Pharmacological and phytochemical evaluation of seven plants used for microbial-related ailments in South African traditional medicine

By

McMaster Vambe

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg Campus

April, 2018

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Pharmacological and phytochemical evaluation of seven plants used for microbial-related ailments in South African traditional medicine

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our abilities and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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M. Vambe, A. O. Aremu, J. C. Chukwujekwu, J. F. Finnie, J. Van Staden 2018. Antibacterial screening, synergy studies and phenolic content of seven South African medicinal plants against drug-sensitive and -resistant microbial strains. South Africa Journal of Botany 114, 250-259. The publication covers part of Chapters 2 and 3.

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ABSTRACT

Since antiquity, man has always believed in the healing properties of plants. The age-old practice of phytotherapy is now justified by numerous phytochemical and pharmacological studies which substantiate the presence of biologically active compound(s) in some medicinal plants. Quite often, ethnopharmacological studies provide important leads for the development of different types of plant-based therapeutic drugs. New effective antibiotics are urgently needed to combat multiple and extensively drug-resistant bacterial strains that are currently threatening public health globally. The rich floral diversity in southern Africa and the resultant extensive chemical diversity provide encouraging prospects for discovering novel pharmacologically important phytocompounds within this region. Against this background, the current study was designed to evaluate the *in vitro* antibacterial properties, phenolic profiles and mutagenic potentials of extracts obtained from seven South African plants used traditionally to manage bacterial infections and where the active principles were unknown. The study also aimed at identifying the principal antibacterial compound(s) in selected plants that demonstrated potent and broad-spectrum antibacterial properties.

Aqueous and organic solvent extracts of the seven selected plants namely *Bolusanthus speciosus, Cucumis myriocarpus, Ekebergia capensis, Protea caffra, Prunus africana, Searsia lancea* and *Solanum panduriforme* were screened for antibacterial (minimum inhibitory concentration, MIC) properties against a panel of seven bacterial strains (*Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae,* multiple drug-resistant (MDR) *E. coli,* MDR *K. pneumoniae,* drug-sensitive *Staphylococcus aureus* and penicillin-resistant *S. aureus*) using the microdilution technique. The extracts were also screened for antigonococcal properties using microdilution and agar disk-diffusion techniques. In addition, combinations of

the different plant extracts, as well as plant extracts with four antibiotics (ampicillin, cefotaxime, chloramphenicol and penicillin) were evaluated for antibacterial synergistic interactions against MDR Gram-negative bacterial strains (*E. coli* and *K. pneumoniae*) using the checkerboard titration and time-kill bio-assays. Preliminary phytochemical analyses for phenolics in aqueous methanol (50% MeOH) plant extracts were conducted using spectrophotometric methods. In addition, specific phenolic acids in 80% MeOH extracts of the plants were quantified with the use of ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The mutagenic properties of plant extracts that demonstrated noteworthy antibacterial activities (MIC<1 mg/ml) were assessed using the Ames Salmonella/Microsome assay involving two tester strains, TA98 and TA102 (without S9 metabolic activation).

The most potent antibacterial activities were exhibited by the MeOH bark extracts of *B. speciosus* which yielded MIC values of 0.039 and 0.078 mg/ml against *S. aureus* (penicillin-resistant and drug-sensitive strains, respectively). Dichloromethane (DCM) leaf extracts of *S. lancea* yielded an MIC value of 0.63 mg/ml against five of the seven test bacterial strains including MDR *E. coli* and MDR *K. pneumoniae. B. speciosus* bark (MeOH and DCM), *P. caffra* seeds (DCM) and twigs (MeOH) also demonstrated broad-spectrum antibacterial activities. *B. speciosus* MeOH bark extracts, as well as the DCM leaf extracts of *P. africana* and *S. lancea* demonstrated moderate antigonococcal properties (MIC range: 0.31-0.63 mg/ml). The checkerboard assay detected antibacterial synergistic interactions in combinations of chloramphenicol with each of *B. specious* leaf MeOH extracts, *P. africana* leaf MeOH and DCM extracts against MDR *E. coli* (fractional inhibitory concentration index ≤ 0.5). However, the time-kill assay did not detect any significant synergistic interactions in any of these three aforementioned combinations. Total phenolic content in the plant extracts investigated ranged

from 2.38 to 62.73 mg gallic acid equivalents (GAE)/g dry matter. Variations in the quantities of flavonoids, condensed tannins, hydroxybenzoic and hydroxycinnamic acids among these plant extracts were also observed. In generally, higher levels of phenolic compounds were detected in *S. lancea* (leaves), *P. africana* (leaves) and *P. caffra* (leaves, twigs), while lower levels were detected in *B. speciosus* (barks), *C. myriocarpus* (fruits) and *S. panduriforme* (fruits, leaves, roots, stem). Based on the Ames test, all test plant extracts were non-mutagenic against *Salmonella typhimurium* tester strain TA98 and TA102 (without S9 metabolic activation).

S. lancea leaves and *P. caffra* twigs demonstrated broad-spectrum antibacterial properties and as a result, a detailed analysis of their phytochemical constituencies was conducted. Antibacterial activity directed fractionation led to the isolation of two compounds from the aqueous (80%) MeOH leaf extracts of *S. lancea*. The isolated compounds were characterised using NMR data and both of them demonstrated noteworthy antibacterial activities (MIC range: 0.016-0.25 mg/ml) against *E. faecalis* and *S. aureus.* 1-Tetracosanol (43.98%), 1-nonadecanol (37.5%), eicosane (7.67%), 1,7-di-*iso*-propylnaphthalene (4.23%), 1-pentadecanol (3.04%), 2,6-di-*iso*-propylnaphthalene (1.96%) 1,3-*iso*-propylnaphthalene (1.62 %) were identified by GC-MS in an active *S. lancea* leaf ethyl acetate sub-fraction. The sub-fraction inhibited the growth of both *E. faecalis* and *S. aureus* at an MIC value of 0.25 mg/ml. 1-Tetracosanol and 1-nonadecanol, which accounted for more than 80% of the total phytochemical constituents of the fraction, are known antibacterial compounds which most likely contributed to the antibacterial properties of *S. lancea* leaves. Gas chromatography-mass spectrometry (GC-MS) analysis also revealed the presence of 15 compounds in three fractions obtained from a methanolic *P. caffra* twig extract. Two of the identified compounds, 1-adamantanecarboxylic acid and levoglucosan, are often incorporated into antimicrobial

moieties to improve the efficacy of the therapeutic molecule, or as carbon skeletons. Two known antibacterial compounds, namely 1-heptacosanol and 1-nonadecanol were also among the compounds detected in the methanolic *P. caffra* twig extract.

Overall, the present study reaffirmed that botanical medicines can potentially be used to manage drug-sensitive and drug-resistant bacterial infections often prevalent in humans. The study also provided the scientific rationale for the use of *P. caffra, C. myriocarpus* and *S. panduriforme* in South African folk medicine.

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4NQO	4-Nitroquinoline-N-Oxide
AIDS	Acquired immune-deficiency syndrome
ATTC	American Type Culture Collection
CFU	Colony forming unit
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
GC-MS	Gas Chromatography-Mass Spectrometry
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HNMR	Proton Nuclear Magnetic Resonance
INT	*p*-Iodonitrotetrazolium chloride
MDR	Multiple drug-resistant
MeOH	Methanol
MH	Mueller-Hinton
MHz	MegaHertZ
MIC	Minimum inhibition concentration
NMR	Nuclear Magnetic Resonance
PE	Petroleum ether
PMB	Pietermaritzburg
RCPGD	Research Centre for Plant Growth and Development
RNA	Ribonucleic acid
SAAB	South African Association of Botanists
SASSB	South African Society of Systematic Biology
TLC	Thin Layer Chromatography
UKZN	University of KwaZulu-Natal

CHAPTER 1:Introduction

1.1. South African biodiversity and medical bio-prospecting

Approximately 80% of the South African population depends on medicinal plants as supplements or alternatives to synthetic pharmaceutical drugs **(NATTRASS, 2008)**. These phytomedicines are often prescribed by herbalists or 'witch doctors' as remedies for both spiritual and health care problems **(VAN WYK, 2008)**. Extensive research into botanical medicines over the past few decades has resulted in the herbal and natural plant products industry becoming a prominent feature of South Africa's agribusiness (**MANDER AND MCKENZIE, 2005; MANDER et al., 2007; VAN WYK, 2008)**. This perhaps explains why in 1998 the South African government embarked on a 'Proudly South African' campaign in a bid to promote the trading of its natural products on the international market **(MAKUNGA et al., 2008)**. African potato (*Hypoxis hemerocallidea*), devil's claw (*Harpagophytum procumbens*) and rooibos (*Aspalathus linearis*) are some classical examples of commercially important South African medicinal plants that have attained international status in recent times (**VAN WYK, 2015)**.

South Africa's rich biodiversity continues to captivate the attention of both international and local ethnopharmacologists (**VAN STADEN, 2008)**. **VAN WYK (2008)** observed that since the year 1995, there has been a steady increase in the number of ethnobotanical publications, citations and patents on South African medicinal plants. Some of the over 3 000 South African medicinal plant species (**VAN WYK et al., 1997)** including *Bolusanthus speciosus, Helichrysum aureonitens, Helichrysum caespitium, Veronia colorata* and *Warburgia salutaris* have been shown to have potent antibacterial activities (**VAN VUUREN, 2008; MULAUDZI et al., 2011)**. Antibacterial synergistic studies also indicate that antibiotic resistance modifying molecules

could potentially be obtained from South African medicinal plants including *Combretum edwardsii*, *Combretum krausii* (**CHUKWUJEKWU AND VAN STADEN**, **2016)**, *Centratherum punctatum* (**CHUKWUJEKWU et al.**, **2018)**, *Merwilla plumbea* (**NCUBE et al.**, **2012)**, to mention but a few. The extensive floral diversity within this country (**GERMISHUIZEN AND MEYER**, **2003)** provides a potential rich source of structurally diverse biologically active molecules which could be useful in combating both drug-sensitive and -resistant microbial strains.

1.2. Aims and significance of the study

The current study primarily aimed at identifying the principal antibacterial molecules in some South African medicinal plants with potent and broad-spectrum antibacterial properties. South Africa has a rich floral diversity which has been under-explored with regards to medical bioprospecting. Even though some South African medicinal plants have been shown to have potent antibacterial properties, the principal antibacterial compounds within some of these plants are unknown. Little is also currently known about the susceptibility of drug-resistance bacterial strains to extracts from these plants. Furthermore, the interspecies synergistic interactions of South African medicinal plant extracts, as well the interactions of these plant extracts with conventional antibiotics remain largely unknown. Findings from this study may provide further justification for the traditional use of indigenous plants in South Africa, reveal novel sources of potent and extended-spectrum antibacterial compounds, as well as provide leads for the discovery and development of new antibacterial compounds with potential use in both mono- and/ or combination therapies.

CHAPTER 2: Literature review

2.1. Literature review on selected medicinal plants

Plants are known to produce a myriad of structurally diverse secondary metabolites, some of which have significant medicinal properties (**GURIB-FAKIM**, **2006)**. By the mid-70's, approximately 75% of all pharmaceutical drugs present were developed as a direct result of extensive ethnopharmacological research (**FARNSWORTH AND BINGEL**, **1977)**. Artemisinin, aspirin, camptothecin, quinine and taxol are some examples of the over 7 000 popular therapeutic drugs developed from efficacious plant extracts **(TSHIBANGU et al., 2002)**.

Pharmacological and phytochemical evaluations of South African medicinal plants have led to a marked increase in the commercialization of herbal products from this country (**MAKUNGA et al.**, **2008; MOTEETEE AND VAN WYK**, **2011)**. Buchu water (**STREET AND PRINSLOO**, **2012)**, marula creams, marula jams (**SHACKLETON et al.**, **2003)** and *umkalor* **(VAN WYK, 2011)** are but a few classical examples of pharmaceutical and cosmetic products developed from South African medicinal plants. However, given that only a small proportion of South African medicinal plants have been tested pharmacologically (**PIETERS AND VLIETINCK**, **2005)**, there still exist encouraging prospects of discovering several novel bioactive compounds from this country's rich floral diversity. For the current study, Google scholar (https://scholar.google.com) was used to search for South African medicinal plants used traditionally to manage different bacterial infections and where the active principles had not yet been identified. Based on these criteria and material availability, seven traditional medicinal plants namely *Bolusanthus speciosus*, *Cucumis myriocarpus*, *Ekebergia capensis*, *Prunus africana*, *Protea caffra*, *Searsia lancea* and *Solanum panduriforme* were selected (Figures 2.1-2.7). The ethnomedicinal uses of these plants are summarized in Table 2.1.

Table 2.1: Ethnomedicinal applications of seven South African traditional medicinal plants as recorded in the current literature.

Species, authority Family	Part used	Voucher number	Traditional uses	References
Bolusanthus speciosus (Bolos) Harms Fabaceae	Bark	NU0042512	Abdominal pains, emetic and tuberculosis	**PALGRAVE (1977)**
	Bark Leaves Stems		Blood cleansing, kidney ailments and sexually transmitted infections	**MAGOBO (1990)**
	Roots		Emetic and abdominal pains	**AMUSAN et al. (2002)** **VENTER AND VENTER (2002)**
Cucumis myriocarpus (Naud) E. Mey Curcubitaceae	Fruits	NU0042511	Gonorrhoea and syphilis	**SEMENYA et al. (2013a)**
Ekebergia capensis Sparrm Maliaceae	Bark	NU0042516	Malaria, tonic, chronic coughs, headaches, dysentery, boils, dermatological diseases, laxative, emetic, sexually-transmitted diseases	**HUTCHINGS et al. (1996)** **GRIERSON AND AFOLAYAN (1999)** **KOCH et al. (2005)**
Prunus africana (Hook) Kalkman Rosaceae	Stems Bark	NU0042513	Allergies, diarrhoea, gastrointestinal ailments, kidney and prostate gland diseases	**PUJOL (1990)**
	Leaves		Abdominal pains, laxative, skin diseases, prostatitis	**GANGOUE-PIEBOJI et al. (2009)**
	Not specified		Anti-inflammatory, aphrodisiac, appetite stimulants, chest pains, heart burn, fevers, kidney ailments, madness, malaria, urinary tract infections, wound dressing	**NEUWINGER (2000)** **NDENECHO (2009)**
Protea caffra Meisn Proteaceae	Bark	NU0048533	Bleeding stomach ulcers and diarrhoea	**COETZEE AND LITTLEJOHN (2000)**
	Roots		Healing broken bones	**ZUKULU (2012)**
	Seed		Chlamydia	**SEMENYA et al. (2013a)**
Searsia lancea Barley Anacardiaceae	Wood	NU0042514	Skin tanning	**WATT AND BREYER-BRANDWIJK (1962)**
	Leaves		Colds, fever, papules and pustules	**HUTCHINGS et al. (1996)**
	Roots		Diarrhoea and gall sickness	**VAN DER MERWE et al. (2001)**
Solanum panduriforme E. Mey Solanaceae	Fruits	NU0042515	Gonorrhoea and wounds	**BRUSCHI et al. (2011)** **ERASMUS et al. (2012)**
	Roots		Diarrhoea, ingestion, pelvic pains, stomach aches, snake bites, tooth aches and ulcers	**BRUSCHI et al. (2011)** **HUTCHINGS et al. (1996)**

2.1.1. *Bolusanthus speciosus* Harms

2.1.1.1. Botany and ethnomedicinal applications

B. speciosus belongs to the Fabaceae family and is the only known member of the genus *Bolusanthus* (**ERASTO et al., 2004)**. This deciduous tree which grows up to 10 m in height is indigenous to Africa's Sub-Saharan regions where it thrives mainly on bushveld with alkaline soils (**VAN WYK AND VAN WYK, 1997)**. In South Africa, this tree is locally known as tree wisteria (English); *vanwykshout* (Afrikaans); *mogaba* (Northern Sotho); *motsokophala* (Tswana); *mukambana* (Venda) and *umholo* (Zulu) (**ELISHA et al., 2015)**. In Limpopo, South Africa, the bark, leaves and stems of *B. speciosus* are commonly used by the VhaVenda people to treat kidney and venereal diseases (**MAGOBO, 1990)**. Root infusions are also used as an emetic, while the stem bark is used to manage abdominal pains, emetism and tuberculosis (**PALGRAVE, 1977)**.

2.1.1.2. Ethnopharmacology and phytochemistry

Several studies validated the traditional use of *B. speciosus*. For instance, **MULAUDZI et al. (2011)** revealed and confirmed that the plant has potent antibacterial activities against pathogens that cause abdominal pains and gonorrhoea. The authors also revealed that the plant has noteworthy antifungal and anti-HIV properties **MULAUDZI et al. (2013)** demonstrated that *B. speciosus* stems have potent anti-inflammatory activities. In other studies, organic solvent extracts from the leaves of *B. speciosus* exhibited strong inhibitory effects against the growth of *Bacillus anthracis* (**ELISHA et al., 2015)** and weak antimycobacterial activities (**ELISHA et al., 2017)**.

Figure 2.1: *Bolusanthus speciosus* growing in the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Some of the compounds isolated from *B. speciosus* (Table 2.2.) have been demonstrated to have noteworthy antimicrobial activities. **BOJASE et al. (2001b)** used the Thin Layer Chromatography (TLC) bioautography technique to investigate the antibacterial properties of flavonoids isolated from the stem bark. The authors observed that both 4,7,2C-trihydroxy-4C-methoxy isoflavanol and 5,7,3C,4C-tetrahydroxy-5C-(2-epoxy-3-methylbutyl) isoflavanone had weak activities against Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial strains. Based on the agar overlay technique, Bolusanthin II, 5,7,2′,4′-tetrahydroxy-8,3′-di(γ,γ-dimethylallyl) isoflavanone, 5,7,2′-trihydroxy-4′- methoxy-6,5′-diprenylisoflavanone and 5,7,4′- trihydroxy-6,3′- di (γ,γ-dimethylallyl) isoflavanone has good activities against *B. subtilis* and *S. aureus*, as well as moderate inhibitory activities against *E. coli*, *Candida mycoderma* and *Saccharomyces cerevisiae* (**BOJASE et al., 2002)**. Bolusanthin III, Bolusanthin IV and Isogacaonine also have noteworthy antimicrobial and antioxidant activities (**ERASTO et al., 2004)**.

Although the chemistry and biological activities of *B. speciosus* are well documented, the synergistic interactions between its extracts and antibiotics, as well as the efficacy of these extracts and their bioactive constituents against drug-resistant bacterial strains remains largely unknown.

Table 2.2: Putative bioactive compounds isolated from different organs of *Bolusanthus speciosus* as recorded in the current literature.

Plant organ	Compound	Chemical group	Reference
Leaves	11α-allycytisine, anagyrine, 5,6-dehydrolupanine, 13-hydroxyanagyrine, ß-*iso*-parteine, lupanine, N-methylcytisine, sparteine	Alkaloids	**ASRES et al. (1986)**
Root bark	Bolucarpan A-D, bolusanthin II	Flavonoids	**BOJASE et al. (2001b)**
Root bark	Bolusanthin II, bolucarpan A, bolucarpan B, bolucarpan D	Flavonoids Ptero-carpans	**BOJASE et al. (2002)**
Root wood	Bolusanthin III, bolusanthin IV, derrone, 7,3'-dihydroxy-4'-methoxyisoflavone, gancaonin C, genistein, 7-hydroxy-4-methoxyisoflavone, isogancaonin C, lupiwighteone, medicarpan, wighteone	Flavonoids	**ERASTO et al. (2004)**
Seeds	Biochanin, bolusanthin, genistein, orobol, pratensein, 3'-O-methylorobol, 3-O-methylpratensein	Flavonoids	**ASRES et al. (1985)**
Stem bark	Bolusanthols A-C	Flavonoids	**BOJASE et al. (2001a)**
Stem bark	5,7,3'-trihydroxy-4'-methoxy-5'-ɣ,ɣ-dimethylallylisoflavanone, 5,7,2'-trihydroxy-4'-methoxy-6,5'-di(ɣ,ɣ-dimethylallyl) isoflavanone, 5,7,2',4'-tetrahydroxy-8,3'di(ɣ,ɣ-dimethylallyl) isoflavanone 4,2,3',4'-tetrahydroxy-6,7- methylenedioxyisoflavonol, 4,7,2'-trihydroxy-4'-methoxyisoflavanol, derrone, 5,7,3',4'- tetrahydroxy-5'-ɣ,ɣ-dimethylallyl-isoflavanone, 5,7,4'-trihydroxy-6,3'-di(ɣ,ɣ-dimethylallyl) isoflavanone, 5,7,3',4'-tetrahydroxy-5'-(2,3-epoxy-3-methylbutyl) isoflavanone	Flavonoids	**BOJASE et al. (2001b)**

2.1.2. *Cucumis myriocarpus* Naud

2.1.2.1. Botany and ethnomedicinal applications

C. myriocarpus Naud is an annual climber which flowers in summer and grows up to 2 m in length (**SHAIK et al., 2011)**. This scandent herb is a member of the Curcubitaceae family and has two subspecies: subsp. *leptodermis* (Schweick.) found only in South Africa, and subsp. *myriocrpus* which is found in different parts of the world (**KIRKBRIDE, 1993)**. In South Africa, this plant is commonly known as the bitter apple, wild cucumber (English), *agurkie*, *bitterappeltjie*, *komkommertjie* (Afrikaans) and *sendelenja* (Zulu) (**HUTCHINGS et al., 1996)**.

A survey by **SEMENYA et al. (2013c)** revealed that the Bapedi people of South Africa use the fruits of this plant to treat gonorrhoea and syphilis. Fruits are also used to treat boils and as a purgative or emetic agent **(MOTEETEE AND VAN WYK, 2011; USMAN et al., 2015)**. However, the plant contains high concentration of toxic cucurbitacins (**SIMMONDS et al., 2000; MASHELA et al., 2008)** and as such, extreme caution should be taken when using this herb as a vegetable (**NKGAPELE AND MPHOSI, 2012)** or when administering it to patients. Research indicates that consumption of the plant often leads to photosensitization in sheep and blindness in cattle **(MCKENZIE et al., 1988; SIMMONDS et al., 2000; KESSELL et al., 2015)**. Over dosages of root and ripe fruit decoctions may also lead to sudden death in humans (**BOTHA AND PENRITH, 2008)**.

Figure 2.2: *Cucumis myriocarpus* growing in the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The insert shows the fruit of *C. myriocarpus*.

2.1.2.2. Ethnopharmacology and phytochemistry

Compared to other African traditional medicinal plants, the ethnobotanical applications of wild cucumber are quite restricted, perhaps due to its bitterness and toxicity. Cucurbitacins which are highly concentrated in the roots and fruits are among some of the bitterest substance known to man **(POFU et al., 2010)**. The fruits also contain elevated quantities of curcumin, leptodermin, as well as cucurbitin A and D which are highly toxic **(MASHELA, 2002; MPHAHLELE et al., 2012)**.

The potential use of this plant as a bionematicide has been extensively investigated **(MASHELA, 2002; MAFEO AND MASHELA, 2010; POFU et al., 2010)**. However, a review of the current literature indicates that only one study (**MUEDI**, **2005)** was conducted to assess the pharmacological properties of *C. myriocarpus*. The authors reported that the fruits have anthelmintic properties as well as inhibitory effects against *Bacillus litcheniformis*, *Bacillus laterosporus* and *Bacillus chitinosporus* (MIC range: 0.78-3.13 mg/ml). Further studies are warranted to particularly validate the traditional uses of this medicinal plant, as well as to identify the bioactive compounds it produces.

2.1.3. *Ekebergia capensis* Sparrm

2.1.3.1. Botany and ethnomedicinal applications

The genus *Ekebergia* consists of African trees belonging to the Trichiliae tribe of the Meliaceae family (**MULHOLLAND AND IOURINE**, **1998)**. *E. capensis* is a fairly large deciduous tree (60 m tall) native to Africa's tropical and subtropical regions (**POOLEY**, **1993; HEMP**, **2006)**. In South Africa, this tree is known locally as the Cape-ash (English), *essenhout* (Afrikaans), *mmidibidi* (Nothern Sotho) and *umnyamati* (Zulu) (**GRIERSON AND AFOLAYAN**, **1999; SMITH et al.**, **2010; GRAINGER et al.**, **2011)**.

