± 5.30

Cytotoxic activity exhibited by the different crude extracts, the $M_sA_sH_s$ subfraction and plumbagin was not comparable between cell lines which proves that different cell lines display differential sensitivity towards these extracts/compounds. It is also evident that both the resting and stimulated human lymphocyte results display higher variance than that of the C_2C_{12} cells, which may be due to experimental procedures coupled to the fact that human lymphocytes are non-adherent cells whereas the C_2C_{12} cell line is adherent. This is one of the disadvantages and potential problems with the cell viability assays, especially in non-adherent cells, as the washing steps may lead to a loss of cells.

According to the American National Cancer Institute guidelines, the IC_{50} for crude extracts should be less than 30 µg/ml after 72 h exposure for the toxicity to be useful as an antineoplastic agent (Suffness and Pezzuto 1990). CWA exhibited IC_{50} values greater than 30 µg/ml against all three cell lines and was therefore the only relatively non-toxic extract. Only one report could be found on the cytotoxicity of *P. capense* extracts, where low cytotoxicity was observed in human monocytic THP-1 cells (> 50 µg/ml) (Kaou et al. 2008). It should be noted that the study by Kaou et al. (2008) was performed on crude extracts of the aerial parts of *P. capense*, which may contain different compounds compared to the root-bark material as used in the current study.

There are several *Piper* species which have been shown to possess cytotoxic activity. *Piper chaba* roots exhibited potent cytotoxic activity when tested with the brine shrimp lethality bioassay (Naz et al. 2009). *Piper methysticum* G. Forster exhibited cytotoxic activity against ovarian tumour and leukaemia cancer cell lines (Tabudravu and Jaspars 2005) which was ascribed to the presence of cis-yagonin, flavokavain A and flavokavain B in these extracts. Cytotoxicity has also been proven for extracts of the fruit of *Piper longum* against mouse Ehrlich ascites carcinoma (Hullatti et al. 2006). Cytotoxicity has been reported for chloroform extracts of *Piper aborescens* against KB cell culture system and P-388 lymphocytic leukaemia and aduncamide isolated

from *Piper aduncum* against KB nasopharyngeal carcinoma cells (Parmar et al. 1997).

Cytotoxic compounds/subfractions isolated include three cytotoxic pyridone alkaloids from *Piper aborescens* (Duh et al. 1990) and five dihydrochalcones from *Piper aduncum* (Orjala et al. 1994) and five fractions from *Piper longum* fruits, one of which was identified as β-sitosterol (Hullatti and Murthy 2010).

Much work has been done on plumbagin. Reports of plumbagin anticancer properties are known in fibrosarcoma, P388 lymphocytic leukaemia, colon cancer, and hepatoma, some of which have been studied *in vivo* (Srinivas et al. 2004). Cytotoxicity has been reported in MCF-7, Bowes cells, Raji, Calu-1, HeLa and Wish tumour cell lines (Nguyen et al. 2004; Lin et al. 2003). Plumbagin is an apoptosis-inducing naphthoquinone that also promote necrosis through free-radical formation (Montoya et al. 2004). The latter is true for napthoquinones in general (Montoya et al. 2004; Inbaraj and Chignell 2004). The current study confirmed the *in vitro* cytotoxicity of plumbagin.

Other napthoquinones have previously been demonstrated as being potential anti-cancer agents as they possess desirable cytotoxic activity in *vitro* and appears to be relatively safe *in vivo* (Montoya et al. 2004). Plumbagin causes cell death by two distinct mechanisms, which are likely to occur simultaneously, resulting in potent cytotoxic activity (Montoya et al. 2004). Firstly redo cycling causes the formation of semiquinone radicals, which in turn leads to the generation of superoxide anions and H_2O_2. These ROS are involved with the cell death process. The second mechanism is reaction with reduced glutathione which depletes the antioxidant ability of the cell.

3.4 Stability

Stability testing was performed in order to determine whether $M_SA_SH_S$ retained its antimicrobial activity over time. The $M_SA_SH_S$ subfraction maintained antibacterial activity against *S. aureus* after 30 days at both 25℃ and 40℃ (Table 3-9). Interestingly, there was an increase in antifungal activity of the $M_SA_SH_S$ after 30 days at both storage temperatures. It has been reported that antibacterial compounds can be converted to more active compounds during storage (Stafford et al. 2005), and it would seem possible that this could be true for antifungal activity of the $M_SA_SH_S$ subfraction.