The Cape-ash is used widely in African traditional medicine. For instance, the Zulu people of South Africa use stem wood decoctions as an oxytocic agent, while root decoctions are used to treat acne, boils, coughing, dysentery, gastritis, hyperacidity and sores (**WATT AND BREYER-BRANDWIJK**, **1962; PUJOL**, **1990)**. In Kenya, the Sabaot herbalists use leaf macerations to manage headaches, fevers, coughing and skin diseases, while the Agĩkũyũ and Kikuyu traditional healers use the stem bark and roots to treat diarrhoea (**IRUNGU et al.**, **2014)**. Kenyans also use the stem bark to manage gonorrhoea and tuberculosis (**KIMUTAI et al.**, **2015)**. In different parts of Africa, the plant is used to treat several ailments including malaria, chest pains, respiratory infections and also, as a purgative parasiticide (**WATT AND BREYER-BRANDWIJK**, **1962; HUTCHINGS et al.**, **1996; MUREGI et al.**, **2004; IRUNGU et al.**, **2014)**.

2.1.3.2. Ethnopharmacology and phytochemistry

The traditional uses of *E. capensis* are, to some extent, substantiated by empirical evidence. Leaf extracts of the plant have inhibitory activities against *Cryptococcus neoformans* (MIC = 0.4 mg/ml), a fungi that cause respiratory infections (**YORK et al.**, **2012)**. **LALL AND MEYER (1999)** also demonstrated that acetone extracts from the bark have noteworthy activities against both drug-resistant and -sensitive *Mycobacterium tuberculosis* strains. The plant also has potent antifungal, antigonococcal (**MULAUDZI et al.**, **2011)** and broad-spectrum antibacterial properties (**RABE AND VAN STADEN**, **1997; KIMUTAI et al.**, **2015)**. Apart from antimicrobial properties, work by other researchers revealed that the plant also has noteworthy antihypertensive (**KAMADYAAPA et al.**, **2009)**, antimalarial(**CLARKSON et al.**, **2004; MUREGI et al.**, **2004; IRUNGU et al.**, **2014)** and antiviral properties (**BAGLA et al.**, **2012)**.

Figure 2.3: *Ekebergia capensis* growing in the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Although empirical evidence suggests that *E. capensis* has extended-spectrum antibacterial activities, the principal bioactive compounds remain largely unknown. Some phytochemical studies on this plant, however, revealed the presence of limonoids and triterpenoids (Table 2.3), some of which demonstrated promising antiplasmodial properties (**NISHIYAMA et al.**, **1996; MULHOLLAND AND IOURINE**, **1998; MURATA et al.**, **2008)**. These compounds have, however, not yet been evaluated for antibacterial and other pharmacological properties.

Table 2.3: Putative bioactive compounds isolated from different organs of *Ekebergia capensis* as recorded in the current literature.

Plant organ	Compounds	Chemical group	References
Stem bark	Ekeberin D4, D5, (3R,22R)-2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene, (2R,3R,22R)-2-hydroxymethyl-2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetraene	Triterpenoids	**MURATA et al. (2008)**
Stem bark	7-deacetoxy-7-oxogedunin Ekeberin C1-C3	Limonoids	
Seeds	Capensolactones l-3	Limonoids	**MULHOLLAND AND IOURINE (1998)**
Stem bark	2,3,22,23-tetrahydroxy-2,6,10,15,19,23-hexamethyl-6,10,14,18-tetracosatetrae ne and 2-hydroxymethyl-2,3,22,23-tetrahydroxy-6,10,15,19,23-pentamethyl-6,10,14,18-tetracosatetraene	Acyclic triterpenoids	**NISHIYAMA et al. (1996)**

2.1.4. *Prunus africana* (Hook. F.) Kalkman

2.1.4.1. Botany and ethnomedicinal applications

In South Africa, *P. africana* is commonly known as the African almond, African cherry, bitter almond (English), *bitteramandelhout*, *nuweamandelhout*, *rooi-stinkhout, wilde-kersieboom*, (Afrikaans), *inkokhokho*, *inyazangoma-elimnyama*, *ngubozinyeweni*, *umlalume* (Zulu), *inyazangoma*, *umkhakhazi* (Xhosa and Zulu) and *mulala-maanga* (TshiVhenda) **(PALMER AND PITMAN, 1961; POOLEY, 1993)**. This dicotyledonous, evergreen and highland forest tree (30-40 m tall) belongs to the Rosaceae family and thrives mostly in tropical and subtropical islands and countries across the African and American continents **(BRENAN, 1978)**. In South Africa, isolated populations of the African almond are found in the Eastern Cape, Free State, Gauteng, Kwazulu-Natal, North West, Limpopo and Mpumalanga provinces (**SCOTT-SHAW**, **1999)**.

P. africana plays a very significant role in South African folk medicine so much that there are growing concerns that the plant may soon be extinct due to overexploitation (**GANGOUE-PIEBOJI et al.**, **2009)**. Traditional healers prescribe different parts of the plant to manage fevers, malaria, stomach pains, kidney diseases, high blood pressure, allergies, prostate gland disease, insanity and as a purgative agent **(STEWART, 2003; BII et al., 2010)**. This plant is also used traditionally to treat diseases that are known or suspected to be caused by bacterial infections such as diarrhoea, inflammations, gonorrhoea, dermatological, respiratory and urethral tract infections (**GANGOUE-PIEBOJI et al.**, **2009)**. According to **SHENOUDA et al. (2007)**, the plant is also used by African traditional medical practitioners to manage different types of cancer.

Figure 2.4: A specimen photograph of *Prunus africana* courtesy of http://sizeofwales.org.uk.

2.1.4.2. Ethnopharmacology and phytochemistry

Although *P. africana* leaves are used widely in folk medicine, it seems most pharmacological studies focused mainly on screening its stem bark for antimicrobial activities. **ELDEEN et al. (2005)** demonstrated that ethanol extracts of the bark and leaves have noteworthy inhibitory activities against both Gram-positive (*B. subtilis* and *S. aureus)* and Gram-negative (*E. coli* and *K. pneumoniae*) bacterial strains. Ethanol extracts of the bark also exhibited noteworthy activities against ß-lactam-resistant *Enterococcus hirae* and *Staphylococcus saprophyticus* with MIC values of 0.6 and 0.3 mg/ml, respectively (**GANGOUE-PIEBOJI et al., 2009)**. Studies by **BII et al. (2010)** provided further empirical evidence suggesting that the plant has antibacterial compounds effective against drug-resistant bacterial strains. The authors demonstrated that methanolic extracts of the bark had good inhibitory activities against drug-sensitive *S. aureus*, *Trichophyton mentagrophyte*, *Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MIC range: 0.039-0.156 mg/ml). They also observed that the same extracts (methanol bark) were not active against *Cryptococcus neoformans* and *Candida albicans*. **MWITARI et al. (2013)** confirmed some of these results and postulated that the responsible bioactive compounds had bactericidal and immunopotentiation effects.

Prostate gland hypertrophy and benign prostrate hyperplasia (BPH) are manageable using commercially available medicines prepared from *P. africana* root and stem bark extracts **(SUNDERLAND AND OBAMA, 1999; SCHIPPMANN et al., 2002)**. Pharmacological and phytochemical studies indicates that these barks contain phytosterols (an anti-inflammatory agent that inhibit the accumulation of proinflammatory prostaglandins in the prostate), pentacyclic triterpenes (anti-edema or decongesting action) and ferulic esters of long chain fatty alcohols (lowers the level of prolactin and inhibit the accumulation of cholesterol in the prostate) (**BOMBARDELLI AND MORAZZONI, 1997)**. The plant also produces another anti-BPH

compound known as myristic acid (**HASS et al., 1999)**. Phytochemical studies by **NTUBA-JUA et al. (2018)** and **NGULE et al. (2014)** revealed the presence of tannins, flavonoids, terpenoids, saponins, phenolics and steroids in *P. africana* barks. The presence of these compounds could probably justify the traditional use of the plant to treat different bacterial infections.

P. africana has several ethnomedicinal applications and hence is a potential source of different bioactive molecules. While the plant's anti-BPH properties are well documented, little is currently known about its antibacterial compounds. Research indicates that there is much potential in discovering antibacterial compounds effective against both drug-resistant and -sensitive bacterial strains from this medicinal plant (**GANGOUE-PIEBOJI et al., 2009; BII et al., 2010)**.

2.1.5. *Protea caffra* Meisn

2.1.5.1. Botany and ethnomedicinal applications

P. caffra is a dicotyledonous shrub that flowers in summer and has an average height of 3 m (**DU PREEZ AND VENTER, 1990)**. In South Africa, it is known as the common sugarbush, Natal sugarbush, highveld protea (English), *gewone suikerbos*, *waboom*, (Afrikaans), *isadlunge*, *indlunge*, *isiqwanwe* (Isixhosa), *uhlinkihane* (Zulu) *tshididiri*, *tshidzungu* (TshiVhenda), *mahlako*, *mogalagala*, *segwapi*, *sekila* and *tshidzungu* (Sotho) (**PATERSON-JONES, 2000, 2007; RAIMONDO et al., 2009; WYBENGA, 2010)**. The common sugarbush grows mainly on rocky or mountainous south-facing slopes of the northern and eastern parts of South Africa and has also encroached into some parts of Mozambique and Zimbabwe **(STRUGNELL, 2002; BAMFORD et al., 2010)**.

The bark of *P. caffra* is used by some South Africa traditional healers to treat bleeding stomach ulcers and diarrhoea (**COETZEE AND LITTLEJOHN, 2000)**. Warm infusions of the stem bark and root bark are taken orally or in the form of enemas to treat diarrhoea **(RAIMONDO et al., 2009)**. According to **ZUKULU (2012)**, the roots are used to prepare *umhlabelo*, a decoction used to help heal broken bones. The fruit and bark are used to relieve people from dizziness, while dried seeds are used in decoctions to manage different psychological disorders (**VAN WYK AND GERICKE, 2000)**. According to **SEMENYA et al. (2013b)**, *P. caffra* seeds are also used to treat chlamydia, a sexually transmitted disease.

2.1.5.2. Ethnopharmacology and phytochemistry

Literature review indicates that the pharmacological properties and phytochemical profiles of *P. caffra* are currently unknown. Extensive pharmacological studies are warranted to ascertain if the traditional uses of this plant are based on mere folklore or its bioactive secondary metabolites.

Figure 2.5: *Protea caffra* growing in the Walter Sisulu National Botanical Garden, Johannesburg, South Africa.

2.1.6. *Searsia lancea* (L.f) Barkley

2.1.6.1. Botany and ethnomedicinal applications

S. lancea (synonym *Rhus lancea*) belongs to the mango family, Anacardiaceae, which is the fourth largest tree family in southern Africa (**Van Wyk and Van Wyk, 1997)**. In South Africa, this plant is known locally as '*karee*' (Afrikaans and English), '*moshabele*' (seTswana) and '*umhlakotshane*' (Xhosa) (**Van der Merwe et al., 2001; Lange et al., 2012; Kabongo-Kayoka et al., 2016)** and in north America as the 'Africa sumac' (**Karlik and Winer, 2001)**. This evergreen tree can achieve a height of 7 m and grows mainly in temperate and tropical regions around the world (**Rayne and Mazza, 2007)**.

S. lancea has a wide range of ethnobotanical applications in South Africa. The Basotho people, for example, use the leaves and fruits to manage diabetes, skin infections, herpes sores (**Seleteng Kose et al., 2015)**, dizziness due to anemia, heart problems and high blood pressure (**Nair et al., 1983; Aganga and Mosase, 2001; Moteetee and Van Wyk, 2011)**. The leaves are also used traditionally to treat fevers, colds, headache, papules and pustules (**Magobo, 1990)**. The Thiaping people on the other hand use the stem wood for tanning purposes (**Watt and Breyer-Brandwijk, 1962)**.

2.1.6.2. Ethnopharmacology and phytochemistry

Extracts from *S. lancea* have been shown to demonstrate a wide range of pharmacological properties. **Mayekiso et al. (2009)** observed that the leaves have good anti-mycobacterial properties. The bark and roots have noteworthy antibacterial and anthelmintic properties (**McGaw et al., 2007)**. **Mulaudzi et al. (2012)** demonstrated that the leaves have potent antibacterial and anti-inflammatory properties.

Figure 2.6: *Searsia lancea* growing in the Botanical Garden, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Several members of the *Searsia* genus are used in folk medicine around the world (**RAYNE AND MAZZA**, **2007; AHMED et al.**, **2014)** and literature strongly suggests that this genus is a potential rich source of novel anti-infection agents (**RAYNE AND MAZZA**, **2007; AHMED et al.**, **2014)**. Given the widespread traditional use of karee, it is conceivable that this plant may contain a wide range of novel pharmacological compounds.

2.1.7. *Solanum panduriforme* E. Mey

2.1.7.1. Botany and ethnomedicinal applications

S. panduriforme is a small, perennial herbal plant that shares the same family (Solanaceae) with tomatoes, potatoes and tobacco **(RAIMONDO et al., 2009)**. This herb has an average height of 0.5 m and flowers in summer (**PLOWES AND DRUMMOND**, **1990; BURROWS AND WILLIS**, **2005)**. According to **KIRBY (2013)**, the local South African names given to this plant include bitter apple (English), *bitterappel* (Afrikaans), *mohato*, *setlwanwe*, *morolane* (Setswana), *intuma*, *intumemcane* (Zulu) and *ndulwane* (Tsonga). In South Africa, the bitter apple is mainly found in the Free State, Gauteng, North West, KwaZulu-Natal, Limpopo and North West provinces (**HUTCHINGS et al.**, **1996)**.

The bitter apple has important ethnoveterinary and ethnobotanical applications in South Africa. In the Free State, the fruit sap from this plant, known locally as *mohato*, is used to relieve cattle of diarrhoea (**VAN DER MERWE et al.**, **2001)**. In folk medicine, however, the fruits are used to treat wounds, while roasted and ground roots are used to treat several gastrointestinal ailments such as diarrhoea, chronic pain, ingestion and ulcers **(BRUSCHI et al., 2011)**. Chewing the root helps to relieve pelvic pains, while roasted roots and fruit sap decoctions are used to manage toothaches (**HUTCHINGS et al.**, **1996)**. The Bapedi people reportedly manage toothaches by burning the plant's fruits and inhaling the smoke (**SEMENYA et al.**, **2013b)**. A survey by

ERASMUS et al. (2012) revealed that macerated fruit decoctions are used in Limpopo to treat gonorrhoea.

2.1.7.2. Ethnopharmacology and phytochemistry

Although *S. panduriforme* is used widely in South African traditional medicine to treat bacterial infections, studies indicate that the plant generally has weak antibacterial activities. **STEENKAMP et al. (2007)** reported that the methanol extracts from the fruits of *S. panduriforme* exhibited weak inhibitory activity against *Staphylococcus epidermidis* and no active against *S. aureus*. Studies by **MORE et al. (2008)** also indicated that extracts from the roots of this plant which, as indicated above, are used to manage toothache did not have any inhibitory activities on several pathogens that cause human oral health diseases namely *Actinobacillus actinomycetemcomitans*, *Actinomyces naeslundii*, *Actinomyces israilii*, *C. albicans*, *Porphyromonus gingivalis*, *Privotella intermedia* and *Streptococcus mutans*. Additionally, **GREEN et al. (2010)** found that the plant had very weak anti-mycobacterial activities (MIC ≥ 10 mg/ml). However, using the disk-diffusion technique, **MULAUDZI et al. (2015)** demonstrated that the plant has potent antigonococcal properties (74% inhibition). This plant could therefore be further explored as a potential source of antigonococcal molecules.

Figure 2.7: A specimen picture of *Solanum panduriforme* courtesy of www.ispotnature.org.

2.2. Antimicrobial resistance and the essence of combination therapy

The misuse and widespread use of antibiotics in medicine have caused several pathogenic bacterial strains to mutate and acquire additional survival strategies (**POOLE, 2001)**. These bacterial mutations have often instigated the evolution of several kinds of antibiotic resistant bacterial strains which are now a menace to public health globally. Particularly, multidrug-resistant (MDR) bacterial strains remain a serious cause of concern since they cause treatment failures and impose huge economic burdens especially in developing countries (**DE ANGELIS et al., 2014; BALLANI AND BABBY, 2016)**. This predicament is exacerbated by the precipitous rate at which antibiotic resistant bacterial strains are evolving which far surpasses the rate at which new antibiotics are being developed (**COATES et al., 2002)**. Unlike the 'Golden era' of antibiotic discovery (mid 1940s-early 1970s) during which several classes of antibiotics were discovered, only two new classes of antibiotics were developed in the last 40 years (**SINGH AND BARRETT, 2006; MUNDY et al., 2016)**. Furthermore, there is also an increasing concern that newly developed antibiotics might have shorter life spans because most MDR mutants often resist the effects of the antibiotics they have been previously exposed to as well as other chemically similar or unrelated antibacterial agents (**COATES et al., 2002)**.

Most MDR bacterial infections can successfully be managed using combination therapies. As a result, modern therapeutic regimes usually consist of combinations of antibiotics with other antibiotics, non-bactericidal chemicals or plant extracts (**WILLIAMSON, 2001; ANDRIES et al., 2005)**. Combination therapies are often favoured in cases where the combined antibacterial effects of two antibacterial agents used in a given combination supersedes the sum effects of either agent used alone (**KHAN et al., 2009)**. Apart from curtailing the growth and development of antimicrobial resistant mutants, synergistic therapies are known to potentiate

the efficacy of antibiotics, increase the antibacterial spectrum and the bioavailability of antibacterial agents into bacterial cells (**ZHAO et al.**, **2002; TRIPODI et al.**, **2007; CHUKWUJEKWU AND VAN STADEN**, **2016)**.

A few years ago, **GANDHI et al. (2006)** discovered *Mycobacterium tuberculosis* strains resistant to all known antibiotics in KwaZulu-Natal, South Africa, which perhaps served as early warning signs of an imminent 'untreatable bacterial infections' era. The need to accelerate the rate at which novel therapeutic drugs are being developed cannot therefore, be overemphasized. Thus, researchers including ethnobotanists should strive to, among other things, discover more clinically relevant highly efficacious antibacterial compounds and novel antibacterial resistance modifying compounds from natural sources especially plants. Given that some medicinal plants such as *Berberis fremontii* have been reported to produce both bactericidal compounds and antibacterial resistance inhibitors (**STERMITZ et al.**, **2000)**, understanding the intricate nature of combination therapies involving medicinal plant extracts will inevitably help achieve these two important research goals.

CHAPTER 3: Antibacterial screening

3.1. Introduction

Alkaloids, flavonoids, essential oils, phenolic acids, saponins and tannins are amongst some of the high-value phytocompounds responsible for the therapeutic properties of several medicinal plant extracts (**WALLACE**, **2004; ERDELYI et al.**, **2005; KULKARNI AND DHIR**, **2010; CHANDRASEKARA AND SHAHIDI**, **2011; XIAO et al.**, **2011)**. The presence and concentration of these bioactive compounds often vary from one taxonomic group to the other, within organs of the same plant and seasonally (**LONGANGA et al.**, **2000; VAN HEERDEN et al.**, **2003; SICILIANO et al.**, **2004; LUCKNER**, **2013)**. Compelling empirical evidence also indicates that medicinal plants with distinctively unique chemical compositions can have similar pharmacological properties (**MULAUDZI et al.**, **2011; ERASMUS et al.**, **2012)**. Additionally, some medicinal plants are used to treat a wide range of both infectious and non-infectious maladies (**MAGOBO**, **1990; HUTCHINGS et al.**, **1996; NEUWINGER**, **2000)**. This obviously explains why medicinal plants form an integral part of the *Materia medica* utilised in both folk and conventional medicine.

The exploitation of South Africa's extensive floral diversity (**GERMISHUIZEN AND MEYER**, **2003)**, with respect to medical bioprospecting, could lead to the discovery of numerous antibacterial molecules with clinical significance. The current study was designed to evaluate the *in vitro* antibacterial properties of extracts from seven South African traditional medicinal plants.

3.2. Materials and methods

3.2.1. Plant collection

The plant materials were collected between July and September 2015. *B. speciosus*, *E. capensis*, *P. africana* and *S. lancea* were collected from the University of KwaZulu-Natal (UKZN) Pietermaritzburg (PMB) Botanical garden, while *P. caffra* was collected from the Walter Sisulu South African National Biodiversity Institute (SANBI) (27º 50´ 40.7´´ E 26º 05´13.8´´S). Seeds of *C. myriocarpus* and *S. panduriforme* were obtained from Bradfield, Bulawayo, Zimbabwe (20º 10´40´´S 28º 35´ 06´´ E) and grown in the UKZN PMB Botanical garden. The selected plants were positively identified by the Curator (University of KwaZulu-Natal Bews Herbarium), after which voucher specimens were prepared and deposited in the UKZN Bews Herbarium. The voucher specimen numbers are presented in Table 2.1. Once collected, the plant materials were oven dried for 3-5 days at 50 ºC, ground and stored in brown paper bags at room temperature.

3.2.2. Source of chemicals

Organic solvents (dichloromethane, methanol, and petroleum ether) were purchased from Radchem laboratory supplies (PTY) Ltd (Alberton, South Africa). The antibiotics and dimethylsulfoxide (DMSO) were supplied by Sigma-Aldrich Co. (Steinheim, Germany). Carbon dioxide generator, chocolate agar, Mueller and Hinton (MH) growth medium (broth and agar), soluble haemoglobin, and Vitox were bought from Oxoid Ltd (Hampshire, United Kingdom).

3.2.3. Extraction of plant for biological activities

At a ratio of 10:1 (10 ml/g), dry powdered samples were mixed with different solvents (water, methanol = MeOH, dichloromethane = DCM, and petroleum ether = PE) and stirred in a rotary

shaker (Edmund Bühler, Tübingen, German) for 12 h at 150 rpm at room temperature, after which they were sonicated for 1 h on ice (Julabo GMBH, Germany). Organic solvent extracts were filtered using Whatman No. 1 filter paper under vacuum and later concentrated using a rotary evaporator (Heldolph vv 2000, Germany) at 35 ^{o}C. Concentrated organic solvent extracts were transferred into glass pill vials and air-dried in front of a fan. All water extracts were freeze-dried. The resulting 64 dried extracts were kept in closed glass pill vials in the dark at 10 ^{o}C until required for further use.

3.2.4. Antigonococcal activity

3.2.4.1. Preparations of gonococcal stocks

A loopful of *Neisseria gonorrhoeae* American type culture collection (ATCC) 49226 stored at -70 ^{o}C was inoculated into 5 ml Mueller Hinton (MH) broth and incubated overnight at 37 ^{o}C in an orbital shaker water bath (50 rpm). Two microliters of the overnight culture were spread uniformly on sterile chocolate agar (Oxoid GC agar base), supplemented with 2% (w/v) haemoglobin together with 1% (v/v) of Vitox, and incubated in 5% carbon dioxide (CO_2) incubators for 24 h at 37 ^{o}C. The plates were then kept at 4 ^{o}C and the gonococcal stocks sub-cultured once every month to maintain bacterial cell viability.

3.2.4.2. Disk-diffusion bioassay

The disk-diffusion technique (**CLSI**, **2009)** was used to determine the antigonococcal activities of the 64 plant extracts. Five milliliters of sterile MH broth were inoculated with a colony of *N. gonorrhoeae* and incubated overnight at 37 ^{o}C in an orbital shaker water bath (50 rpm). Two microliters of the diluted overnight cultures [$\approx$ 5 x 10^5 colony forming units per milliliter (cfu/ml)] were then spread uniformly on chocolate agar and incubated in 5% CO_2 incubators for 24 h at 37 ^{o}C. Stock sample solutions were prepared by resuspending

samples in 20% DMSO to a concentration of 100 mg/ml. The working sample solutions were made by diluting the stock solutions with sterile distilled water to a concentration of 10 mg/ml. Five microliters of each resuspended plant extract (10 mg/ml) were impregnated onto sterile Whatman filter paper disks (6 mm diameter) and dried under a stream of sterile air. Ciprofloxacin (0.5 µg/disk) was used as a positive control, while 5 µl of distilled water and 5 µl of 2% DMSO served as the negative controls. The impregnated disks were then aseptically placed on top of the *N. gonorrhoeae* cultures growing on chocolate agar and incubated in 5% CO_2 incubators for 24 h at 37 ^{o}C. The assay was repeated twice, in quadruplicate for each sample.

Plant extracts with antigonococcal properties were indicated by the appearance of clear zones of bacterial growth inhibition around each disk after a 24 h incubation. The efficacy of each plant extract was then determined by comparing the diameters of the test plant extract with the positive control and expressing them as inhibition percentages, as shown below;

Percentage (%) inhibition = (Diameter of the sample ÷ diameter of positive control)×100

The results were expressed as average inhibition percentage ± standard error. The greater the inhibition percentage, the more efficacious the plant extract was considered to be.

3.2.4.3. Antigonococcal microdilution bioassay

Minimum inhibitory concentration (MIC) values of the 64 plant extracts were determined using 96-well microplates (Greiner Bio-one, Germany) as previously described by **ELOFF (1998)**. A colony of *N. gonorrhoeae* was inoculated into 5 ml sterile MH broth and incubated overnight at 37 ^{o}C in an orbital shaker water bath (50 rpm). One hundred microliters of each resuspended plant extract (10 mg/ml) were 2-fold serially diluted with sterile water, in triplicate, down a

96-well microplate. A similar 2-fold serial dilution of ciprofloxacin (1 mg/ml) was used as a positive control, while sterile distilled water and 2% DMSO were used as the negative controls. One hundred microliters of diluted bacterial cultures were then added to each well to give a final inoculum of approximately 5 x 10^5 cfu/ml and then incubated in 5% CO_2 incubators for 24 h at 37 ^oC. Bacterial growth was detected by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated for 1 h at 37 ^oC. Bacterial growth was indicated by a red-pink colour, whereas clear wells indicated inhibition of bacterial growth by the test plant extract. MIC values were recorded as the lowest concentration of the extract showing a clear well. To confirm results, the assay was repeated twice.

3.2.5. Antibacterial activity

3.2.5.1. Bacterial stock preparation

Bacterial stocks were cultured on MH agar. A colony of each strain was inoculated into 5 ml sterile MH broth and incubated overnight at 37 ^oC in an orbital shaker water bath (50 rpm). Twenty milliliters of hot sterile MH agar were transferred into each petri dish, allowed to cool and set. Each test bacterial stock strain (overnight cultures) was then streaked on MH agar and incubated at 37 ^oC for 24 h. The plates were stored at 4 ^oC and the bacterial stocks sub-cultured monthly to maintain bacterial cell viability.

3.2.5.2. Microdilution bioassay

Minimum inhibitory concentration (MIC) values of extracts from seven traditional medicinal plants were determined using 96-well microplates (Greiner Bio-one, Germany) as previously described by **ELOFF (1998)**. A colony of each bacterial strain was inoculated into 5 ml sterile MH broth and incubated overnight at 37 ^oC in an orbital shaker water bath (50 rpm). One

hundred microliters of each resuspended sample (10 mg/ml) were 2-fold serially diluted with sterile water, in triplicate, down a 96-well microplate. A similar 2-fold serial dilution of neomycin (0.1 mg/ml) was used as a positive control against each test bacterial strain (*E. coli* ATCC 11775, *Enterococcus faecalis* ATCC 19433 , *Klebsiella pneumoniae* ATCC 13883, MDR *E. coli* ATCC 25218, MDR *K. pneumoniae* ATCC 70603, drug-sensitive *S. aureus* ATCC 12600 and penicillin-resistant *S. aureus* ATCC 11632). Sterile MH broth, distilled water and 2% DMSO were used as the negative controls. One hundred microliters of each diluted, test bacterial strain were added to each well to give a final inoculum of approximately $5x10^5$ cfu/ml, after which the plates were covered with parafilm and incubated for 24 h at 37 ^oC. The detection of bacterial growth and interpretation of results were done as described in **Section 3.2.4.3** above. The assay was done twice to confirm the results.

3.2.6. Antibacterial synergy

3.2.6.1. Checkerboard titration method

The antibacterial interactions of different plant extract combinations, as well as combinations of plant extracts with antibiotics (ampicillin, cefotaxime, chloramphenicol and penicillin) were assessed in 96-well microplates using the 8 x 8 microdilution checkerboard analysis technique (**RAND et al., 1993)**. Fifty microliters of water were added to each well after which 50 µl of plant extract X were added in row A and serially diluted down to row I. Fifty microliters of plant extract Y or a given antibiotic were added in decreasing concentrations from column 1 to 8. Once the test antibacterial agents were mixed together, each microplate well was then inoculated with 100 µl of the test bacterial strain (MDR *E. coli*, MDR *K. pneumoniae* or *N. gonorrhoeae*), to give a final inoculum of approximately 5 x 10^5 cfu/ml. The plates were covered with parafilm and incubated for 24 h at 37 ^oC. However, in the case of *N. gonorrhoeae,* incubation was done in 5% CO_2 incubators for 24 h at 37 ^oC, during which

the microplates were not covered with parafilm. For each combination, the fractional inhibitory concentration index (FICI) was calculated as follows: $FICI = FIC_X + FIC_Y$, where FIC_X was the MIC of antibacterial agent X when used in combination with antibacterial agent Y, divided by the MIC of antibacterial agent X when used alone. The results were interpreted thus; $FICI \leq 0.5$ (synergy); $0.5 < FICI \leq 1.0$ (additive) and $1.0 < FICI \leq 4.0$ (no interaction) and $FICI > 4.0$ (antagonism) (**VAN VUUREN AND VILJOEN, 2011)**.

3.2.6.2. Time-kill bioassay

Time-kill assays for plant extract + antibiotic combinations that demonstrated noteworthy antibacterial synergism using the checkerboard assay were performed using the broth microdilution technique (**WHITE et al., 1996)**. This assay was done thrice, in triplicate for each sample. Sterile MH broth was used as the negative control, while test plant extracts and antibiotics were each used as the positive controls. After the antibiotic and plant extract were mixed in their respective ratios, 500 µl of the mixture were then added to 500 µl of MH broth in a sterile Eppendorf tube and a 2-fold serial dilution of the mixture performed to obtain the MIC and 0.5 x MIC. Five hundred microliters of the test bacterial strain were then added to give a final inoculum of approximately 5×10^5 cfu/ml. The inoculum was then incubated for 90 min at 37 ^oC in an orbital shaker water bath (50 rpm). Ten microliter aliquots were taken from the inoculum after incubation, added to 990 µl of sterile MH broth and serially diluted (100 fold) in MH broth. Twenty-five microliters of the final dilution were plated on MH nutrient agar to determine the 0 h counts. This procedure was repeated at 3, 6 and 24 h after the 0 h viable colony counts were taken and the plates were incubated for 24 h at 37 ^oC. The mean viable colony counts (cfu/ml) were expressed as $\log_{10}$ units. A 2 $\log_{10}$ difference in mean cfu between the positive control (antibiotic) and a given antibacterial combination was considered indicative of noteworthy synergistic interactions between the test antibacterial

agents used in the combination mixture. Data were presented as mean ± standard error of the triplicate determinants.

3.3. Results and discussion

3.3.1. Antigonococcal activity

The intricate chemo-diversity found in phytocompounds, position medicinal plants as impeccable sources for novel pharmaceutical drugs. Against this background, medicinal plant extracts were screened in the current study for antigonococcal activity using two traditional antibacterial susceptibility testing methods, namely the disk-diffusion and microdilution techniques. Based on the agar disk-diffusion assay, all 64 tested plant extracts did not demonstrate antigonococcal activities at the tested concentration(s). However, when the same extracts were screened using the microdilution assay, four plant extracts, namely *S. lancea* DCM leaf extracts (MIC = 0.313 mg/ml), *B. speciosus* MeOH and DCM bark extracts (MIC's = 0.625 mg/ml) and *P. africana* leaf DCM extracts (MIC = 0.625 mg/ml), demonstrated moderate antigonococcal activities (Table 3.1). The difference observed in the results obtained from these two bioassays could be attributed to several factors. A scrutiny of these results reveals that antibacterial activity was not observed in the disk-diffusion assay due to either a total lack of antigonococcal property in some of the tested plant extracts or that the antibacterial activity was masked. The latter could have been due to the use of a plant extract concentration (10 mg/ml) that was too low to effect notable antigonococcal activities. Although the disk-diffusion technique is one of the inexpensive and less laborious antibacterial susceptibility testing methods currently available (**RELLER et al., 2009)**, this procedure suffers with the demerit of not always producing reproducible results for several plant extracts (**ELOFF, 1998)**. This is primarily because some phytochemicals, especially non-polar ones, do not diffuse readily in polarized agarose gels (**RIOS et al., 1988)**. Hence, bioactive compounds in

B. speciosus, *S. lancea* and *P. africana* plant extracts probably failed to diffuse through the chocolate agar during the disk-diffusion incubation period to effect noticeable antigonococcal activity. This in turn probably led to the pseudo-negative results observed.

The antigonococcal property of *B. speciosus* has previously been reported (**MULAUDZI et al., 2011)**. Based on the available literature, this is the first report on the antigonococcal properties of *S. lancea* and *P. africana*. These two are classic examples of plants, not only with extended-spectrum antibacterial activities, but also having sundry pharmacological activities (**PUJOL, 1990; MCGAW et al., 2007; KADU et al., 2012; MULAUDZI et al., 2012)**. Such plants obviously owe their extensive pharmacological properties to their rich array of bioactive compounds. The current study provides vital scientific information that could be utilised by traditional healers to especially, improve the provision of rural primary health care. Instead of using the usual herbal preparations from plants such as *Callilepis salicifolia*, *Jatropha zeyheri*, *Cotyledon orbiculata*, *Catharanthus roseus* and *Senna italica* to manage gonorrhoea (**ERASMUS et al., 2012)**, herbalists could now alternatively use the leaves of *S. lancea* and *P. africana.* This could perhaps be one of the rare incidences in which such vital ethnobotanical information flows from the scientists to herbalists, more often than not, it is the other way around. The use of several alternative plant species to manage gonorrhoea does not only help delay the development of pathogenic drug-resistance, but also helps to conserve our biodiversity by preventing the over-exploitation of one or a few popular traditional medicinal plant species known to have antigonococcal properties.

Table 3.1: Minimum inhibition concentration (MIC, mg/ml) values of extracts obtained from seven South African traditional medicinal plants against *Neisseria gonorrhoeae*.

Plant species	**Plant Organ**	**MIC (mg/ml)**			
		Water	**MeOH**	**DCM**	**PE**
Bolusanthus speciosus	Bark	>2.5	**0.625**	**0.625**	2.5
Cucumis myriocarpus	Fruits	>2.5	>2.5	>2.5	>2.5
	Roots	>2.5	>2.5	>2.5	>2.5
	Leaves	>2.5	>2.5	>2.5	>2.5
Ekebergia capensis	Leaves	>2.5	>2.5	>2.5	>2.5
Prunus africana	Leaves	>2.5	2.5	**0.625**	2.5
	Twigs	>2.5	>2.5	>2.5	>2.5
Protea caffra	Bark	>2.5	>2.5	>2.5	>2.5
	Flowers	>2.5	>2.5	>2.5	>2.5
	Leaves	>2.5	>2.5	>2.5	>2.5
	Seeds	>2.5	>2.5	>2.5	>2.5
	Twigs	>2.5	>2.5	>2.5	>2.5
Searsia lancea	Leaves	>2.5	2.5	**0.313**	2.5
Solanum panduriforme	Fruits	>2.5	>2.5	>2.5	>2.5
	Roots	>2.5	>2.5	>2.5	>2.5
	Stems	>2.5	>2.5	>2.5	>2.5

DCM= Dichloromethane, MeOH = Methanol; PE = Petroleum ether.

*MIC values bold-written are considered to have noteworthy antigonococcal activity.

3.3.2. Antibacterial activity

Indigenous knowledge often provides important leads in the discovery of novel pharmaceutical drugs. In the present study extracts from seven South African medicinal plants selected based on their ethnomedicinal uses were screened for antibacterial activities against a panel of seven bacterial strains. The antibacterial MIC values of all test plant extracts are presented in Table 3.2. Plant extracts were classified as having significant (MIC ≤ 0.1 mg/ml), moderate (100<MIC≤ 0.625 mg/ml) or weak (MIC >0.625 mg/ml) antibacterial activities according to **KUETE (2010)**. The most potent antibacterial activity was therefore exhibited by the MeOH extracts of *B. speciosus* bark which achieved MIC values of 0.039 and 0.078 mg/ml against *S. aureus* (penicillin-resistant and drug-sensitive, respectively). Since season and climate have a bearing on the chemical profiles of most plants (**KADU et al., 2012)**, it is imperative that further studies be conducted to ascertain if the antibacterial properties displayed by *B. speciosus* in the present study were due to the compounds previously isolated by **BOJASE et al. (2002)** and **ERASTO et al. (2004)**. The MeOH leaf extracts of *S. lancea*, as well as PE seed extracts of *P. caffra* also yielded moderate antibacterial activities against *S. aureus* (MIC = 0.12 mg/ml) and *E. coli* (MIC = 0.25 mg/ml), respectively. Ten other plant extracts also achieved noteworthy activity (MIC = 0.31 mg/ml) against different test bacterial strains including the penicillin-resistant *S. aureus* (Table 3.2).

DCM leaf extracts of *S. lancea* yielded an MIC value of 0.63 mg/ml against five of the seven test bacterial strains including MDR *E. coli* and MDR *K. pneumoniae*. *B. speciosus* bark (MeOH and DCM), *P. caffra* seeds (DCM) and twigs (MeOH) as well as *S. lancea* leaves (MeOH) also demonstrated extended-spectrum antibacterial properties. The present findings indicate that medicinal plant extracts could potentially be used to manage a wide range of infections caused by both Gram-positive and Gram-negative MDR bacterial strains. Medicinal

plants with potent, broad-spectrum antibacterial properties such as *B. speciosus* could serve as new sources of novel antimicrobial compounds in the near future. If fully exploited, such medicinal plants could therefore play a crucial role in addressing the prevalent pharmaceutical drug shortages. Interestingly, *P. caffra*, an ornamental plant rarely used in folk medicine, also demonstrated noteworthy antibacterial activity. Notably, most organic solvent seed extracts from this plant showed promising activity against *E. coli* and *K. pneumoniae* (Gram-negative strains) with MIC values in the range of 0.25-0.63 mg/ml (Table 3.2). The moderate antibacterial activities exhibited by *P. caffra* in the current study could perhaps explain the use of its seeds to manage chlamydia (a bacterial infection) by some traditional healers in South African (**SEMENYA AND POTGIETER, 2014)**. *P. caffra* as well as the whole Proteaceae family and *Protea* genus should be fully explored as potential sources of medicinally important antibacterial compounds.

In the present investigation, 50% of the screened plant extracts demonstrated noteworthy activities against the Gram-positive *E. faecalis* making this the most susceptible bacterial strain. The least susceptible bacterial strain was however, MDR *K. pneumoniae*. Gram-negative bacterial strains have thick hydrophilic peptidoglycan layers which impedes the influx of most, especially hydrophobic, antibacterial agents into their cells (**REECE et al., 2011)**. Consequently, Gram-negative bacterial strains are generally less susceptible to antibacterial agents than Gram-positive strains which are characterized by thinner peptidoglycan layers. It was therefore notable that, of the 64 plant extracts evaluated only *S. lancea* leaf DCM and MeOH extracts displayed moderate activities against Gram-negative, MDR *K. pneumoniae* (MIC's = 0.63mg/ml). In addition, *S. lancea* was the only one, out of the seven test plants, that demonstrated noteworthy activity against most Gram-negative bacterial strains (*E. coli*, *K. pneumoniae*, MDR *K. pneumoniae*).

All aqueous extracts evaluated in the present study had MIC values >2.5 mg/ml and were therefore excluded in Table 3.2. It is not, however, uncommon for aqueous extracts to exhibit no antibacterial activities during bioassays yet aqueous decoctions and infusions are widely used in folk medicine. This is largely because during most ethnobotanical studies plant extracts are prepared under highly controlled laboratory conditions which are often quite different from the conditions under which traditional medicines are prepared. Boiling, mixing different herbs, as well as mixing herbs with non-plant materials during preparations of traditional medicines often activate and/or increase the bioavailability of phytocompounds in the extract (**CANO AND VOLPATO, 2004; AZIZAH et al., 2009)**. Additionally, it is a well-known fact that the principal bioactive compounds in some medicinal plant extracts are not necessarily microbiocidal or microbiostatic in nature, but rather immune-regulators which generally boost the human immune system and hence help it fight pathogenic invasions (**JEONG et al., 2003; ZWICKEY et al., 2007; KHODADADI, 2016)**.

Table 3.2: Minimum inhibitory concentration values (MIC, mg/ml) of medicinal plant extracts screened against drug-sensitive and -resistant bacterial strains.

Family **Species**	**Plant organ**	**MIC (mg/ml)**													
		MeOH							**DCM**						
		Ec	***Ef***	***Kp***	***Sa***	***Ec D***	***Kp D***	***Sa D***	***Ec***	***Ef***	***Kp***	***Sa***	***Ec D***	***Kp D***	***Sa D***
Fabaceae															
Bolusanthus speciosus	Bark	2.5	**0.31**	1.25	**0.078**	**0.63**	1.25	**0.039**	2.5	**0.31**	2.5	**0.078**	2.5	2.5	**0.078**
Curcubitaceae															
Cucumis myriocarpus	Fruits	1.25	**0.63**	2.5	1.25	>2.5	>2.5	>2.5	2.5	1.25	2.5	1.25	>2.5	>2.5	1.25
	Leaves	>2.5	2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	1.25	>2.5	>2.5	>2.5	>2.5	>2.5
	Roots	>2.5	2.5	>2.5	2.5	>2.5	2.5	>2.5	2.5	1.25	>2.5	2.5	2.5	2.5	2.5
Maliaceae															
Ekebergia capensis	Leaves	2.5	2.5	>2.5	>2.5	2.5	>2.5	2.5	2.5	**0.31**	2.5	2.5	2.5	2.5	2.5
Proteaceae															
Protea caffra	Bark	1.25	**0.63**	>2.5	**0.31**	>2.5	>2.5	>2.5	1.25	>2.5	2.5	1.25	>2.5	2.5	1.25
	Flowers	1.25	1.25	1.25	2.5	2.5	2.5	2.5	1.25	**0.63**	1.25	**0.63**	2.5	1.25	>2.5
	Leaves	2.5	**0.63**	1.25	1.25	2.5	2.5	**0.63**	2.5	>2.5	1.25	2.5	>2.5	2.5	2.5
	Seeds	**0.63**	1.25	**0.31**	1.25	1.25	2.5	1.25	**0.63**	**0.63**	**0.31**	1.25	2.5	1.25	1.25
	Twigs	1.25	**0.31**	**0.63**	**0.31**	1.25	1.25	**0.63**	2.5	2.5	2.5	2.5	>2.5	>2.5	>2.5
Rosaceae															
Prunus africana	Leaves	2.5	**0.63**	1.25	1.25	1.25	2.5	**0.63**	2.5	>2.5	1.25	2.5	**0.63**	2.5	2.5
	Twigs	2.5	**0.63**	1.25	**0.31**	2.5	2.5	**0.31**	2.5	2.5	>2.5	2.5	2.5	2.5	2.5
Anacardiaceae															
Searsia lancea	Leaves	1.25	**0.63**	1.25	**0.12**	1.25	**0.63**	1.25	1.25	**0.63**	**0.63**	**0.63**	**0.63**	**0.63**	1.25
Solanaceae															
Solanum panduriforme	Fruits	1.25	2.5	2.5	1.25	>2.5	2.5	1.25	2.5	**0.63**	2.5	>2.5	>2.5	>2.5	2.5
	Leaves	>2.5	2.5	>2.5	2.5	>2.5	>2.5	2.5	>2.5	2.5	2.5	1.25	>2.5	>2.5	>2.5
	Roots	1.25	2.5	1.25	>2.5	2.5	1.25	1.25	1.25	**0.63**	1.25	1.25	2.5	1.25	1.25
	Stems	2.5	2.5	2.5	>2.5	>2.5	>2.5	2.5	1.25	>2.5	>2.5	1.25	>2.5	>2.5	>2.5

Table 3.2: Continued.

Family Species	Plant organ	MIC (mg/ml)						
		PE						
		Ec	***Ef***	***Kp***	***Sa***	***Ec D***	***Kp D***	***Sa D***
Fabaceae								
Bolusanthus speciosus	Bark	2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
Curcubitaceae								
Cucumis myriocarpus	Fruits	2.5	1.25	2.5	1.25	>2.5	>2.5	2.5
	Leaves	2.5	2.5	>2.5	1.2	>2.5	>2.5	>2.5
	Roots	2.5	2.5	>2.5	2.5	>2.5	>2.5	2.5
Maliaceae								
Ekebergia capensis	Leaves	2.5	2.5	2.5	2.5	2.5	>2.5	>2.5
Proteaceae								
Protea caffra	Bark	2.5	**0.63**	2.5	2.5	2.5	2.5	>2.5
	Flowers	1.25	**0.31**	1.25	**0.63**	2.5	1.25	2.5
	Leaves	2.5	>2.5	1.25	**0.63**	2.5	2.5	2.5
	Seeds	**0.25**	2.5	1.25	2.5	2.5	1.25	2.5
	Twigs	2.5	2.5	2.5	2.5	2.5	1.25	1.25
Rosaceae								
Prunus africana	Leaves	2.5	>2.5	1.25	**0.63**	2.5	2.5	2.5
	Twigs	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
Anacardiaceae								
Searsia lancea	Leaves	2.5	2.5	1.25	**0.47**	1.25	1.25	**0.63**
Solanaceae								
Solanum panduriforme	Fruits	>2.5	1.25	>2.5	2.5	>2.5	>2.5	1.25
	Leaves	>2.5	2.5	>2.5	2.5	>2.5	>2.5	2.5
	Roots	2.5	>2.5	2.5	2.5	2.5	>2.5	>2.5
	Stems	>2.5	1.25	2.5	2.5	>2.5	>2.5	>2.5
Neomycin (positive control)	µg/ml	1.56	>100	1.56	0.39	6.25	6.25	25

Ec = Escherichia coli; *Ef = Enterococcus faecalis*; *Kp = Klebsiella pneumoniae*; *Sa = Staphylococcus aureus*; *Ec D* = Multidrug resistant *E. coli*; *Kp D* = Multidrug-resistant *K. pneumoniae, Sa D* = Penicillin resistant *S. aureus*.

*MIC values bold-written indicate noteworthy antibacterial activity.

3.3.3. Antibacterial synergy

Understanding the antibacterial synergistic interactions in medicinal plant-based combination therapies will undoubtably provide important leads in the discovery of novel antibacterial resistance modifying compounds from plants. In the current study, the antibacterial synergistic interactions within different plant extracts, as well as between plant extracts and antibiotics were investigated. The evaluated microbial strains were indeed multidrug-resistant (Table 3.3) given that the MIC breakpoints for all tested antibiotics used in this study against *E. coli* and *K. pneumoniae* were > 8 µg/ml (**EUCAST**, **2016)**. The FICI values of all the antibacterial combinations evaluated in this study are presented in Tables 3.4-3.6. All plant extract combinations evaluated against *N. gonorrhoeae* (Table 3.4) yielded FICI values greater than 0.5 and hence exhibited no synergistic interactions (**VAN VUUREN AND VILJOEN**, **2011)**. Similarly, all plant extract + antibiotic combinations screened against the MDR *K. pneumoniae* showed no antibacterial synergistic interactions (Table 3.5). Both *N. gonorrhoeae* and *K. pneumoniae* are typical Gram-negative bacterial strains which, as already alluded to, are comparatively less susceptible to antibacterial agents than Gram-positive bacterial strains.

However, 3 out of 32 combinations (antibiotic + plant extract) evaluated against the MDR *E. coli* demonstrated noteworthy antibacterial synergism with FICI values ranging from 0.38 to 0.5 (Table 3.6). It is interesting to note that in all three combinations there was a 4-7.8-fold reduction in the MIC values of the test antibiotic (chloramphenicol). This observation indicated that in all these combinations, certain plant extract constituents made the test bacterial strain much more susceptible to chloramphenicol. These bioactive compounds together with chloramphenicol probably exerted multi-target effects on the test bacterial strain. Chloramphenicol inhibits bacterial protein synthesis resulting in the premature death of

bacterial cells (**SIIBAK et al., 2009)**. Some antibacterial agents however, inhibit bacterial cell wall formation, while others block important metabolic pathways such as those involved in DNA replication, RNA synthesis and reproduction (**CHANDA AND RAKHOLIYA, 2011)**. Chances are also that chloramphenicol and the bioactive compounds found in the tested plant extracts had similar antibacterial mechanisms. Hence the two antibacterial agents probably made the test bacterial strain more vulnerable by preventing the formation of several important bacterial proteins such as enzymes, cytochromes and DNA-binding proteins.