Stability was further tested by comparing the chromatographic "fingerprints" of $M_SA_SH_S$, from before and after the stability testing, obtained from HPLC analysis (Figure 3-25). Changes in the relative concentrations of the major compound of $M_SA_SH_S$ was evident from the different HPLC-analyses. These changes in concentration of the major compound could be due to oxidation, formation of a breakdown product or polymerisation or esterification of the active compound(s) (Stafford et al. 2005). As it is known that the major peak in the chromatogram was plumbagin and knowing the chemical instability of quinone-like compounds it is highly probable that either a dimerisation or oxidation product had formed from the high concentration of plumbagin in the original $M_SA_SH_S$ subfraction.

Obi et al. (2002) have shown that when root material of *P. capense* is autoclaved at 121℃ for 15 min, no significant differences in antimicrobial activity is observed. This suggests that the antimicrobial compound(s) in the root material of *P. capense* is highly heat-resistant. For some plants however the opposite is true in that heat degrades active compounds. Some plant extracts have been reported to either lose antibacterial activity, retain antibacterial activity, and others to have increased antibacterial activity after storage (Stafford et al. 2005).

Table 3-9: Antimicrobial activity ($MIC_{p\text{-}INT}$) of $M_sA_sH_s$ against *S. aureus* and *C. albicans* on Day 0 and Day 30 at 25℃ and 40℃.

Microorganism	$MIC_{p\text{-}INT}$ (mg/mL)		
	Temperature	Day 0	Day 30
S. aureus	25℃	0.029	0.029
	40℃	0.029	0.029
C. albicans	25℃	0.029	< 0.007
	40℃	0.029	< 0.007

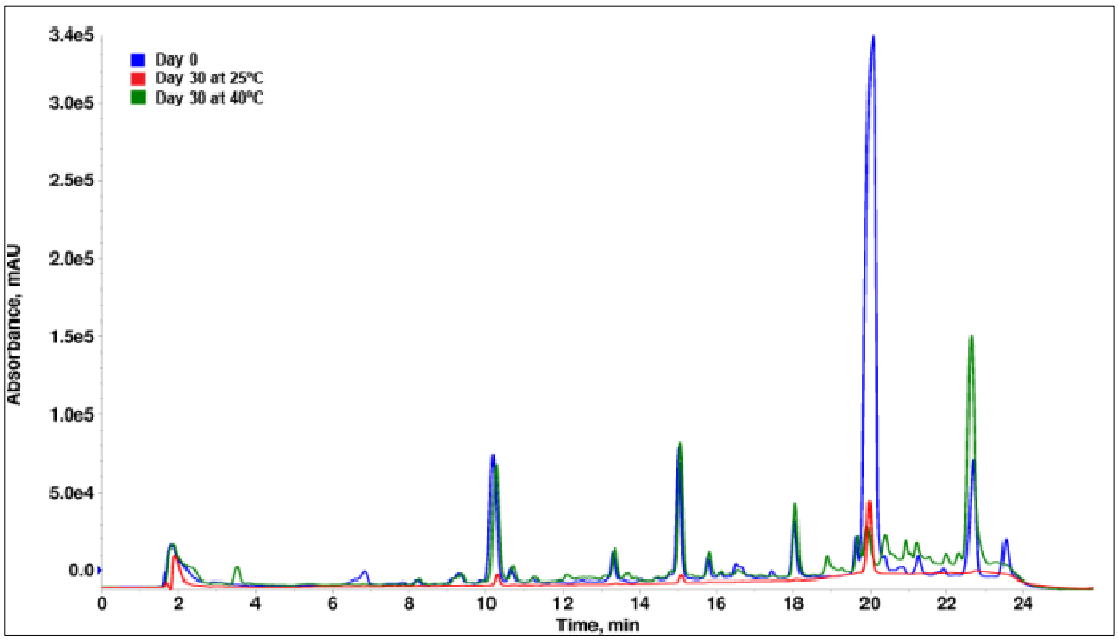


Figure 3-25: HPLC chromatogram of the $M_SA_SH_S$ subfraction recorded at a wavelength of 214 nm. The mobile phase was run as a gradient consisting of water (0.1% formic acid) and methanol (0.1% formic acid). The blue solid line represents a freshly prepared sample (Day 0), the solid red line represents the sample stored at 25ºC for 30 days and the solid green line represent the sample stored at 40ºC for 30 days.