Table 3.3: Minimum inhibitory concentration (MIC, mg/ml) values of selected plant extracts and *antibiotics against the multidrug-resistant (MDR) strains of *Escherichia coli* and *Klebsiella pneumoniae*.

	MIC mg/ml	
Plant extract	**MDR *E. coli***	**MDR *K. pneumoniae***
Bolusanthus speciosus bark MeOH	0.625	1.25
Protea caffra flower DCM	NT	1.25
Protea caffra flower PE	NT	1.25
Protea caffra seed DCM	NT	1.25
Protea caffra seed MeOH	1.25	NT
Protea caffra seed PE	NT	1.25
Protea caffra twig MeOH	1.25	1.25
*Protea caffra tw*ig PE	NT	1.25
Prunus africana leaf DCM	0.625	NT
Prunus africana leaf MeOH	1.25	NT
Searsia lancea leaf DCM	0.625	0.625
Searsia lancea leaf MeOH	1.25	0.625
Searsia lancea leaf PE	1.25	0.625
Solanum panduriforme root DCM	NT	1.25
Solanum panduriforme root MeOH	NT	1.25
*Ampicillin	5	5
*Cefotaxime	0.02	0.3
*Chloramphenicol	0.156	0.3
*Penicillin	0.625	5

DCM= Dichloromethane, MeOH = Methanol, PE= Petroleum ether, NT = Not tested.

Table 3.4: Antigonococcal synergistic interactions among different plant extract combinations.

Plant Combinations	MIC (mg/ml)		FIC	FICI	Outcome
	Individual	Combination			
Palm + Bsbm	2.5/ 0.625	2.5/ 0.125	1/ 0.25	1.25	No interaction
Palm + Bsbd	2.5/ 0.625	1.25/ 0.313	0.5/ 0.5	1	Additive
Palm + Bsbp	2.5/ 2.5	1.25/ 0.625	1/ 0.002	1.002	Additive
Palm + Sllm	2.5/ 2.5	2.5/ 0.005	1/0.002	1.002	Additive
Palm + Slld	2.5/ 0.313	1.25/ 0.313	0.5/ 1	1.5	No interaction
Palm + Sllp	2.5/ 2.5	0.625/ 2.5	0.25/ 1	1.25	No interaction
Pald + Bsbm	0.625/ 0.625	0.125/ 0.313	0.2/ 0.5	0.7	Additive
Pald + Bsbd	0.625/ 0.625	0.313/ 0.313	0.5/ 0.5	1	Additive
Pald + Bsbp	0.625/ 2.5	0.625/ 0.313	1/ 0.125	1.1	No interaction
Pald + Sllm	0.625/ 2.5	0.039/ 2.5	0.06/ 1	1.1	No interaction
Pald + Slld	0.625/ 0.313	0.156/ 0.156	0.25/ 0.5	0.75	Additive
Pald + Sllp	0.625/ 2.5	0.313/ 1.25	0.5/ 0.05	0.6	Additive
Palp + Bsbm	2.5/ 0.625	2.5/ 0.6	1/ 1	2	No interaction
Palp + Bsbd	2.5/ 0.625	0.625/ 0.625	0.25/ 1	1.25	No interaction
Palp + Bsbp	2.5/ 2.5	1.25/ 1.25	0.5/ 0.5	1	Additive
Palp + Sllm	2.5/ 2.5	1.25/ 2.5	0.5/ 1	1.5	No interaction
Palp + Slld	2.5/ 0.313	1.25/ 0.078	0.5/ 0.25	0.75	Additive
Palp + Sllp	2.5/ 2.5	2.5/ 0.005	1/ 0.002	1	Additive
Sllm + Bsbm	2.5/ 0.625	2.5/ 0.156	1/ 0.25	1.25	No interaction
Sllm + Bsbd	2.5/ 0.625	0.625/ 0.625	0.25/ 1	1.25	No interaction
Sllm + Bsbp	2.5/2.5	2.5/1.25	1/0.5	1.5	No interaction
Slld + Bsbm	0.313/ 0.625	0.156/ 0.156	0.5/ 0.25	0.75	Additive
Slld + Bsbd	0.313/0 .625	0.156/ 0.078	0.5/ 0.13	0.625	Additive
Slld + Bsbp	0.313/2.5	0.078/2.5	0.25/1	1.25	No interaction
Sllp + Bsbm	2.5/ 0.625	2.5/ 0.156	1/ 0.25	1.25	No interaction
Sllp + Bsbd	2.5/ 0.625	2.5/ 0.315	1/ 0.5	1.5	No interaction
Sllp + Bsbp	2.5/2.5	2.5/0.315	1/ 0.126	1.13	No interaction

Bsbd = *Bolusanthus speciosus* bark dichloromethane extract, Bsbm = *Bolusanthus specious* bark methanol extract, *Bsbp* = *Bolusanthus specious* bark petroleum ether extract, Pald = *Prunus africana* leaf dichloromethane extract, Palm = *Prunus africana* leaf methanol extract, Palp = *Prunus africana* leaf petroleum ether, Slld = *Searsia lancea* leaf dichloromethane, Sllm = *Searsia lancea* leaf methanol extract, Sllp = *Searsia lancea* leaf petroleum ether, FIC = fractional inhibition concentration, FICI = fractional inhibition concentration index, MIC = minimum inhibition concentration.

Table 3.5: Antibacterial interactions within combinations of selected conventional antibiotics and plant extracts against multidrug-resistant *Klebsiella pneumoniae*.

Antibacterial combinations	MIC (mg/ml)		FIC	FICI	Outcome
	Individual	Combination			
Bsbm + Amp	1.25/ 5	0.625/ 2.5	0.5/ 0.5	1	Additive
Bsbm + Cef	1.25/ 0.313	0.625/ 0.078	0.5/ 0.25	0.75	Additive
Bsbm + Chl	1.25/ 0.313	0.625/ 0.078	0.5/ 0.25	0.75	Additive
Bsbm + Pen	1.25/ 5	1.25/ 1.25	1/ 0.25	1.25	No interaction
Pcfd + Amp	1.25/ 5	0.313/ 5	0.25/ 1	1.25	No interaction
Pcfd + Cef	1.25/ 0.313	0.625/ 0.157	0.5/ 0.5	1	Additive
Pcfd + Chl	1.25/ 0.313	0.313/ 0.313	0.25/ 1	1.25	No interaction
Pcfd + Pen	1.25/ 5	1.25/ 0.313	1/ 0.06	1.06	No interaction
Pcfp + Amp	1.25/ 5	1.25/ 2.5	1/ 0.5	1.5	No interaction
Pcfp + Cef	1.25/ 0.313	0.313/0.157	0.25/0.52	0.77	Additive
Pcfp + Chl	1.25/ 0.313	1.25/ 0.313	1/ 1	2	No interaction
Pcfp + Pen	1.25/ 5	0.6/ 2.5	0.48/ 0.5	0.98	Additive
Pcsd + Amp	1.25/ 5	0.156/ 5	0.12/ 1	1.12	No interaction
Pcsd + Cef	1.25/ 0.313	1.25/ 0.039	1/ 0.125	1.13	No interaction
Pcsd + Chl	1.25/ 0.313	0.039/ 0.313	0.031/ 1	1.03	Additive
Pcsd + Pen	1.25/ 5	0.625/ 5	0.5/ 1	1.5	No interaction
Pcsp + Amp	1.25/ 5	0.625/ 0.625	0.5/ 0.13	0.625	Additive
Pcsp + Cef	1.25/ 0.313	0.313/ 0.313	0.25/ 1	1.25	No interaction
Pcsp + Chl	1.25/ 0.313	0.625/ 0.157	0.5/ 0.5	1	Additive
Pcsp + Pen	1.25/ 5	1.25/ 0.313	1/ 0.06	1.06	No interaction
Pctm + Amp	1.25/ 5	0.625/ 5	0.5/ 1	1.5	No interaction
Pctm + Cef	1.25/ 0.313	1.25/ 0.02	1/ 0.06	1.06	No interaction
Pctm + Chl	1.25/ 0.313	0.313/ 0.157	0.25/ 0.5	0.75	Additive
Pctm + Pen	1.25/ 5	1.25/ 0.313	1/ 0.06	1.06	No interaction
Pctp + Amp	1.25/ 5	1.25/ 0.625	1/ 0.12	1.12	No interaction
Pctp + Cef	1.25/ 0.313	1.25/ 0.001	1/ 0.003	1	Additive
Pctp + Chl	1.25/ 0.313	0.039/ 0.313	0.031/ 1	1.03	Additive
Pctp + Pen	1.25/ 5	0.313/ 2.5	0.25/ 0.5	0.75	Additive
Sllm + Amp	0.625/ 5	0.039/ 5	0.06/ 1	1.06	No interaction
Sllm + Cef	0.625/ 0.313	0.158/ 0.157	0.25/ 0.5	0.75	Additive
Sllm + Chl	0.625/ 0.313	0.02/ 0.313	0.03/ 1	1.03	Additive
Sllm + Pen	0.625/ 5	0.313/ 1.25	0.5/ 0.2	0.7	Additive
Slld + Amp	0.63/ 5	0.313/ 2.5	0.5/ 0.5	1	Additive
Slld + Cef	0.63/ 0.313	0.313/ 0.313	0.5/ 1	1.5	No interaction
Slld + Chl	0.63/ 0.313	0.63/ 0.313	1/ 1	2	No interaction
Slld + Pen	0.63/ 5	0.16/ 5	0.25/ 1	1.25	No interaction
Sllp + Amp	0.63/ 5	0.315/ 5	0.5/ 1	1.5	No interaction
Sllp + Cef	0.63/ 0.313	0.63/ 0.039	1/ 0.1	1.12	No interaction
Sllp + Chl	0.63/ 0.313	0.315/ 0.157	0.5/ 0.5	1	Additive
Sllp + Pen	0.63/ 5	0.16/ 2.5	0.25/ 0.5	0.75	Additive

Table 3.5: Continued.

Antibacterial combinations	MIC (mg/ml)		FIC	FICI	Outcome
	Individual	Combination			
Sprd + Amp	1.25/ 5	0.313/ 5	0.25/ 1	1.25	No interaction
Sprd + Cef	1.25/ 0.313	0.625/ 0.078	0.5/ 0.25	0.75	Additive
Sprd + Pen	1.25/ 5	0.313/ 2.5	0.25/ 0.5	0.75	Additive
Sprm + Amp	1.25/ 5	1.25/ 5	0.5/ 1	1.5	No interaction
Sprm + Cef	1.25/ 0.313	0.313/ 0.57	0.25/ 0.5	0.75	Additive
Sprm + Chl	1.25/ 0.6	0.625/ 0.157	0.5/ 0.52	1.02	Additive
Sprm + Pen	1.25/ 5	0.625/ 5	0.5/ 1	1.25	No interaction

Bsbm= *Bolusanthus speciosus* bark methanol extract, Pcfd = *Protea caffra* flower dichloromethane extract, *Pcsd = Protea caffra* seed dichloromethane extract, Pcsp = *Protea caffra* seed petroleum ether extract, Pctm = *Protea caffra* twig methanol extract, Pctp = *Protea caffra* twig petroleum ether extract, Slld = *Searsia lancea* leaf dichloromethane leaf extract, Sllm = *Searsia lancea* leaf methanol extract, Sllp = *Searsia lancea* leaf petroleum ether extract, Amp = ampicillin, Cef = cefotaxime, Chl = chloramphenicol, Pen = penicillin, FIC = Fractional inhibition concentration, FICI = Fractional inhibition concentration index, MIC = minimum inhibition concentration.

Table 3.6: Antibacterial interactions within combinations of selected conventional antibiotics and plant extracts against multidrug-resistant *Escherichia coli.*

Antibacterial combinations	MIC (mg/ml)		FIC	FICI	Outcome
	Individual	Combination			
Bsbm + Amp	0.625/ 5	0.313/ 2.5	0.5/ 0.5	1	Additive
Bsbm + Cef	0.625/ 0.002	0.625/ 0.001	1/ 0.5	1.5	No interaction
Bsbm + Chl	0.625/ 0.156	0.156/ 0.02	0.25/ 0.125	**0.375**	**Synergy**
Bsbm + Pen	0.625/ 0.625	0.625/ 0.313	1/ 0.5	1.5	No interaction
Pcsm + Amp	1.25/ 5	0.625/ 5	0.5/ 1	1.5	No interaction
Pcsm + Cef	1.25/ 0.002	0.625/ 0.001	0.5/ 0.5	1	Additive
Pcsm + Chl	1.25/ 0.156	1.25/ 0.156	1/ 1	2	No interaction
Pcsm + Pen	1.25/ 0.625	0.313/ 0.625	0.25/ 1	1.25	No interaction
Pctm + Amp	1.25/ 5	0.625/ 1.25	0.5/ 0.25	0.75	Additive
Pctm + Cef	1.25/ 0.002	0.078/ 0.002	0.062/ 1	1.06	No interaction
Pctm + Chl	1.25/ 0.156	0.039/ 0.156	0.031/ 1	1.03	No interaction
Pctm + Pen	1.25/ 0.625	0.156/ 0.313	0.125/ 0.5	0.625	Additive
Palm + Amp	1.25/ 5	0.156/ 2.5	0.125/ 0.5	0.625	Additive
Palm + Cef	1.25/ 0.002	0.625/ 0.002	0.5/ 1	1.5	No interaction
Palm + Chl	1.25/ 0.156	0.313/ 0.02	0.25/ 0.128	**0.378**	**Synergy**
Palm + Pen	1.25/ 0.625	1.25/ 0.313	1/ 0.5	1.5	No interaction
Pald + Amp	0.625/ 5	0.625/ 1.25	1/ 0.25	1.25	No interaction
Pald + Cef	0.625/ 0.002	0.313/ 0.002	0.5/ 1	1.5	No interaction
Pald + Chl	0.625/ 0.156	0.156/ 0.039	0.25/ 0.25	**0.5**	**Synergy**
Pald + Pen	0.625/ 0.625	0.313/ 0.625	0.5/ 1	1.5	No interaction
Sllm + Amp	1.25/ 5	1.25/ 0.078	1/ 0.016	1.02	No interaction
Sllm + Cef	1.25/ 0.002	1.25/ 0.001	1/ 0.5	1.5	No interaction
Sllm + Chl	1.25/ 0.156	0.625/ 0.078	0.5/ 0.5	1	Additive
Sllm + Pen	1.25/ 0.625	1.25/ 0.313	1/ 0.5	1.5	No interaction
Slld + Amp	0.625/ 5	0.625/ 0.156	1/ 0.031	1.03	No interaction
Slld Cef	0.625/ 0.002	0.156/ 0.002	0.25/ 1	1.25	No interaction
Slld + Chl	0.625/ 0.156	0.625/ 0.001	1/ 0.006	1.01	No interaction
Slld + Pen	0.625/ 0.625	0.039/ 0.625	0.062/ 1	1.06	No interaction
Sllp + Amp	1.25/ 5	0.313/ 2.5	0.25/ 0.5	0.75	Additive
Sllp + Cef	1.25/ 0.002	0.039/ 0.002	0.031/ 1	1.03	No interaction
Sllp + Chl	1.25/ 0.156	1.25/ 0.078	1/ 0.5	1.5	No interaction
Sllp + Pen	1.25/ 0.625	0.156/ 0.625	0.125/ 1	1.13	No interaction

Bsbm = Bolusanthus speciosus bark methanol extract, *Pcsm = Protea caffra* seed methanol extract, Pctm = *Protea caffra* seed methanol extract, Palm = *Prunus africana* leaf methanol extract, Pald = *Prunus africana* leaf dichloromethane extract, Slld = *Searsia lancea* leaf dichloromethane extract, Sllm = *Searsia lancea* leaf dichloromethane extract, Sllp = *Searsia lancea* leaf petroleum ether extract, Amp = ampicillin, Cef = cefotaxime, Chl = chloramphenicol, Pen = penicillin, FIC = fractional inhibition concentration, FICI = fractional inhibition concentration index, MIC = minimum inhibition concentration. *FICI values bold-written indicate noteworthy antibacterial synergistic interactions.

Findings from the evaluation of the three above-mentioned antibacterial synergistic pairs using the time-kill assay did not concur with those obtained from the checkerboard assay. The time-kill curves for these combinations are presented in Figures 3.1-3.3. According to **ELIOPOULOS AND MOELLERING (1996)**, noteworthy antibacterial synergy is defined by a ≥ 2 $\log_{10}$ decline in cfu/ml by the antibacterial combination when compared with the positive control (antibiotic) after a 24 h incubation period, ≥ 2 $\log_{10}$ increase in cfu/ml indicates antagonism, while a < 2 $\log_{10}$ change shows no interactions. After 24 h of interaction the $\log_{10}$ cfu/ml difference, between the antibiotic and combination treatments for these antibacterial combinations ranged from -0.6 to 0.3, an indication that, according to the criteria stipulated above, there were no significant interactions within all three combinations examined. There was a 0.2 $\log_{10}$ and 0.3 $\log_{10}$ cfu/ml augmented killing of the MDR *E. coli* in combination treatments compared to the antibiotic (most active constituent) alone in combinations of chloramphenicol with each of *B. speciosus* MeOH bark extract and *P. africana* DCM leaf extract respectively, indicating some minor antibacterial synergistic interactions (Figures 3.1 and 3.2). In both cases, the two assays detected synergism, however the time-kill kinetics indicated that the interactions were insignificant. The checkerboard assay therefore accurately detected synergism in both combinations, but apparently overestimated the extent to which the two antibacterial agents interacted. **PETERSEN et al. (2006)** reported that such exaggerations are often a direct result of the interactions between bacteriostatic and bactericidal agents in combination therapies. Although the checkerboard MIC technique is one of the traditional methods used to evaluate antibiotic interactions, the results from this technique are not always reproducible. After observing major inconsistences in results obtained from the same checkerboard treatments, **RAND et al. (1993)** recommended the use of at least five replicates per each checkerboard assay and further suggested that more than 80% agreement among replicates is required for accurate

interpretations of results. Furthermore, the authors cited uneven inoculum concentrations, reader and pipette errors as some of the root causes of such discrepancies.

Whereas the checkerboard assay, mainly assesses the bacteriostatic nature of antibacterial agents, the time-kill assay determines the rate and extent to which these agents kill the test bacterial strains (**MUNDY et al., 2016)**. In clinical studies, these two methods are often used together, with the less laborious checkerboard assay being used mainly for preliminary screening purposes and the time-kill assay to give a more accurate estimation of the degree of antibacterial synergy. This is largely because the time-kill kinetic graphs gives a more vivid picture of changes in bacterial populations after exposure to antibiotics over time and has the added advantage of showing us whether the antibacterial effects are concentration-dependent or time-dependent (**PFALLER et al., 2004)**.

From the foregoing argument, it is evident that using the checkerboard assay alone will not always guarantee accurate interpretation of antibacterial synergism. Observations from the current study reaffirms the above statement, especially in combinations of chloramphenicol with *P. africana* leaf methanolic extract where the checkerboard assay detected noteworthy antibacterial synergism (FICI = 0.5) but the time-kill assay detected minor antibacterial antagonism within the same combinations (a -0.6 2 $\log_{10}$ difference between the combination and antibiotic treatment) (Figure 3.3). Antibacterial antagonism here implied that certain plant extract constituents interfered with the action of the test antibiotic (chloramphenicol) or the other way around. Such deleterious interactions undoubtably negate the therapeutic essence of antibacterial combination therapies. The current findings suggest that ethnobotanical evaluations of plant-based antibacterial synergistic interactions should not be done using the

checkerboard bioassay alone, but confirmatory time-kill assays should also be done, as applicable in clinical trials.

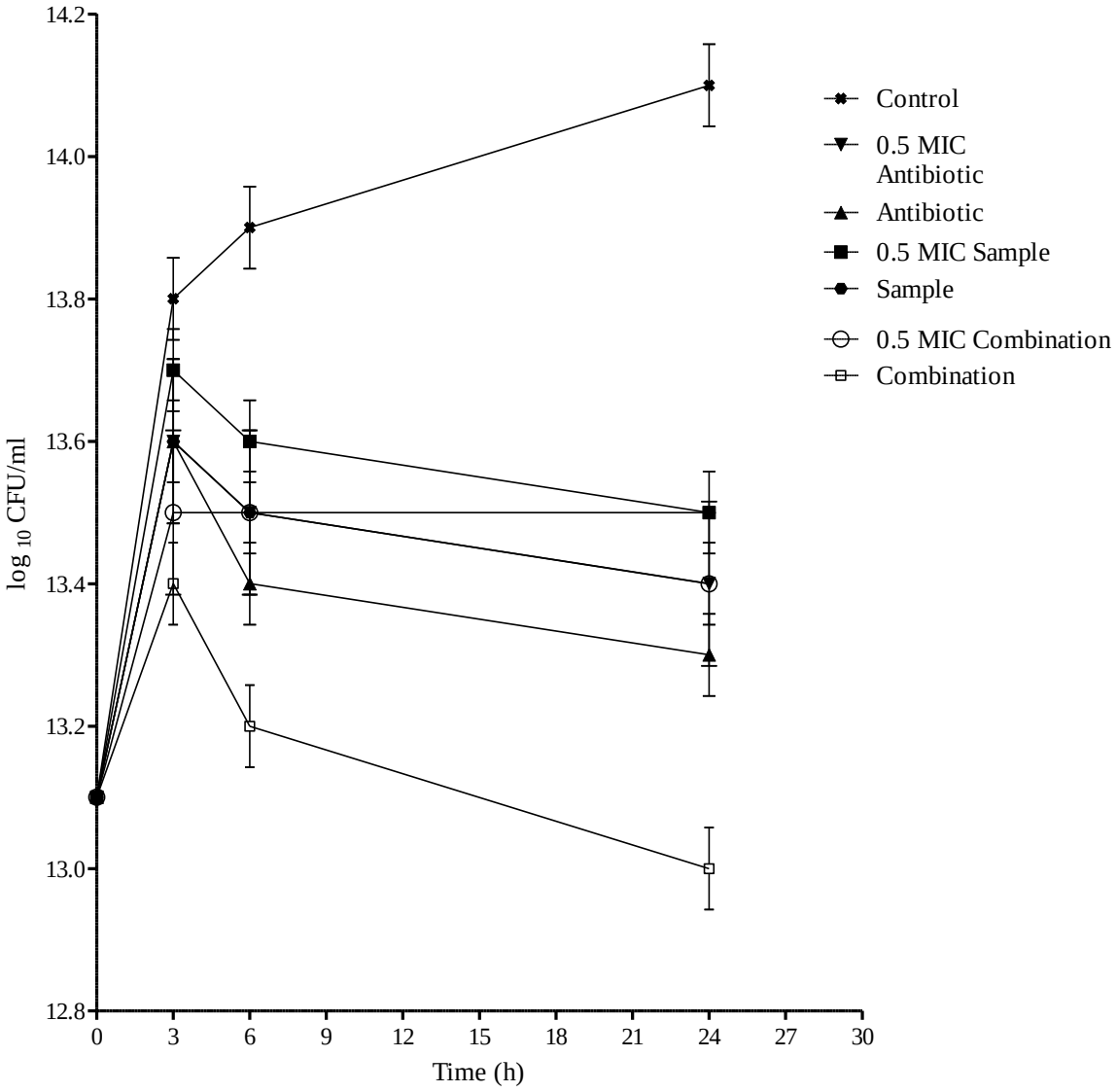

Figure 3.1: Time-kill curves showing the antibacterial interactions within combinations of chloramphenicol and *Bolusanthus speciosus* bark methanol extract against multidrug-resistant *Escherichia coli*.

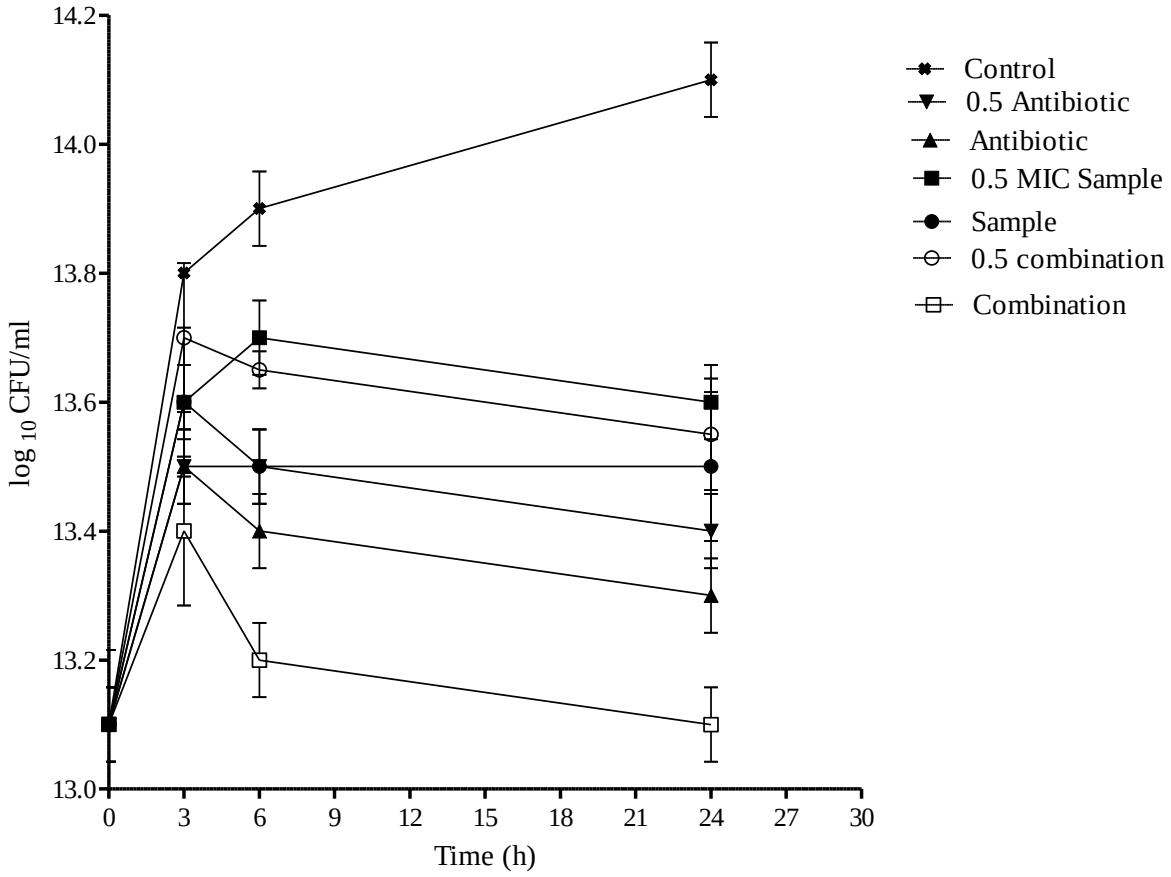

Figure 3.2: Time-kill curves showing antibacterial synergistic effects of combinations of chloramphenicol and dichloromethane extracts of *Prunus africana* leaves against multidrug-resistant *Escherichia coli*.

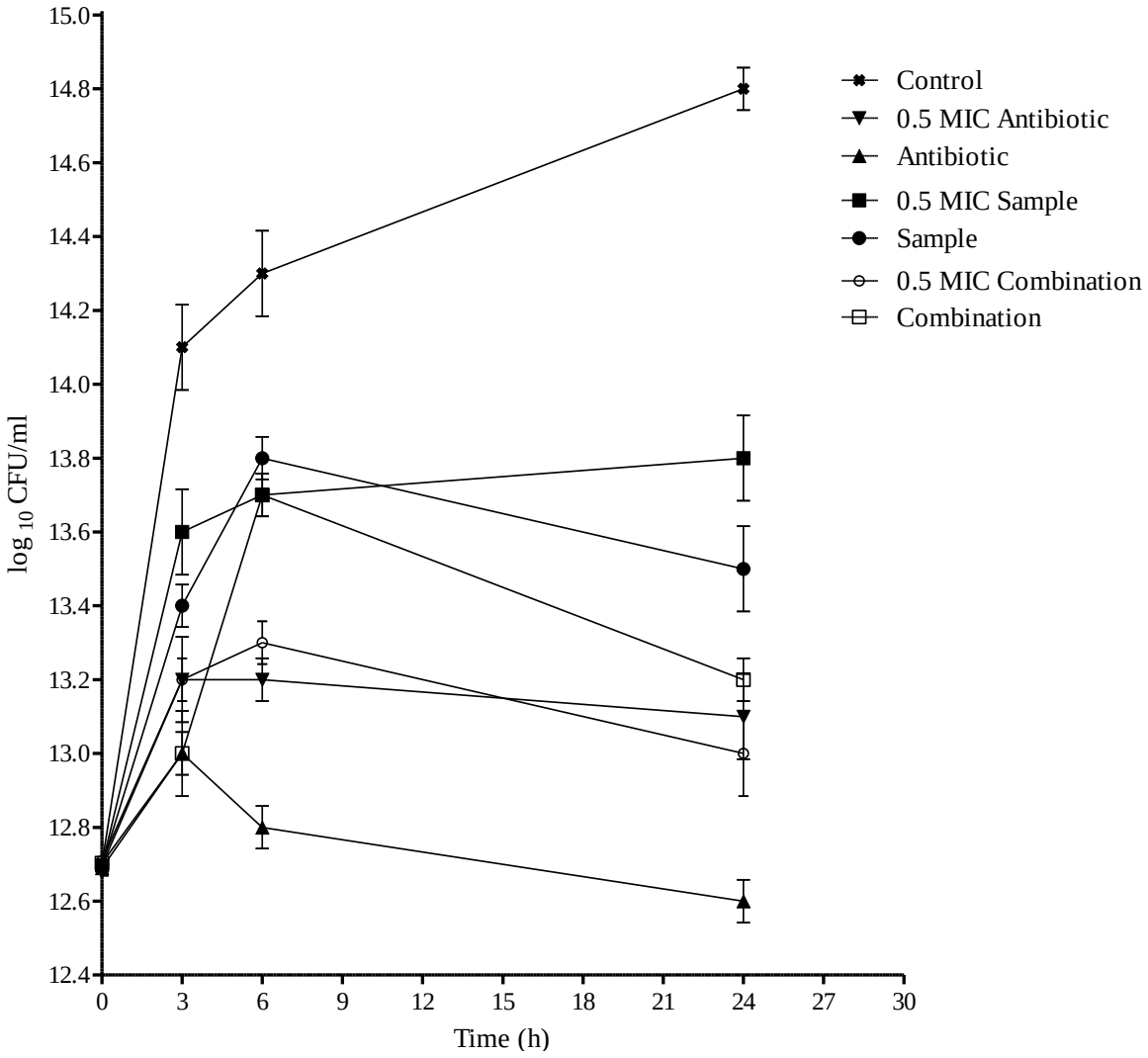

Figure 3.3: Time-kill curves showing antibacterial interactions between chloramphenicol and *Prunus africana* methanol leaf extract against multidrug-resistant *Escherichia coli*.

3.4. Conclusions

The study reveals for the first time the antigonococcal properties of *S. lancea* and *P. africana.* However, the safety of these plants should be further evaluated via cytotoxicity and genotoxicity tests. The current findings also indicate that highly efficacious and broad-spectrum antibacterial agents could potentially be obtained from traditional medicinal plants such as *B. speciosus.* Extracts from *B. speciosus, P. africana* and *S. lancea* demonstrated promising activities against MDR *E. coli* and MDR *K. pneumoniae* reaffirming the potential use of medicinal plants as sources of novel antibacterial agents effective against MDR, Gram-negative bacterial strains. *P. caffra,* a plant with a scanty ethnomedicinal history was among the test plant species that demonstrated broad-spectrum antibacterial activities. This plant could be used as a taxonomic marker for the discovery of new pharmacological compounds within the Proteaceae and *Protea* taxa. The weak antibacterial synergistic interactions detected by the time-kill assay in combinations of chloramphenicol with *B. specious* and *P. africana* reiterates the inherent potential of medicinal plant extracts in improving the activity of clinically inactive antibiotics. Finally, results from the current study lay special emphasises on the sensitivity of the time-kill assay as compared to the checkerboard assay with respect to antibacterial synergistic interactions within antibiotic and medicinal plant extract combinations.

CHAPTER 4: Phenolic profiles and mutagenic properties of selected plants

4.1. Introduction

Flavonoids, tannins and phenolic acids are among phytochemical secondary metabolites that often act as 'chemical barriers' to pathogenic invasions in plants (**MAZID et al., 2011)**. Empirically, these bioactive phytocompounds form the principal mainstay of herbal medicine. Consequently, quantitative phytochemical analysis has become an integral part of ethnopharmacology. Different colourimetric techniques including the aluminium chloride, butanol-hydrochloric acid as well as the Folin and Ciocalteu (Folin C) bioassays are often used to determine the presence and quantity of phenolic compounds in medicinal plant extracts. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has also been used widely to identify and quantify phenolic acids in medicinal plant extracts (**KALILI AND DE VILLIERS, 2011)**.

In addition to synthesising a wide range of pharmacologically important compounds, some medicinal plants also produce cytotoxins, genotoxins and mutagens as part of their natural defence mechanisms (**ELGORASHI et al., 2003; TAYLOR et al., 2003; CHUKWUJEKWU et al., 2014)**. Some bioactive phytocompounds, particularly sesquiterpene lactones, also possess potent cytotoxic and genotoxic potentials (**YU et al., 2007; CHATURVEDI et al., 2015)**. It is therefore pertinent that medicinal plants be carefully screened for unwanted toxic effects before their continued use is encouraged. *In vitro* toxicological tests, such as the *Salmonella* microsome assay (Ames test) are usually favoured over animal tests due to their limited use of live animals, cost effectiveness, sensitivity and rapidity (**ASENSIO et al., 2007)**. The current investigation sets out to identify and quantify phenolic compounds present in tested plant organs. The study also aimed at screening test plant extracts for mutagenic properties.

4.2. Materials and methods

4.2.1. Plant collection and preparation of samples

Plant samples were collected and prepared as described in **Section 3.2.1**.

4.2.2. Source of chemicals

Biotin, catechin, Folin C reagents, gallic acid, histidine and 4-nitroquinoline-N-oxide (4NQO) were supplied by Sigma-Aldrich Co. (Steinheim, Germany). Other chemicals utilized in this study included sodium carbonate and sodium nitrate (BDH Chemicals Ltd, England, United Kingdom); ferric ammonium sulphate (Hopkins and Williams Ltd, England, United Kingdom); cyanidin chloride (Carl Roth, GmbH + Co., Karlsruhe, Germany); aluminium chloride and sodium hydroxide (Merck KDaA, Darmstadt, Germany).

4.2.3. Phenolic composition of selected plants

4.2.3.1 Extraction for phenolics

For each plant, 2 g of each dried-powdered material were extracted using 10 ml of aqueous (50%) methanol by sonification in a cold-water bath for 20 min. The extracts were then filtered *in vacuo* through Whatman No. 1 filter papers and used immediately.

4.2.3.2. Assay for total phenolic content

As described by **MAKKAR et al. (2000)**, the Folin C assay was used to determine the total phenolic content in each tested plant organ**.** In triplicate, the reactants were mixed in succession as follows; 50 µl of the sample extract, 950 µl of distilled water, 500 µl of Folin C reagent (1 N) and 2.5 ml of 2% sodium carbonate. The blank contained a similar mixture made up of aqueous (50%) methanol instead of the plant sample, while varying volumes of gallic acid

(0.1 mg/ml) were used as a standard for generating the calibration curve. The reaction mixtures were then incubated at room temperature for 40 min after which absorbance was read at 725 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). The amount of total phenolics in each sample was expressed as gallic acid equivalents (GAE), derived from a standard curve.

4.2.3.3. Assay for condensed tannins

The concentration of condensed tannins (proanthocyanidins) in each test plant organ was determined using the butanol-hydrochloric acid assay (**MAKKAR et al., 2000)**. In triplicates, the reaction mixtures were made up of 3 ml of butanol-hydrochloric acid reagent (95:5 v/v), 500 µl of the plant sample and 100 µl of ferric reagent (2% ferric ammonium sulphate in 2 N hydrochloric acid). The blank contained a similar mixture consisting of 50% methanol instead of the plant extract, while varying volumes of cyanidin chloride (0.1 mg/ml) were used as a standard for generating the calibration curve. The mixtures were vortexed and heated in a boiling water bath (100 ^{o}C) for 1 h. Absorbance was then read at 550 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). The quantity of condensed tannins in each sample was expressed as cyanidin chloride equivalents (CCE), derived from a standard curve.

4.2.3.4. Assay for flavonoids

The quantity of flavonoids in each plant organ was determined using the aluminium chloride assay as previously described by **MAKKAR et al. (2000)**. In triplicates, 200 µl of the plant sample were added to 1 ml of distilled water in a test tube, followed by 75 µl of 5% sodium nitrite, 75 µl of 10% aluminium chloride, 0.5 ml of 1M sodium hydroxide and 0.6 ml water. A similar mixture consisting of 50% methanol instead of the plant extract was used as a blank and catechin (0.1 mg/ ml) was used as a standard. Absorbance of the reaction mixture was then

read immediately (no incubation period) at 510 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). The concentrations of flavonoids were expressed as catechin equivalents (CE), derived from a standard curve.

4.2.3.5. Quantitative analysis of phenolic acids

In triplicates, 30 mg of lyophilized plant material were transferred into a 2 ml Eppendorf tube and extracted with 80% methanol (30 mg/ml) and 20 µl internal standard [10^{-4} mol/L; solution of salicylic acid (3,4,5,6-$^{2}H_4$) and 4-hydroxybenzoic acid (2,3,5,6-$^{2}H_4$)] containing 2 mm ceria-stabilised zirconium oxide beads (Retsch GmbH & Co. KG, Haan, Germany). The mixture was homogenised using an Oscillation ball mill for 3 min at a frequency of 27 Hz/s and an ultrasonicator for 15 min, then centrifuged (26 000 g) at 4 ^{o}C for 10 min. The resultant supernatants were transferred into Eppendorf tubes and re-extracted as previously described above. Both extracts were combined and dried in an oil evaporator for 6-8 h.

The supernatants were filtered through 0.45 mm nylon membrane filters (Alltech, Breda, Netherlands) and analysed using the UHPLC^{TM} system (Waters, Milford MA, USA), linked to a Micromass Quattro API triple quadrupole mass spectrometer (Waters, Milford MA, USA) which was equipped with a Z- spray electron spray ionisation (ESI) source operating in the negative mode as previously described by **GRUZ et al. (2008)**. The UHPLC^{TM} was also connected to a PDA 2996 photo diode array detector (Water, Milford, MA, USA). All phenolic acids were identified and quantified using authentic standards.

4.2.3.6. Data analysis

The quantification of all phenolic compounds was done in triplicate and the results presented as mean ± standard error. Mean values were compared using one-way analysis of variance

(ANOVA) and where statistical significance ($p \leq 0.05$) existed, these values were further separated using the Duncan's multiple range test. Bio-statistical analysis were done using the SPSS version 24.0 for Windows (IBM SPSS Inc., Chicago, IL) and all graphs were done using Graph Pad Prism version 5.0 for Windows (Graphpad Software Inc., San Diego, CA).

4.2.4. Mutagenic properties of selected medicinal plant

4.2.4.1 Ames test

The mutagenic properties of medicinal plant extracts that demonstrated noteworthy antibacterial activities (Tables 3.1 and 3.2) were evaluated using the Ames Salmonella/ microsome assay involving two *Salmonella typhimurium* tester strains, TA98 and TA102, in the absence of S9 metabolic activation (**MARON AND AMES**, **1983; ELGORASHI et al.**, **2003)**. One hundred microliters of each stock tester strain were incubated in 10 ml of Oxoid nutrient broth No. 2 for 16 h at 37 ^{o}C to achieve a cell density $\approx 1 \times 10^{9}$ cfu/ml. In triplicate, 100 µl of 3 dilutions (50, 500 and 5 000 µg/ml) of each plant extract were mixed with 500 µl of phosphate buffer (0.1 mM, pH 7.4), followed by 100 µl of the tester bacterial strain and 2 ml of sterile melted top agar supplemented with 0.5 mM of biotin and histidine (0.5 mM). The mixture was vortexed then transferred into minimal agar plates and allowed to solidify for 2-3 min. One hundred microliters of 50% methanol served as the solvent control, while 2 µg/ plate of 4NQO were used as the positive control. The number of viable colonies in each plate was determined after incubation at 37 ^{o}C for 48 h. The assay was conducted twice and results presented as mean ± standard error number of reverted colonies per plate. Plant samples that induced a 2-fold increase in the number of His^{+} revertants compared to the negative control were considered to be mutagenic. Additionally, samples that exhibited a dose-dependent increase in the number of His^{+} revertants were classified as mutagenic (**CARIELLO AND PIEGORSCH**, **1996)**.

4.3. Results and discussion

4.3.1. Phenolic compositions of selected medicinal plants

Natural phenolic compounds play a significant role in the prevention and management of several infectious and non-infectious diseases (**SHAHIDI AND NACZK**, **1995; SHAHIDI**, **2004)**. This perhaps explains why a considerable number of traditional medicinal plants contain high levels of phenolic compounds (**POLYA**, **2003)**. In the present study, different quantitative phytochemical analysis techniques were employed to determine the quantity of phenolic compounds present in each tested plant organ.

The total phenolic content in the tested plant organs ranged from 5.57-62.73 mg GAE/g DW (Figure 4.1). The highest concentrations of total phenolics, condensed tannins (Figures 4.1 and 4.2), gallic and catechin acids (Table 4.1) were detected in *S. lancea* leaves. It may, therefore, be inferred that the extended-spectrum antibacterial properties exhibited by *S. lancea* leaves (Chapter 3, Tables 3.1 and 3.2) was due to the presence of highly concentrated phenolic compounds in these leaves. Other medicinal plants that contained relatively higher levels of total phenolics and also displayed broad-spectrum antibacterial properties were *P. africana* leaves and *P. caffra* twigs. The leaves of *P. africana* contained more condensed tannins (15.5 mg CCE/g DW) than flavonoids (13.5 mg CE/g DW), while *P. caffra* twigs contained slightly higher amounts of flavonoids (26.7 mg CE/g DW) than condensed tannins (22.1 mg CCE/g DW) as shown in Figures 4.2 and 4.3. Both tested plant organs also contained varying quantities of both hydroxybenzoic and hydroxycinnamic acids (Tables 4.1 and 4.2). The antibacterial properties of phenolic compounds are substantially attributable to the numerous hydroxyl and other functional groups they possess which often binds to the intra- and extracellular components of bacterial cells (**COWAN**, **1999; BORGES et al.**, **2013)**. These lethal interactions often cause irreversible

damage to bacterial cell walls as well as membranes leading to excessive leakages of cyto- and nucleoplasm (**TAYLOR et al., 2005; LOU et al., 2012)**.

It is, however, important to note that not all tested plant organs containing higher levels of total phenolics displayed noteworthy antibacterial properties. *P. caffra* leaves contained the second highest concentration of total phenolics (Figure 4.1) and the overall highest concentrations of flavonoids (Figure 4.3), caffeic, *p*-coumaric, *p*-hydroxybenzoic and *p*-protocatechuic acids (Tables 4.1 and 4.2) but was only active against 38 % of the bacterial strains evaluated in **Chapter 3** (Tables 3.1 and 3.2). Similarly, *E. capensis* leaves, *P. africana* twigs, as well as the bark and flowers of *P. caffra* contained total phenolics in the range of 27.9-52.7 mg GAE/g DW and varying amounts of phenolic acids, but demonstrated promising antibacterial activities (MIC<1 mg/ml) against at most three test bacterial strains (none of which were Gram-negative).

Generally, *B. speciosus* bark, *C. myriocarpus* fruits as well as *S. panduriforme* fruits, leaves, stems and roots contained very low quantities of phenolic compounds. Table 4.1 shows that catechin acid was not detected in all of the test plant organs mentioned above. However, despite containing very low concentrations of phenolics, the bark of *B. speciosus* displayed broad-spectrum and potent antibacterial activities (Table 3.1-3.2). These findings generally concurred with those of **MULAUDZI et al. (2011)**. Tables 4.1 and 4.2 show that the bark of *B. speciosus* also contained some phenolic acids known to have antibacterial properties such as caffeic, ferulic, gallic, *p*-coumaric and sinapic acids (**BARBER et al., 2000; ENGELS et al., 2012; BORGES et al., 2013)**. These phenolic acids probably synergise the antibacterial flavonoids found in this medicinal plant such as Bolusanthin II, Bolusanthin III and

Bolusanthin IV (**BOJASE et al., 2002; ERASTO et al., 2004)**. All the antibacterial compounds produced by this plant should, however, be isolated and identified to substantiate this inference.

The overall weak antibacterial properties displayed by *C. myriocarpus* and *S. panduriforme* (Table 3.1-3.2) could be attributed to lower concentrations of phenolics in extracts obtained from these plants (Figures 4.1-4.3, Tables 4.1-4.2). It should be noted that nearly all phenolic compounds screened in the present study were present in both medicinal plants (Figures 4.1-4.3 and Tables 4.1-4.2). However, since these putative antibacterial compounds occurred in very low quantities, they were perhaps over-diluted to effect notable antibacterial activities. Chances are also that their antibacterial activities were easily antagonised or interfered with by other more concentrated, structurally similar chemical constituents within the medicinal plant extracts. The latter applies especially where the antibacterial mechanism was through competitive inhibition of bacterial enzymes.

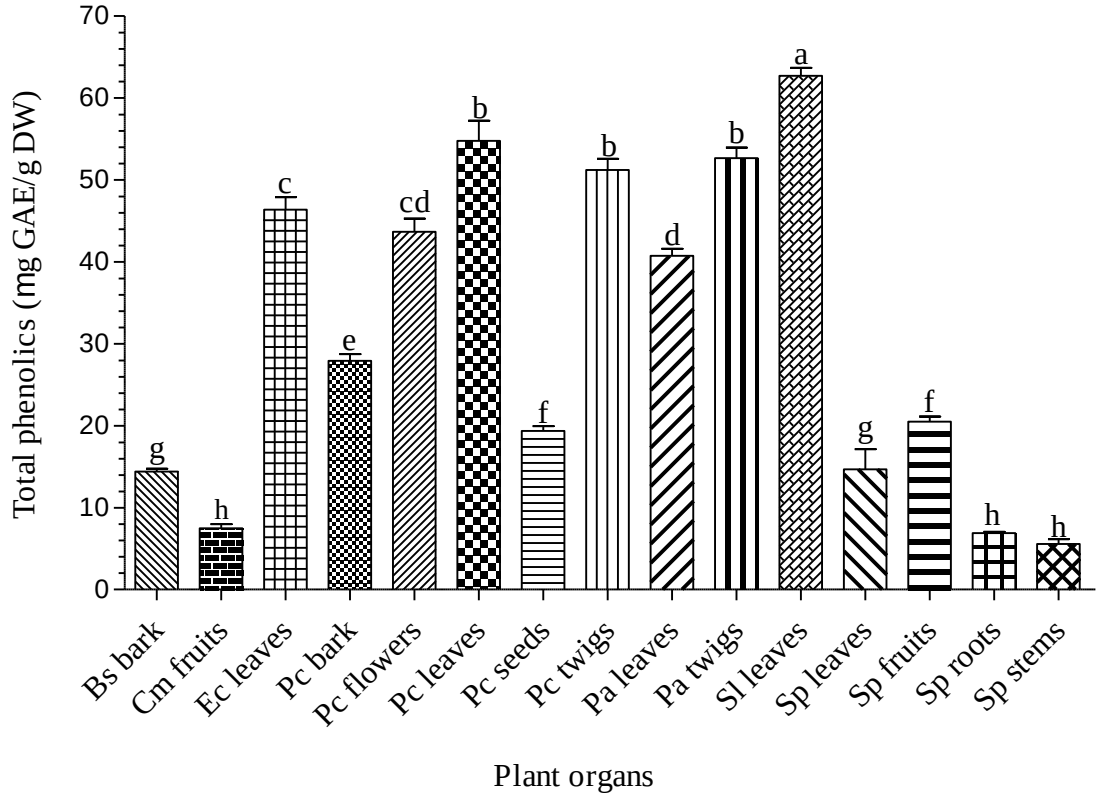

Figure 4.1: Total phenolic content (GAE: Gallic acid equivalents) in 50% methanol extracts of seven South African traditional medicinal plants. Bar represents mean ± standard error, n = 3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test. Bs = *Bolusanthus speciosus*, Cm = *Cucumis myriocarpus*, Ec = *Ekebergia capensis*, Pc = *Protea caffra*, Pa = *Prunus africana*, Sl = *Searsia lancea*, Sp = *Solanum panduriforme*.