As root material or other underground organs from plants often store secondary plant metabolites, the compounds found in these parts are often more stable than those found in other parts e.g. leaves (Stafford et al. 2005). This would appear to be the reason for the stability of the root-bark extracts examined in the current study. As prolonged heating has been found to degrade plumbagin (Hsieh et al. 2005), it is theorised that the retention and increase in antimicrobial activity in the $M_SA_SH_S$ subfraction is probably attributed to a plumbagin derivative that would have the same toxicity mechanism or profile.

Chapter 4

Conclusion

Although traditional healers prepare extracts from *P. capense* as an aqueous infusion, other solvents were also used in order to allow plant compounds of different polarities to be extracted. Of all the crude extracts tested in the study, the crude acetone extracts contained the greatest antimicrobial activity. Antimicrobial activity was observed for Gram-positive bacteria, Gram-negative bacteria as well as a common yeast strain, thus indicating a broad spectrum of antimicrobial activity. Using the broth micro-dilution assay, the $M_sA_sH_s$ subfraction showed the highest antimicrobial activity with an MIC value of 0.029 mg/ml against both *S. aureus* and *C. albicans*. Bioautography of collected HPLC eluent of the $M_sA_sH_s$ subfraction revealed that almost all the antimicrobial activity of $M_sA_sH_s$ was restricted to a major peak eluting at 13.6 min using a reverse phase type column.

Phytochemical screening showed an absence of terpenes/terpenoids and flavonoids in all of the samples with alkaloids and lipids/sterols/steroids only being present in $M_sA_sA_s$. Phenolic compounds were detected in six of the subfractions (CWE, CME, CAE, M_sA_s, $M_sA_sA_s$ and $M_sA_sH_s$) and amino acids/peptides in four of the subfractions (CWE, CME, M_sA_s and $M_sA_sA_s$). Of all of the samples tested, $M_sA_sA_s$ appeared to be most complex with six phytochemical groups identified whereas CHE was the least complex with only one compound detected. Quinones were present in all of the samples, and appeared to be responsible for the antimicrobial activity.

IR spectroscopy led to the identification of a carbonyl and hydroxyl functional group in the major compound in the $M_sA_sH_s$ subfraction, and also indicated that this major compound has an aromatic character. When the $M_sA_sH_s$ subfraction was further enriched for the major compound and analysed by LC-MS/MS in

both the positive and the negative modes, the molecular formula of $C_{11}H_8O_3$ was identified for the major compound. This formula and the chemical structure were confirmed using GC-MS, where the mass spectra of the major compound in $M_sA_sH_s$ had a 98% certainty match with 5-hydroxy-2-methyl-1,4-naphtalenedione, a molecule also known as plumbagin. The aromatic nature and presence of carbonyl and hydroxyl groups of the compound of interest was confirmed with the quinone character of plumbagin. This is the first report of the presence of this compound in the *Piper* genus.

TLC analysis revealed the presence of antioxidant activity in CWE, CME, CAE, M_sA_s as well as $M_sA_sA_s$. This activity was confirmed using the DPPH and ABTS radical scavenging assays.

Cytotoxicity was determined against C_2C_{12} cells as well as resting and PHA stimulated lymphocytes where CME, CAE, CHE, $M_sA_sH_s$ and plumbagin were found to exhibit significant cytotoxic activity.

The stability of the antimicrobial $M_sA_sH_s$ subfraction was determined over 30 days at both 25℃ and 40℃. This activity of M $_sA_sH_s$ appeared stable at both temperatures against *S. aureus*. An increase in antifungal activity from 29 µg/ml to < 7 µg/ml was also obtained against *C. albicans*. It would appear as if the antifungal compounds are converted to more active antifungal compound(s) over time.

The compound responsible for the antimicrobial activity in the *P. capense* root bark was isolated and characterised and found to be 5-hydroxy-2-methyl-1,4-naphtalenedione also known as plumbagin. The identity of the compound was confirmed by comparison to authentic plumbagin. The antimicrobial activity was also confirmed for the pure standard of plumbagin. Although plumbagin does have a toxic profile systemically, topical application should be explored.

This study has provided scientific support for the ethnomedical use of the root-bark of *P. capense* in the treatment of infectious diseases. The activity is

ascribed to the isolated compound, plumbagin, which has been identified for the first time in this plant genus.

Chapter 5

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