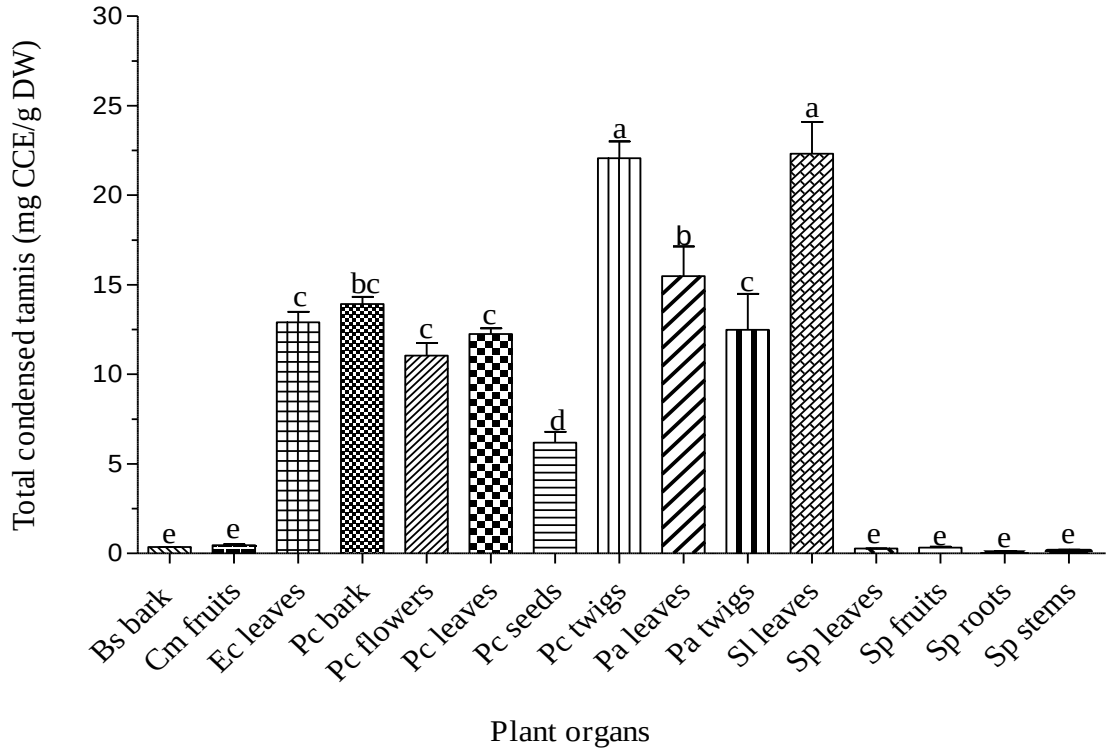

Figure 4.2: Quantity of condensed tannins (CCE: Cyanide chloride equivalents) in 50% methanol extracts of seven South African traditional medicinal plants. Bar represents mean ± standard error, n = 3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test. Bs = *Bolusanthus speciosus,* Cm = *Cucumis myriocarpus,* Ec = *Ekebergia capensis,* Pc = *Protea caffra,* Pa = *Prunus africana,* Sl = *Searsia lancea,* Sp = *Solanum panduriforme*.

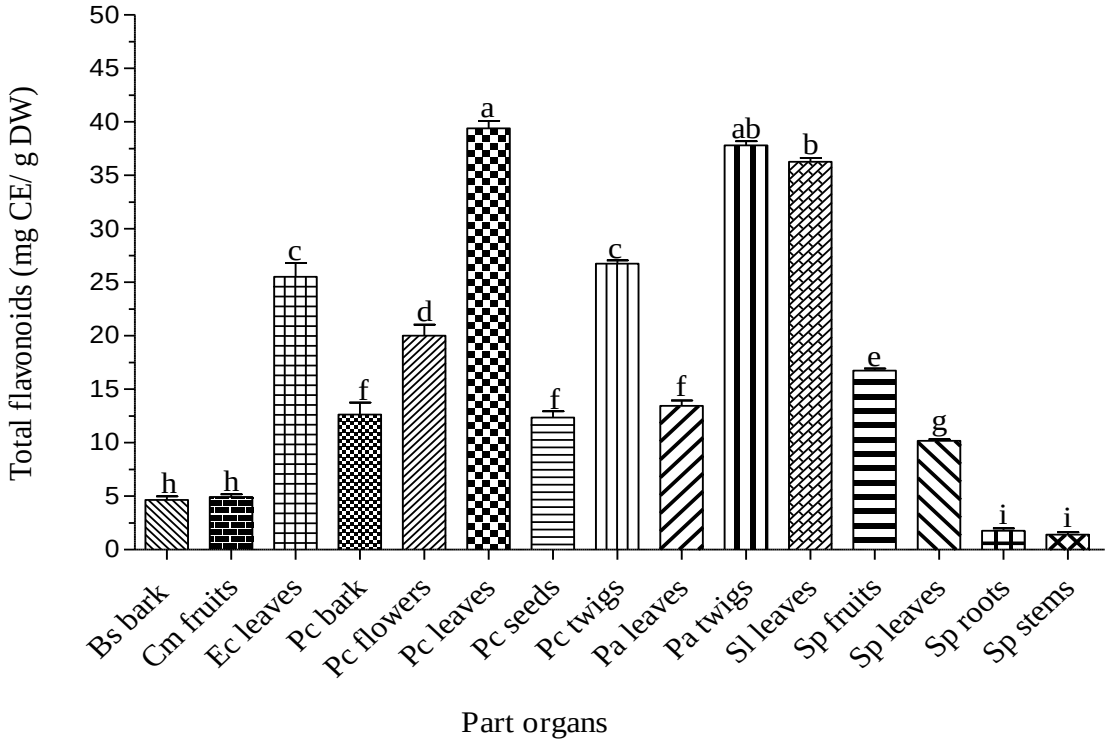

Figure 4.3: Quantity of total flavonoids (CE: Catechin equivalents) in 50% methanol extracts of seven South African traditional medicinal plants. Bar represents mean ± standard error, n = 3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test. Bs = *Bolusanthus speciosus*, Cm = *Cucumis myriocarpus*, Ec = *Ekebergia capensis*, Pc = *Protea caffra*, Pa = *Prunus africana*, Sl = *Searsia lancea*, Sp = *Solanum panduriforme*.

Table 4.1: Quantity (µg/g DW) of hydroxybenzoic acids in 80% methanol plant extracts. Values represent mean ± standard error, n=3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test. <LOD = below level of detection.

		Hydroxybenzoic acids						
Plant species	**Plant part**	**Catechin Acid**	**Gallic Acid**	***p*-Hydroxybenzoic acid**	***p*-Protocatechuic acid**	**Salicylic acid**	**Syringic acid**	**Vanillic acid**
Bolusanthus speciosus	Bark	< LOD	0.14^{d}	3.02± 0.13^{e}	3.82 ±0.07^{i}	0.37 ±0.11^{k}	1.63± 0.04^{fg}	6.55 ±0.19^{e}
Cucumis myriocarpus	Fruits	< LOD	0.23^{d}	22.88 ±0.27^{d}	0.9 ±0.02^{i}	0.95 ±0.14^{j}	0.48± 0.03^{h}	13.5 ±0.15^{cd}
Ekebergia capensis	Leaves	14.45± 033^{c}	2.47 ±0.16^{b}	6.89 ±0.25^{e}	20.56 ±0.71^{gh}	6.57± 0.06^{e}	4.49 ±0.31^{b}	0.98± 0.03^{f}
Protea caffra	Bark	6.83 ±1.4^{d}	0.3±0.3^{d}	1.95 ±0.1^{e}	88.1± 4.4^{c}	0.1 ^{k}	2.4 ±0.4^{cd}	4.7 ±0.2^{e}
	Leaves	17.98 ±0.51^{b}	1.50±0.03^{c}	374.55 ±9.14^{a}	184.35± 4.44^{a}	5.07 ±0.08^{g}	1.22± 0.02^{g}	23.35± 0.13^{b}
	Seeds	< LOD	0.69 ±0.23^{d}	23.74 ±0.37^{d}	44.22 ±1.32^{e}	1.55± 0.04 ^{hi}	0.67± 0.03^{h}	4.8±9 0.2^{e}
	Twigs	13.02 ±2.03^{c}	0.42± 0.02^{d}	4.94 ±0.36^{e}	17.65± 0.76^{h}	1.85± 0.03^{h}	1.28± 0.3^{g}	6.66 ±0.09^{e}
	Flowers	4.08 ±1.3^{f}	1.63 ±0.04^{c}	156.87± 5.03^{b}	50.35 ±1.57^{d}	7.73± 0.36^{d}	1.58 ±0.07^{fg}	10.86 ± 0.7^{d}
Prunus africana	Twigs	6.28 ±0.48^{d}	0.13 ± 0.01^{de}	7.89 ±0.24^{e}	31.78 ± 0.83^{e}	18.81 ± 0.33^{a}	1.53 ±0.05^{g}	14.83 ±0.2^{c}
	Leaves	4.72 ±0.06^{e}	1.55 ±0.03^{c}	8.86± 0.24^{e}	23.52 ±0.77^{g}	11.38± 0.25^{b}	0.29 ±0.01^{h}	4.44± 0.07^{e}
Searsia lancea	Leaves	66.78 ±1.29^{a}	29.5± 0.74^{a}	47.83± 0.76^{c}	123.24± 1.33^{b}	9.92 ±0.39^{c}	1.36 ±0.02^{g}	4.46 ±0.08^{e}
Solanum panduriforme	Fruits	< LOD	2.59± 0.12^{b}	4.91± 0.05^{e}	16.83± 0.15^{h}	1.27± 0.02^{ji}	2.04 ±0.03^{de}	5.97± 0.21^{e}
	Leaves	< LOD	1.35 0.07^{c}	29.27 0.71^{d}	21.88 0.74^{gh}	5.22 ±0.13^{g}	8.09 ±0.36^{a}	82.89 3.4^{a}
	Stems	< LOD	0.29± 0.02^{d}	9.64 ±0.36^{e}	5.56 ±0.03^{i}	7.35± 0.09^{d}	2.69 ±0.11^{c}	21.61± 0.92^{b}
	Roots	< LOD	0.061± 0.01^{d}	2.76 ±0.04^{e}	1.37± 0.03^{i}	5.88± 0.1^{f}	1.96 ±0.11^{ef}	13.9± 0.19^{c}

Table 4.2: Quantity (µg/g DW) of hydroxycinnamic acids in 80% methanol plant extracts. Values represent mean ± standard error, n=3. Plant organs with different letter(s) are significantly ($p \leq 0.05$) different as separated by Duncan's Multiple Range Test. <LOD = below level of detection.

Plant species	**Plant part**	**Hydroxycinnamic acids**				
		Caffeic acid	**Chlorogenic acid**	***p*-Coumaric acid**	**Ferulic acid**	**Sinapic acid**
Bolusanthus speciosus	Bark	0.3 ± 0.01^{i}	< LOD	5.27 ± 0.09^{e}	5.74 ± 0.12^{g}	1.44 ± 0.04^{b}
Cucumis myriocarpus	Fruits	0.16 ± 0.01^{i}	166.47 ± 5.56^{c}	10.71 ± 0.12^{c}	4.8 ± 0.04^{h}	1.47 ± 0.03^{b}
Ekebergia capensis	Leaves	63.12 ± 2.01^{b}	< LOD	9.9 ± 0.37^{cd}	11.51 ± 0.3^{c}	$0.75\pm0.03^{c-e}$
Protea caffra	Bark	5.69 ± 0.22^{h}	0.56 ± 0.03^{j}	1.69 ± 0.06^{g}	14.12 ± 0.39^{b}	0.2^{d-f}
	Leaves	266.37 ± 1.46^{a}	11.67 ± 0.28^{hi}	21.22 ± 0.54^{a}	2.68 ± 0.18^{i}	0.08^{f}
	Seeds	29.09 ± 0.77^{d}	3.34 ± 0.16^{j}	10.03 ± 0.4^{c}	4.89 ± 0.2^{c}	0.09 ± 0.01^{f}
	Twigs	9.36 ± 0.39^{g}	29.91 ± 1.21^{g}	5.3 ± 0.26^{e}	5.68 ± 0.26^{g}	$0.23\pm0.13^{d-f}$
	Flowers	39.36 ± 1.39^{c}	4.82 ± 0.2^{j}	10.77 ± 0.62^{c}	6.92 ± 0.13^{f}	$0.24\pm0.03^{d-f}$
Prunus africana	Twigs	1.17 ± 0.53^{i}	224.5 ± 4.54^{b}	1.89 ± 0.08^{g}	8.91 ± 0.25^{c}	1.39 ± 0.01^{b}
	Leaves	14.02 ± 0.41^{f}	< LOD	16.42 ± 0.31^{b}	10.04 ± 0.36^{d}	0.79 ± 0.04^{cd}
Searsia lancea	Leaves	18.12 ± 0.32^{e}	78.21 ± 1.38^{e}	9.14 ± 0.07^{d}	7.04 ± 0.07^{f}	0.13^{ef}
Solanum panduriforme	Fruits	2.59 ± 0.12^{b}	347.77 ± 4.5^{a}	5.08 ± 0.07^{e}	15.25 ± 0.16^{a}	11.33 ± 0.74^{a}
	Leaves	6.02 ± 0.19^{h}	18.76 ± 0.5^{h}	3.51 ± 0.12^{f}	8.29 ± 0.21^{c}	1.13 ± 0.03^{bc}
	Stems	5.84 ± 0.19^{h}	44.98 ± 1.24^{f}	1.35 ± 0.06^{gh}	5.4 ± 0.16^{gh}	0.78 ± 0.04^{cd}
	Roots	9.86 ± 0.21^{g}	100.4 ± 3.17^{d}	0.67^{h}	6.52 ± 0.15^{f}	$0.59\pm0.03^{c-f}$

4.3.2. Mutagenic properties of selected medicinal plant species

The exorbitant cost of pharmaceutical drugs has seen a considerable number of people turning to complementary and/or alternative herbal medicine. However, since some medicinal plant species are inherently toxic (**TAYLOR et al., 2003)**, the safety of traditional herbal remedies remains a serious cause of concern. The absence of legislative guidelines with respect to the preparation, prescription and administration of herbal medicines in folk medicine (**FENNELL et al., 2004)** exposes patients to risks of medicinal plant poisoning. Often, cases of phyto-poisoning are poorly documented and as such, the actual mortality rates due to medicinal plants remains largely unknown (**MACE et al., 1998)**. It is therefore imperative that all medicinal plants be thoroughly screened for potential toxic effects to ensure their safe application in both western and folk medicine.

Due to limited available resources, only plant extracts that demonstrated noteworthy antibacterial activities (**Chapter 3)** were screened in the present study for mutagenic effects. The samples were not screened for any indirect mutagenic potentials because the exogenous metabolic system (S9 mix) was unavailable. Table 4.3 shows that none of the test plant extracts demonstrated a concentration-dependent increase in the number of His^+ revertants. The average TA98 revertants for all plant extract dilutions ranged from 6.4-60.7 while the TA102 revertants ranged from 172.5-426.7. The corresponding average number of revertants in the negative control (4NQO) were 191.9 and 296.7, respectively. Based on these findings, all tested plant extracts were non-mutagenic against the *Salmonella* tester strains TA98 and TA102 (**CARIELLO AND PIEGORSCH, 1996)**. However, **IRUNGU et al. (2014)** reported on the cytotoxic properties of *E. capensis*, while **TAYLOR et al. (2003)** demonstrated the genotoxic potentials of *P. africana,* suggesting that these two medicinal plants were not entirely safe to administer to patients. **NDHLALA et al. (2010)** also observed that while some medicinal plants

demonstrated non-mutagenic effects against TA98, they were found to be cytotoxic and capable of inducing indirect mutagenesis. It should also be kept in mind that besides mutagenesis, carcinogens can also induce cancerous growth in animals through altering intracellular signals and gene expressions (**VERSCHAEVE et al., 2004)**. The full toxic potentials of medicinal plants can, therefore, only be revealed by subjecting samples to rigorous and diverse toxicological tests.

Based on accessed literature, none of the plant species within the *Bolusanthus* and *Protea* genera have been reported to have potential toxic effects on humans. The current literature, however, indicates that some plant species within the *Cucumis*, *Solanum* and *Searsia* (syn. *Rhus*) genera have potential toxic effects on humans (**NEUWINGER**, **1996; TANNER**, **2000; KAYOKA et al.**, **2016)**. Medicinal plants within these genera, including those evaluated in the present study, should be used with extreme caution, especially in poorly-regulated folk medicine. Although the current data indicates that members of these genera were non-mutagenic, thorough *in vivo* studies are needed before these medicinal plants are declared safe for human consumptions.

Table 4.3: Number of His^+ revertants in *Salmonella typhimurium* strains TA98 and TA102 produced by different medicinal plant extracts (without S9 metabolic activation).

Plant species	Plant extract	Number of His^+ revertants/plate (mg/ml)					
		TA98			TA102		
		5	0.5	0.05	5	0.5	0.05
Bolusanthus speciosus	Bark DCM	16.7 ±7.5	12.7±2.1	12.3±1.2	426.7±33.1	233.3± 15.9	245.3±18.0
	Bark MeOH	19.3±4.5	19.0±6.6	22.3±3.2	330.7±32.2	422.7±17.2	317.3±35.2
Cucumis myriocarpus	Fruit MeOH	7.3±3.6	10.1±1.5	12.4±1.6	178.6±12.5	247.8±19.3	198.2±32
Ekebergia capensis	Leaf DCM	10.3±1.3	15.6±7.3	11.4±3.4	186±33.2	291.3±15.9	315±17.9
Protea caffra	Bark MeOH	9.7±2.3	8.3±3.2	10.7±1.5	248.0±20.1	274.7±11.7	177.3±15.5
	Bark PE	6.4±2.1	9.8±4.1	15.8±6.1	213.5±15.9	172.5±26.2	245.6±21.1
	Flower PE	13.6±5.2	8.8±2.6	12.6±3.2	301±12.1	245.3±22.5	207±12.8
	Seed DCM	8.0±4.6	7.0±3.6	8.7±4.7	269.3±22.0	253.3±28.3	224.0±5.6
	Seed MeOH	9.7±2.1	10.0±2.6	10.7±2.1	225.3±21.1	278.7±8.5	260.0±2.6
	Seed PE	8.7±3.2	11.6±1.9	21.9±7.6	269±33.6	276±18.2	208±25.6
	Twig MeOH	22.0±6.2	29.0 ±8.2	17.3±6.7	192.0±6.2	320.0±22.5	293.3±27.4
	Leaf MeOH	16.7±1.5	15.7±11.0	27.0±11.4	392.0±30.2	290.7±22.0	416.0±43.9
	Leaf PE	7.2±2.3	22.8±7.6	19.5±4.9	233.6±16.5	246±17.7	317.6±14.7
Prunus africana	Leaf DCM	19.3±6.3	23.3±7.2	11.4±2.3	189±23.5	314.1±25.3	236.6±21.3
	Leaf MeOH	18.6±3.2	28.7±6.3	21.6±6.1	223.2±21.6	315.2±20.2	205±20.1
	Leaf PE	24.6±3.1	22.4±8.6	32.7±9.9	338.6±10.6	275.3±7.1	187±12.2
	Twig MeOH	25.3±8.4	21.6±6.1	22.5±7.3	220.6±31.5	198.6±8.1	312.5±29.4
	Twig DCM	13.5±4.2	18±4.5	19.2±9.1	195±12.3	183.1±13.4	205.9±23.6

Table 4.3: Continued.

Plant species	Plant extract	Number of His$^+$ revertants/plate (mg/ml)					
		TA98			TA102		
		5	**0.5**	**0.05**	**5**	**0.5**	**0.05**
Searsia lancea	Leaf DCM	17.3±8.1	23.0±12.2	11.3±3.2	340.9±41.0	234.7±22.0	325.3±10.1
	Leaf MeOH	16.7±2.3	60.7±10.1	38.3±21.0	248.0±16.6	201.3±11.2	181.3±11.7
	Leaf PE	17.4±1.9	45.9±12.8	35.3±12.5	312±21.3	198.3±32.9	174.2±10.2
Solanum panduriforme	Root DCM	22.4±4.5	11.4±1.4	12.3±5.3	264.3±22.6	175±10.6	245.7±33.5
Water (-ve control)			19.1±8.4			145.2±17	
4-NQO (+ve control)			191.9±17.3			296.7±20.6	

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4.4 Conclusions

Phenolic compounds contribute to the therapeutic properties of most traditional medicinal plant species. The current study revealed variations in the total phenolic content, flavonoid, condensed tannin and phenolic acid concentrations in the medicinal plant organs evaluated. The higher concentrations of phenolic compounds detected in *S. lancea* leaves, *P. africana* leaves as well as the twigs of *P. caffra* could perhaps justify the broad-spectrum antibacterial activities displayed by extracts from these plant organs as elucidated in the previous Chapter. Interestingly, the bark of *B. speciosus* contained very low quantities of nearly all phenolic compounds evaluated, yet demonstrated broad-spectrum and the overall best antibacterial activity. Overall, no obvious correlations between antibacterial efficacy and the quantity of phenolics present in the tested plant organs could be established. Apparently, antibacterial activity and spectrum were subject to the type, quality and quantity of phenolic compounds present in each medicinal plant organ. Plant organs that did not demonstrated good antibacterial properties (MIC > 1 mg/ml) but contained higher levels of phenolic compounds such as the leaves, bark and flowers of *P. caffra* and the leaves of *E. capensis* should be screened for other pharmacological activities such as antioxidants, antiviral, antifungal, anti-inflammatory, antimutagenic and anticarcinogenic properties. It was encouraging to note that none of the test plant extracts induced frameshift or point mutations. However, rigorous *in vitro* and *in vivo* toxicological tests are needed to ascertain the safety of all seven medicinal plants assessed in the current investigation.

CHAPTER 5: Identification of putative antibacterial compounds in *Searsia lancea* and *Protea caffra*

5.1. Introduction

The multi-stage process of pharmaceutical drug discovery and development often starts with the identification of the principal bioactive molecules from natural sources such as plants and microbes. In this context, ethnobotany and ethnopharmacology play a crucial role in guiding scientists into discovering novel therapeutic molecules from plants.

The isolation of bioactive phytocompounds dates back to the early 1800's when two French scientists, Caventou and Pelletier, isolated an anti-malarial compound (quinine) from a medicinal plant (*Cinchona officinalis*) commonly used in the Amazon region to treat fevers at the time (**GURIB-FAKIM**, **2006)**. Over the years, ethnobotany and ethnopharmacology have become prominent scientific disciplines focusing on, among other things, validating the traditional use of medicinal plants. Using data generated from ethnobotanical inquiries and ethnopharmacological studies, chemists are able to find novel sources and groups of pharmacologically important phyto-compounds (**TAYLOR et al.**, **2005; MURATA et al.**, **2008; BORGES et al.**, **2013)**.

Appropriate extraction and isolation techniques are key requisites for successful isolation of biologically active compounds from natural sources (**ATANASOV et al.**, **2015)**. The bioactivity-guided fractionation technique has been instrumental in the isolation of active phyto-compounds for decades (**BRUSOTTI et al.**, **2014)**. This technique employs the use of bioassays and column chromatography to isolate compounds in a given sample according to their biological activities and polarity (**PRABHAKAR et al.**, **2006)**. Other chromatographic techniques

such as Thin Layer Chromatography (TLC) are often used to further purify the fractions obtained from column chromatography (**ATANASOV et al., 2015)**. Once the putative pharmacological compounds have been successfully isolated, the next step is their characterization and identification. This is often achieved through 1- and 2-dimensional Nuclear Magnetic Resonance (NMR), Infra-Red (IR), Mass Spectroscopy (MS) and X-ray data analysis (**GURIB-FAKIM, 2006)**. Subsequently, *in vitro* and *in vivo* pharmacological tests are often necessary to determine the efficacy and toxicity of the isolated compounds.

S. lancea and *P. caffra* are used in African traditional medicine to treat a wide range of both infectious and non-infectious diseases (**HUTCHINGS et al., 1996; COETZEE AND LITTLEJOHN, 2000; SEMENYA et al., 2013a)**. Empirical evidence suggests that the leaves of *S. lancea* and *P. caffra* twigs have potent inhibitory properties against a wide range of bacterial strains, including Gram-negative multiple drug-resistant (MDR) mutants (**Chapter 3**). In addition, the data presented in **Chapter 4** (Figures 4.1-4.3 and Tables 4.1-4.2) indicates that both plants contain a wide range of phenolic compounds which possess antibacterial properties. Furthermore, the Ames test results indicated that these two medicinal plants did not have potential mutagenic effects (Table 4.3, **Chapter 4**). Over and above this, little is currently known about the antibacterial compounds in both plants. On this premise, the present study was aimed at identifying the antibacterial compounds from the leaves of *S. lancea* and twigs of *P. caffra*.

5.2. Material and methods

5.2.1. Identification of putative antibacterial compounds in *Searsia lancea*

5.2.1.1. General

Open column chromatography was carried out using Sephadex LH-20 (Sigma-Aldrich). TLC was performed at room temperature using pre-coated silica glass plates (Merck glass plates, 20x20 cm, silica gel F_{254}, 0.25 mm thickness). Detection of spots on chromatograms was done under ultra-violet light (254 and 366 nm). Proton Nuclear Magnetic Resonance (^{1}HNMR) spectroscopy was performed at the School of Chemical and Physical Sciences, University of KwaZulu-Natal, Pietermaritzburg, using a Bruker AV400 spectrometer (400 and 500 MHz). A Perkin-Elmer 241 polarimeter installed with a λ_{589} sodium lamp was used to determine the optical rotations.

5.2.1.2. Plant collection and extraction

The leaves were collected and samples prepared as described in **Section 3.2.1**. Antibacterial compounds were isolated from the leaves of *S. lancea* as previously described by **NAIR et al. (2013)**, with some modifications. The dry powdered leaf sample of *S. lancea* (850 g) was mixed with one litre of 70% aqueous methanol (MeOH) and stirred for 24 h at room temperature. The resultant extract was filtered *in vacuo*, though a Büchner funnel and Whatman No. 1 filter paper. The extraction and filtration were subsequently done thrice, after which the combined extracts were concentrated using a rotary evaporator (Heldolph vv 2000, Germany) at 30 ºC.

5.2.1.3. Liquid-liquid fractionation of the crude plant extract

The concentrated 70% MeOH leaf extract was sequentially extracted with n-hexane (3x250 ml), dichloromethane (3x250 ml) and ethyl acetate (3x400ml). The resultant fractions were separately concentrated to dryness *in vacuo* to give four solvent fractions: hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and MeOH. All four fractions were screened for antibacterial activity against a panel of seven bacterial strains (*E. faecalis*, MDR *E. coli*, *K. pneumoniae*, MDR *K. pneumoniae*, *S. aureus*, penicillin-resistant *S. aureus* and *N. gonorrhoeae*) as described in **Section 3.2.5.2**.

5.2.1.4. Column chromatography

The fraction that gave the best antibacterial activity in **Section 5.2.1.3**. was further purified on a Sephadex LH-20 column using a DCM/EtOAc (1:0-0:1) solvent system followed by an increasing gradient of EtOAc up to 100% and MeOH up to 40%. The resultant fractions were spotted on TLC plates and developed in DCM/EtOAc (4:1). Fractions with similar chemical profiles were combined and subjected to antibacterial susceptibility tests as described in **Section 3.2.5.2**.

5.2.1.5. Preparative Thin Layer Chromatography

The fraction that gave the best antibacterial activity in **Section 5.2.1.4**. was further purified through preparative TLC. The fraction (7.9 mg) was loaded onto a TLC silica glass plate and developed using a DCM/EtOAc (1:1) solvent system. Isolated pure compounds were screened for antibacterial activities as described in **Section 3.2.5.2**. The isolation scheme used in the present study is summarised in Figure 5.1.

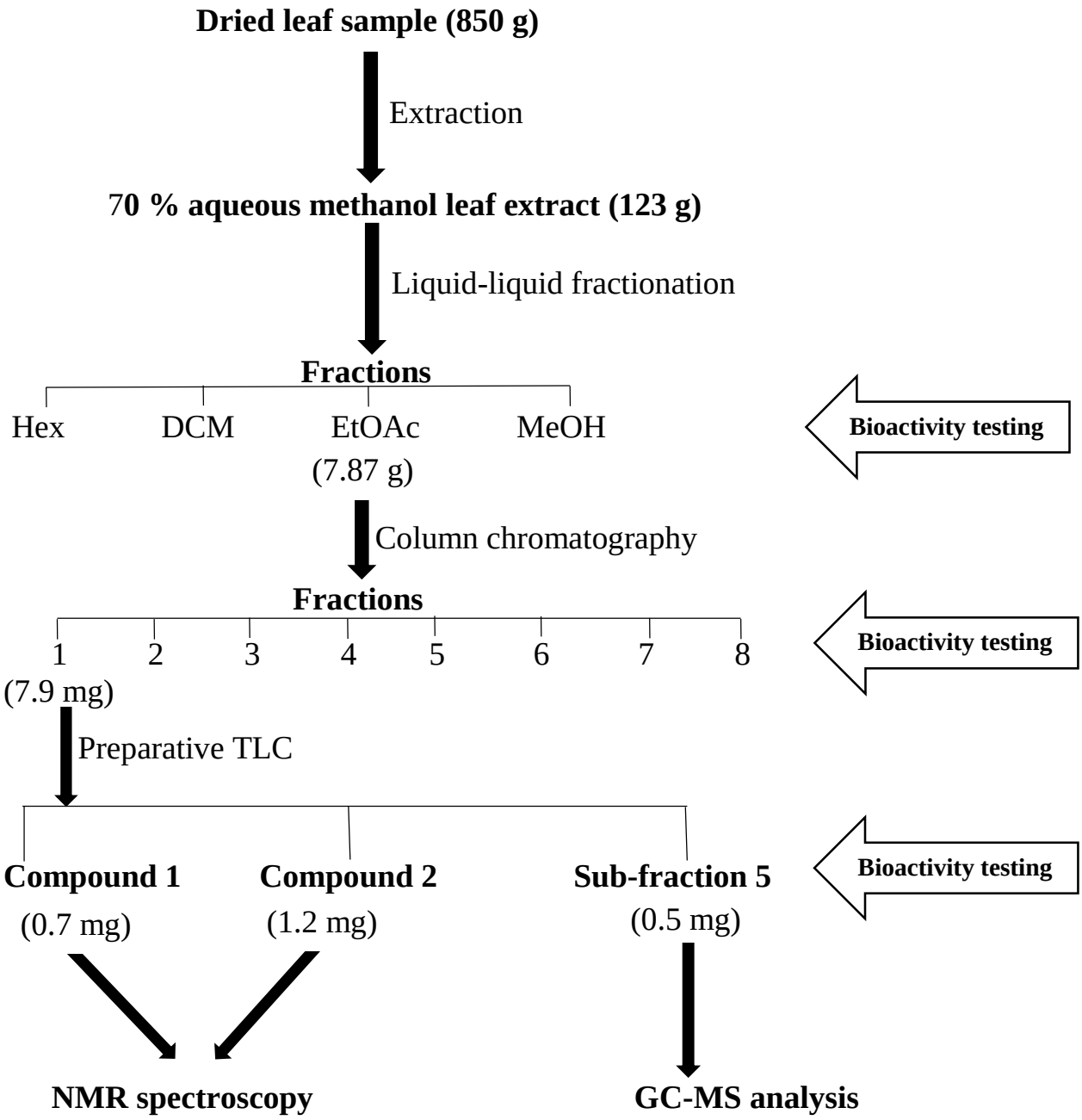

Figure 5.1: Flow diagram illustrating the bio-guided isolation of two antibacterial compounds from aqueous (70%) methanol leaf extracts of *Searsia lancea*. DCM = Dichloromethane, EtOAc = Ethyl acetate, GC-MS = Gas Chromatograph-Mass Spectroscopy, Hex = Hexane, MeOH = Methanol, NMR = Nuclear magnetic resonance, TLC = Thin layer chromatography.

5.2.1.6. Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis was carried out at the School of Chemical and Physical Sciences, University of KwaZulu-Natal, Pietermaritzburg using a Shimadzu QP-2010 SE Gas Chromatography coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent Chemstation software. A Zebron ZB-5MSplus capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) was used. The carrier gas was ultra-pure helium at a flow rate of 1.0 ml/min and a linear velocity of 37 cm/s. Three microlitres of the sample were injected into the column with the injector temperature set at 250 ^{o}C. The initial oven temperature was at 60 ^{o}C which was programmed to increase to 280 ^{o}C at the rate of 10 ^{o}C per min with a hold time of 3 min at each increment. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 ^{o}C, quadrupole temperature 150 ^{o}C, solvent delay 4 min and scan range 50-700 amu.

The compounds were identified by direct comparison of the mass spectrum of the analyte at a particular retention time to that of reference standards found in the National Institute of Standards and Technology (NIST) library. The area percentage of each component was calculated by comparing its average peak area to the total areas obtained.

5.2.2. Identification of putative antibacterial compounds in *Protea caffra*

5.2.2.1. Plant collection and extraction

P. caffra twigs were collected and samples prepared as described in **Section 3.2.1**. The dry powdered samples were mixed with methanol (10 ml/g) and stirred in a rotary shaker (Edmund Bühler, Tübingen, German) for 12 h at 150 rpm at room temperature, after which they were sonicated for 1 h on ice (Julabo GMBH, Germany). The resultant extract was filtered using Whatman No. 1 filter paper under vacuum and later concentrated using a rotary evaporator (Heldolph vv 2000, Germany) at 35 ^{o}C.

5.2.2.2. Liquid-to-liquid fractionation and GC-MS analysis

The concentrated 70% methanol extract was sequentially extracted with acetone (3x100 ml), cold ethyl acetate (10 ^{o}C, 3x50 ml) and hot ethyl acetate (50 ^{o}C, 3x50 ml). The resultant fractions were separately concentrated to dryness *in vacuo* to give four solvent fractions: acetone, cold ethyl acetate, hot ethyl acetate and methanol. The four fractions were screened for antibacterial activity against a panel of eight bacterial strains (*E. faecalis*, *E. coli*, MDR *E. coli*, *K. pneumoniae*, MDR *K. pneumoniae*, *S. aureus*, penicillin-resistant *S. aureus* and *N. gonorrhoeae*) as described in **Section 3.2.5.2**. GC-MS analysis of the four fractions was carried out as described in **Section 5.2.1.6**.

5.3. Results and discussion

5.3.1. Identification of putative antibacterial compounds in *Searsia lancea*

A resultant yield of 123 g was obtained from the extraction of *S. lancea* leaves with 70% MeOH. Liquid-liquid fractionation of that extract resulted in four fractions (DCM, Hex, EtOAc and 70% aqueous MeOH), whose antibacterial activities (MIC) are presented in Table 5.1. It should be noted that the bacterial strains used were selected based on the

antibacterial activities of *S. lancea* organic solvent extracts observed in **Chapter 3** (Tables 3.1 and 3.2). The fractions did not exhibit noteworthy antibacterial activities against *K. pneumoniae* and all three drug-resistant bacterial strains evaluated (MIC's > 2.5 mg/ml), the 70% MeOH fraction was the only one active against *N. gonorrhoeae* (MIC = 0.6 mg/ml). *E. faecalis* and *S. aureus* were susceptible to nearly all fractions (four) evaluated. The most potent fraction with regards to antibacterial activity was, however, the one eluted with EtOAc which achieved an MIC value of 0.078 mg/ml against both *E. faecalis* and *S. aureus*.

Gravity-assisted column chromatography of the EtOAc fraction (7.87 g) resulted in 325 fractions which, based on their TLC profiles, were combined to form eight sub-fractions. As indicated in Table 5.1, it was only the EtOAc fraction 1 that displayed noteworthy antibacterial activities against *E. faecalis* and *S. aureus* (MIC values of 0.031 and 0.061 mg/ml, respectively). Further purification of the EtOAc fraction 1 by preparative TLC led to the isolation of an oily compound (compound **1**) and a white powdered compound (compound **2**).

According to **GIBBONS (2004)**, a natural compound with antibacterial activities (MIC) of 0.064-0.1 mg/ml has clinical relevance. Using this benchmark, the two isolated compounds exhibited good antibacterial activities against *E. faecalis* and *S. aureus* as recorded in Table 5.1. Interestingly, the MIC value of compound **1** (0.016 mg/ml) against *E. faecalis* and *S. aureus* was almost 3 and 4 times more than that the positive control (neomycin) yielded against the same bacterial strains, respectively. Additionally, the clinical MIC breakpoints of some antibiotics (amoxicillin and ampicillin) used to manage *E. faecalis* infections was about half that of compound **1** (**EUCAST**, **2018)**, reiterating that this phytocompound was indeed highly efficacious. **GUNDIDZA et al. (2008)** demonstrated that the essential oils from the leaves

of *S. lancea* had potent antimicrobial and antioxidant activities. The authors speculated that the possible antimicrobial compounds within these essential oils were α-pinene, benzene, δ-3-carene, isopropyl toluene and trans-caryophyllene. The possibility that compound **1**, which was isolated in an oily state in the present study, could be one of the afore-mentioned compounds cannot be completely ruled out. However, if both compound **1** and **2** are unknown, then their chemistry and antibacterial mechanism(s) could possibly inspire scientists to developing new types/classes of antibiotics. These compounds could for instance, be used as the primary active ingredients in antibiotics or used to make semi-synthetic and/or synthetic antibacterial agents.

Table 5.1: Minimum inhibitory concentration values (MIC, mg/ml) of fractions and compounds obtained from the bioactivity-guided isolation of antibacterial compounds from the leaves of *Searsia lancea*.

	MIC (mg/ml)						
Compound/Fraction	***Ec* D**	***Ef***	***Kp***	***Kp* D**	***Sa***	***Sa* D**	***Ng***
DCM	>2.5	**0.15**	>2.5	>2.5	1.25	>2.5	1.25
EtOAc	>2.5	**0.078**	>2.5	>2.5	**0.078**	>2.5	>2.5
Hex	>2.5	1.25	>2.5	>2.5	**0.13**	>2.5	>2.5
70 % aqueous MeOH	>2.5	**0.3**	>2.5	>2.5	**0.1**	>2.5	**0.6**
EtOAc fraction 1	-	**0.031**	-	-	**0.061**	-	-
EtOAc fraction 2	-	>1	-	-	>1	-	-
EtOAc fraction 3	-	>1	-	-	>1	-	-
EtOAc fraction 4	-	>1	-	-	>1	-	-
EtOAc fraction 5	-	>1	-	-	>1	-	-
EtOAc fraction 6	-	>1	-	-	>1	-	-
EtOAc fraction 7	-	>1	-	-	>1	-	-
EtOAc fraction 8	-	>1	-	-	>1	-	-
Sub-fraction 5	>1	**0.25**	>1	>1	**0.25**	>1	>1
Compound **1**	>1	**0.016**	>1	>1	**0.016**	>1	>1
Compound **2**	>1	>1	>1	>1	**0.025**	>1	>1
Neomycin[a] (µg/ml)	>100	5.25	>100	>100	3.9	25	-
Ciprofloxacin[a] (µg/ml)	-	-	-	-	-	-	7

DCM = Dichloromethane, EtOAc = Ethyl Acetate, Hex = Hexane, MeOH = Methanol, - = Not tested, [a] = positive control, Ec D = Multidrug-resistant *Escherichia coli*, *Ef* = *Enterococcus faecalis*, *Kp* = *Klebsiella pneumoniae*, *Kp* D = Multidrug-resistant *Klebsiella pneumoniae*, *Sa* = *Staphylococcus aureus*, *Sa* D = penicillin resistant *Staphylococcus aureus*, *Ng* = *Neisseria gonorrhoeae*.* Values in bold are considered noteworthy.

Both *E. faecalis* and *S. aureus* contribute extensively to the global health and economic burdens associated with drug-resistant bacterial strains. **GOULD (2009)** noted that some methicillin-resistant *S. aureus* (MRSA) mutants acquired advanced resistance to all five major classes of antibiotics (cephalosporins, penicillin, carbapenems, quinolones and aminoglycosides) present at the time. Also, despite current treatment efforts, the morbidity and mortality rates associated with *E. faecalis* infections remain very high due to the prevalence of antibiotic resistance mutants (**BILLINGTON et al., 2014)**. This is indeed a serious cause for alarm given that these two bacterial strains cause serious life-threating diseases such as bacteraemia, meningitis, toxic shock syndrome, septicaemia and several nosocomial infections (**MACPHEE et al., 2013; RUBINSTEIN AND KEYNAN, 2013; GOMEZ et al., 2015; KHAN et al., 2015; POORABBAS et al., 2015; AFZELIUS et al., 2016)**. Developing new classes of antibiotics would help alleviate the socio-economic challenges emanating from drug-resistance. The current findings, therefore, underscore the importance of ethnopharmacology in helping scientists discover new sources and classes of potent antimicrobial agents.

It was interesting to note that antibacterial efficacy improved as the fractions were repeatedly and progressively purified. For instance, there was approximately a 5-fold reduction in the MIC value (inversely, an improvement in antibacterial efficacy) of compound **1** (MIC = 0.016 mg/ml) as compared to the original EtOAc fraction (0.078 mg/ml) from which it was isolated (Figure 5.1 and Table 5.1). This observation suggested that the activities of these antibacterial compounds were probably antagonised by other chemical constituents within the initial crude extracts/fractions. Furthermore, since the weight of compound **1** (0.7 mg) and compound **2** (1.2 mg) constituted approximately 0.01 % and 0.02 % (respectively) of that of the EtOAc fraction (7.87 g) from which they were isolated, these compounds were

perhaps over diluted during the early stages of the isolation procedure to reveal their actual antibacterial efficacy.

5.3.1.2. NMR assignments

Although the structure of compound **1** is still to be determined a high-field ^{1}HNMR spectrum from the small quantity of material (0.7 mg) isolated and purified by silica gel column chromatography was acquired. The 500 MHz ^{1}HNMR spectrum (Figure 5.2) of compound **1** indicated an appreciably clean compound. Three sets of signal resonances were detected in the regions δ 6.7 to 7.3, δ 4.3 to 4.8 and δ 0.4 to 1.7 as depicted in Figures 5.2.1-5.2.3. The expanded spectrum resolved the signals clumped at δ 6.7 to 7.3 into two multiplets, the more deshielded of which was centred at δ 7.19 (2H, m, *J*=2.5, 3.0, 5.0 Hz) and the other at δ 7.12 (2H, m, *J*=1.5, 7.0, 7.5 Hz). The chemical shifts, multiplicities and coupling constants for these signals are characteristic of the protons in an aromatic ring system. The signal at δ 6.76 which resolved to a double doublet also integrated to 2 protons with coupling constants of 6.0 and 12.0 Hz, respectively, can be suggested to belong to a double bond system. The broad singlet at δ 4.57 (1H) is ascribable to an oxygen-related methine proton. Methyl proton signals which resolved to sharp singlets (each 3H) were detected upfield at δ 1.55, 1.28, 1.25 and 1.20, respectively. The remaining proton signals which integrated to 9 protons stretched from δ 0.4 to 0.9 (m) were indicative of methylene and methine protons in the molecule.

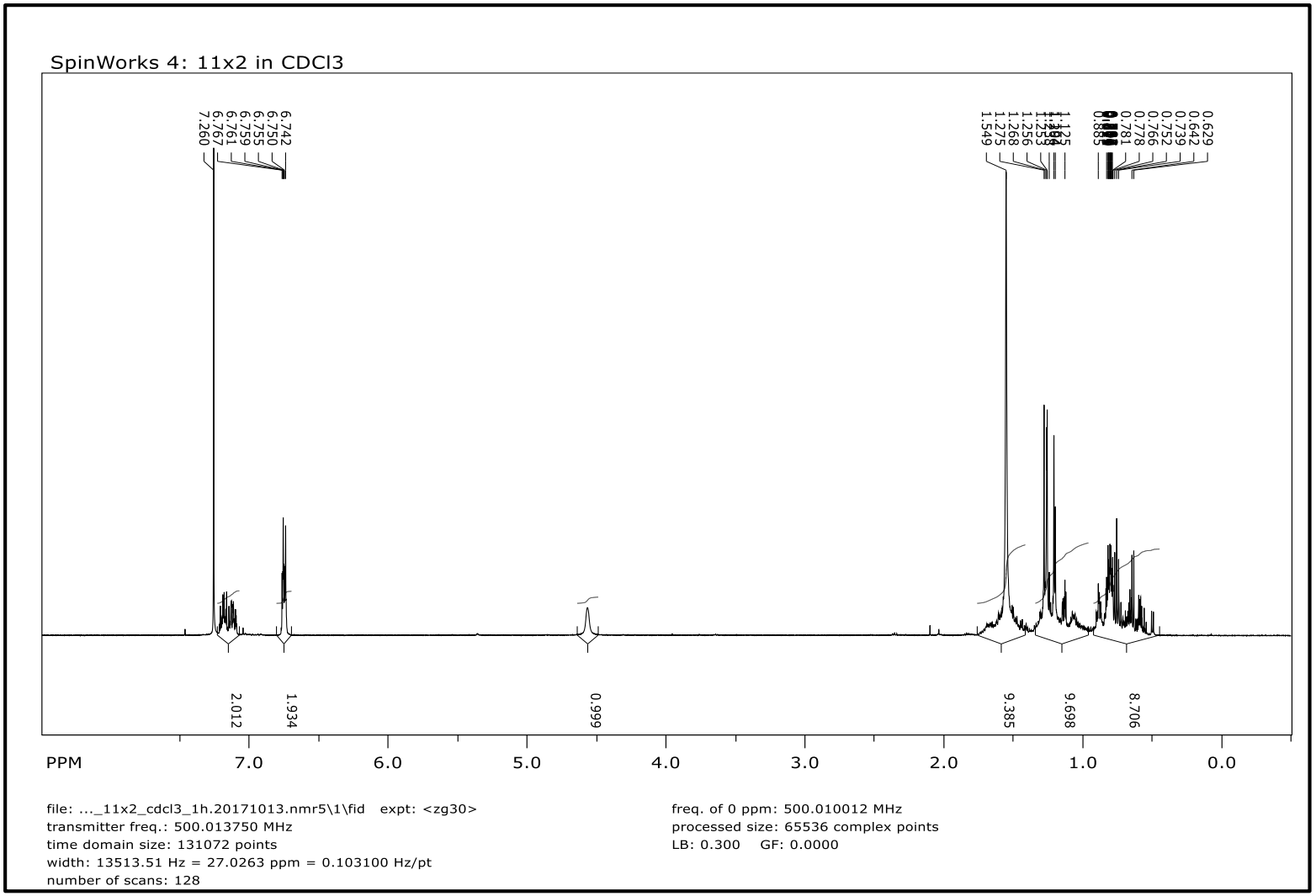

Figure 5.2: ^{1}HNMR spectrum (500 MHz) of compound **1** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

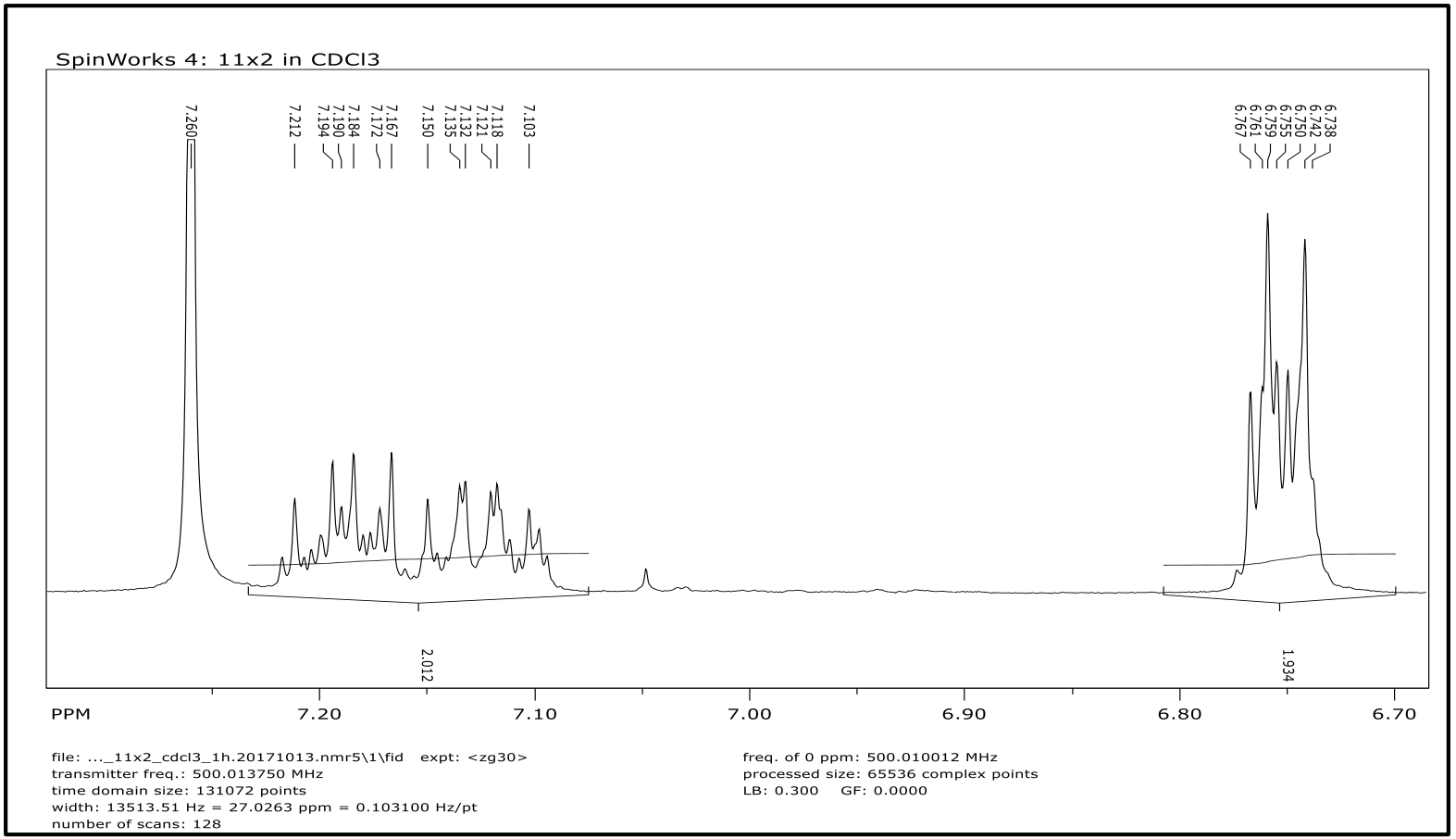

Figure 5.2.1: ^{1}HNMR spectrum (500 MHz) expansion (δ 6.7-7.3) of compound **1** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

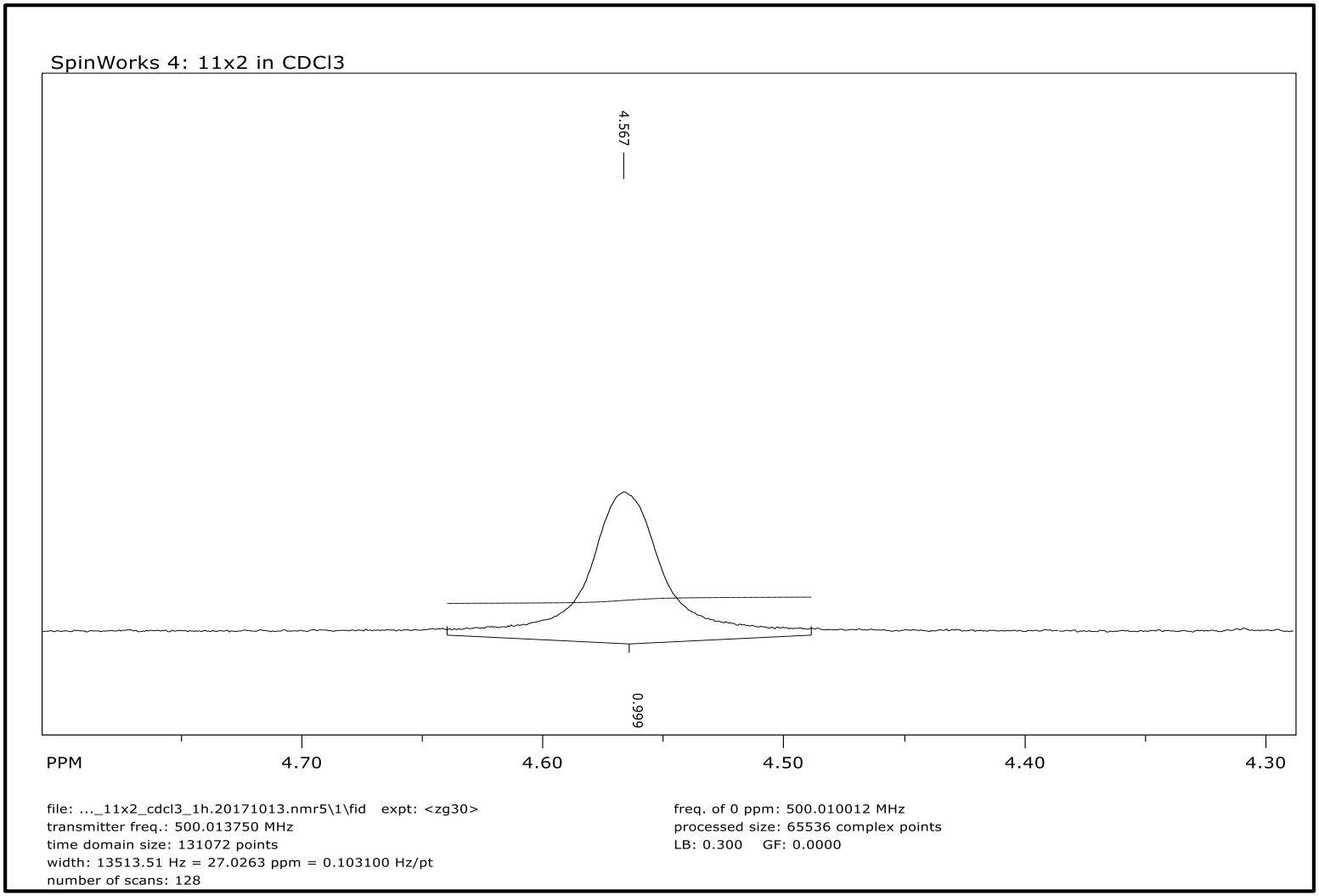

Figure 5.2.2: ^{1}HNMR spectrum (500 MHz) expansion (δ 4.3-4.8) of compound **1** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

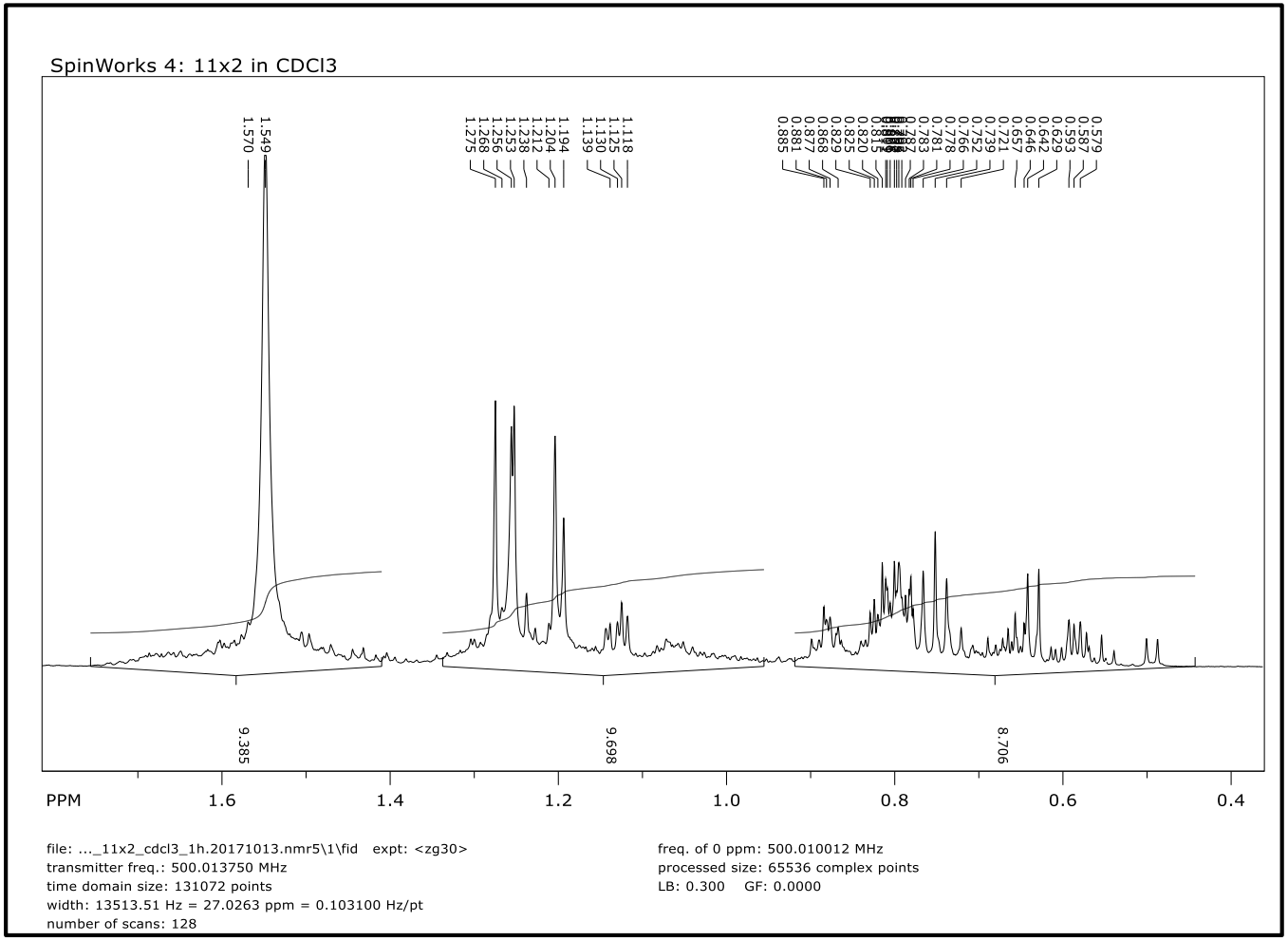

Figure 5.2.3: ^{1}HNMR spectrum (500 MHz) expansion (δ 0.4-1.7) of compound **1** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

Similar to compound **1** described above, compound **2** was isolated in a small quantity (1.2 mg) that restricted extensive analytical and spectroscopic analyses. However, the ^{1}HNMR spectrum obtained at 400 MHz together with its TLC profile [DCM/Hexane (1:1)] confirmed that the compound was satisfactorily clean. Figure 5.3 depicts the high-field NMR spectrum of compound **2** run in deuterated chloroform as solvent. Three sets of signals were detected in the range δ 7.4 to 7.8 (low-field), δ 4.1 to 4.3 (mid-field) and δ 0.8 to 1.8 (high-field) as shown in Figures 5.3.1-5.3.3. The low-field signals were a symmetrical pair of multiplets resonant at δ 7.69 (1H, m, *J*=3.0, 4.0, 7.0, Hz) and δ 7.51 (1H, m, *J*=3.0, 4.0, 7.0, Hz) which suggested them to be protons belonging to an aromatic system. The mid-field region of the spectrum was populated by a septet at δ 4.22 (2H, *J*=6.5, 7.5, 14.0 Hz). Such a spin system is diagnostic of a methine proton flanked by two methyl groups (responsible for the splitting pattern) as well as an oxygen-related functionality (responsible for the deshielding effect). The high-field area of the spectrum had signal resonances as follows: (i) δ 1.69 (1H, quintet, *J*=7.5, 7.5, 15.0 Hz), (ii) δ 1.56 (1H, s), (iii) δ 1.37 (9H, m), and (iv) δ 0.90 (6H, m). The resonance at δ 1.69 can be attributed to a methine proton flanked by two methylene groups, that at δ 0.90 indicated the presence of at least one methyl group given the sharp intensity of the peak, whilst the δ 1.37 signal resonance is indicative of methylene and methine protons.

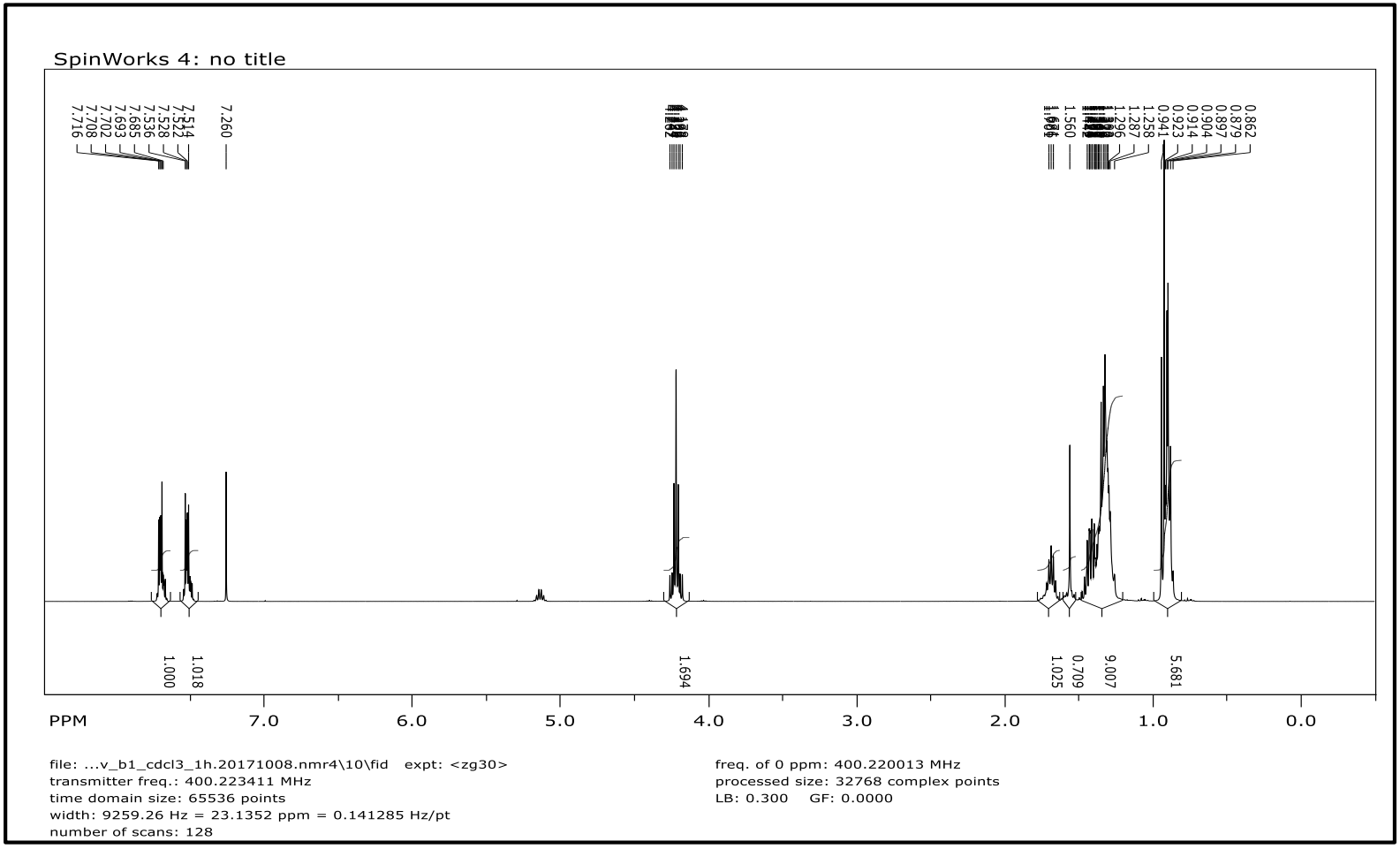

Figure 5.3: ^{1}HNMR spectrum (400 MHz) of compound **2** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

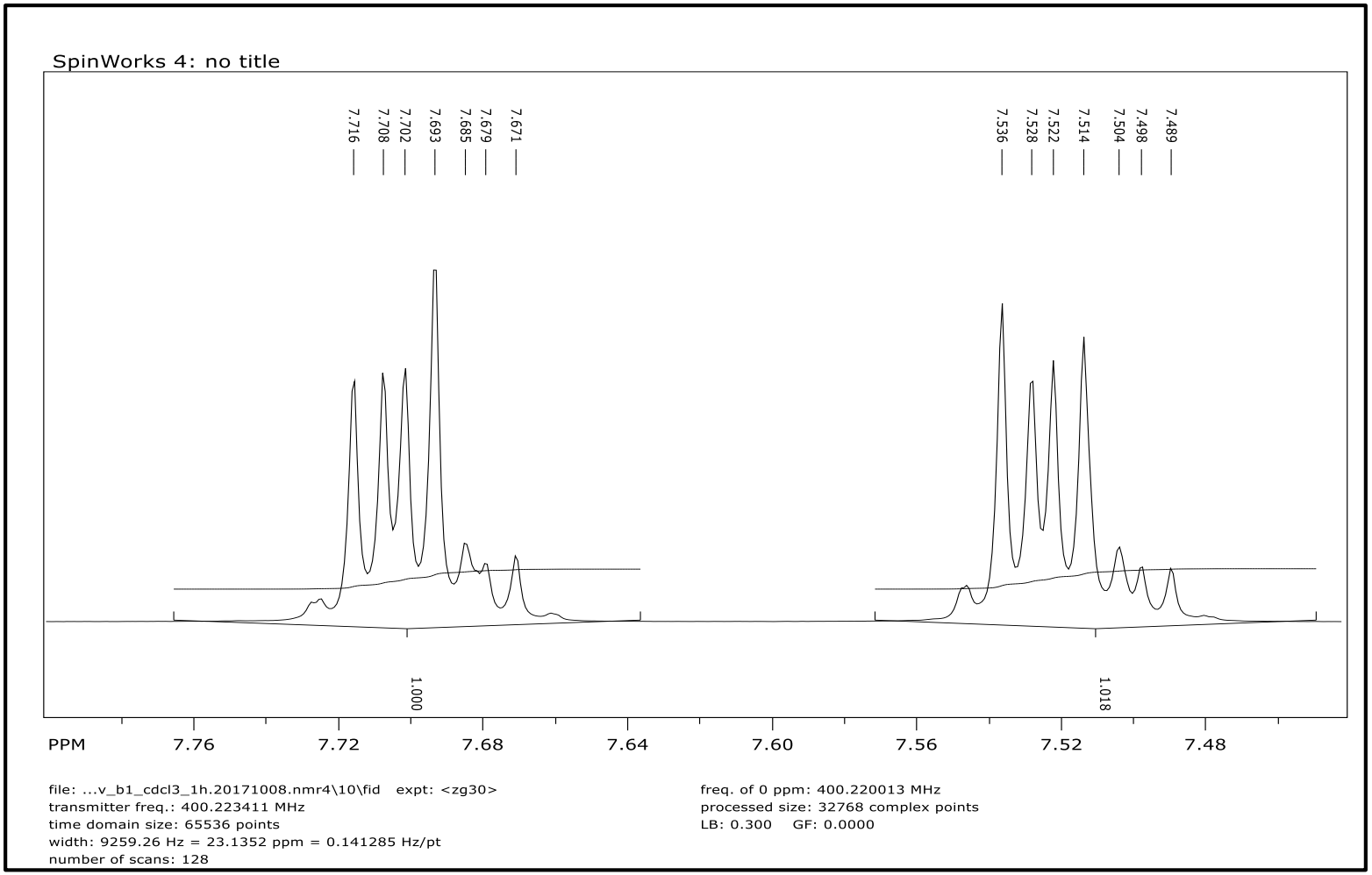

Figure 5.3.1: ^{1}HNMR spectrum (400 MHz) expansion (δ 7.4-7.8) of compound **2** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

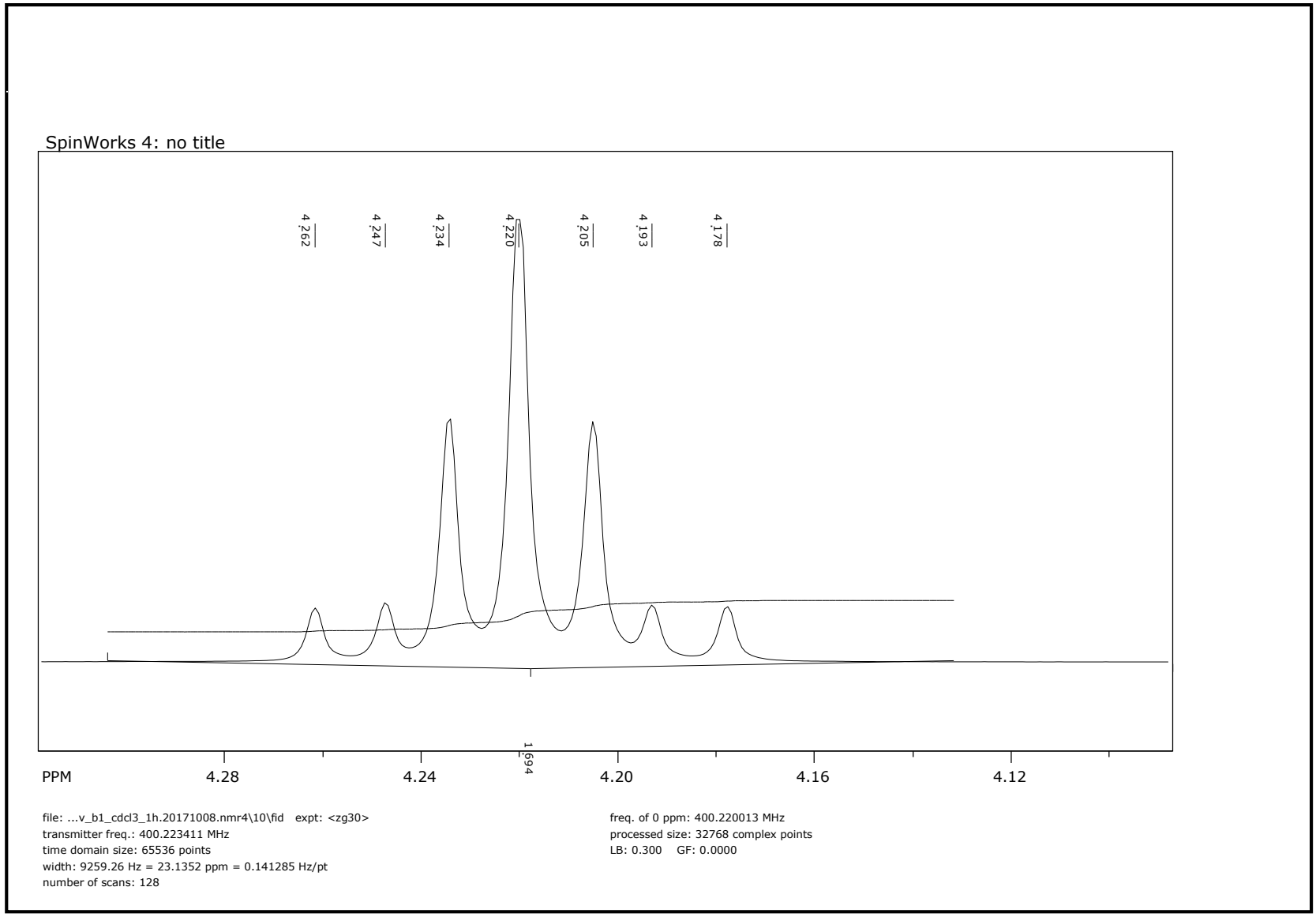

Figure 5.3.2: ^{1}HNMR spectrum (400 MHz) expansion (δ 4.1-4.3) of compound **2** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

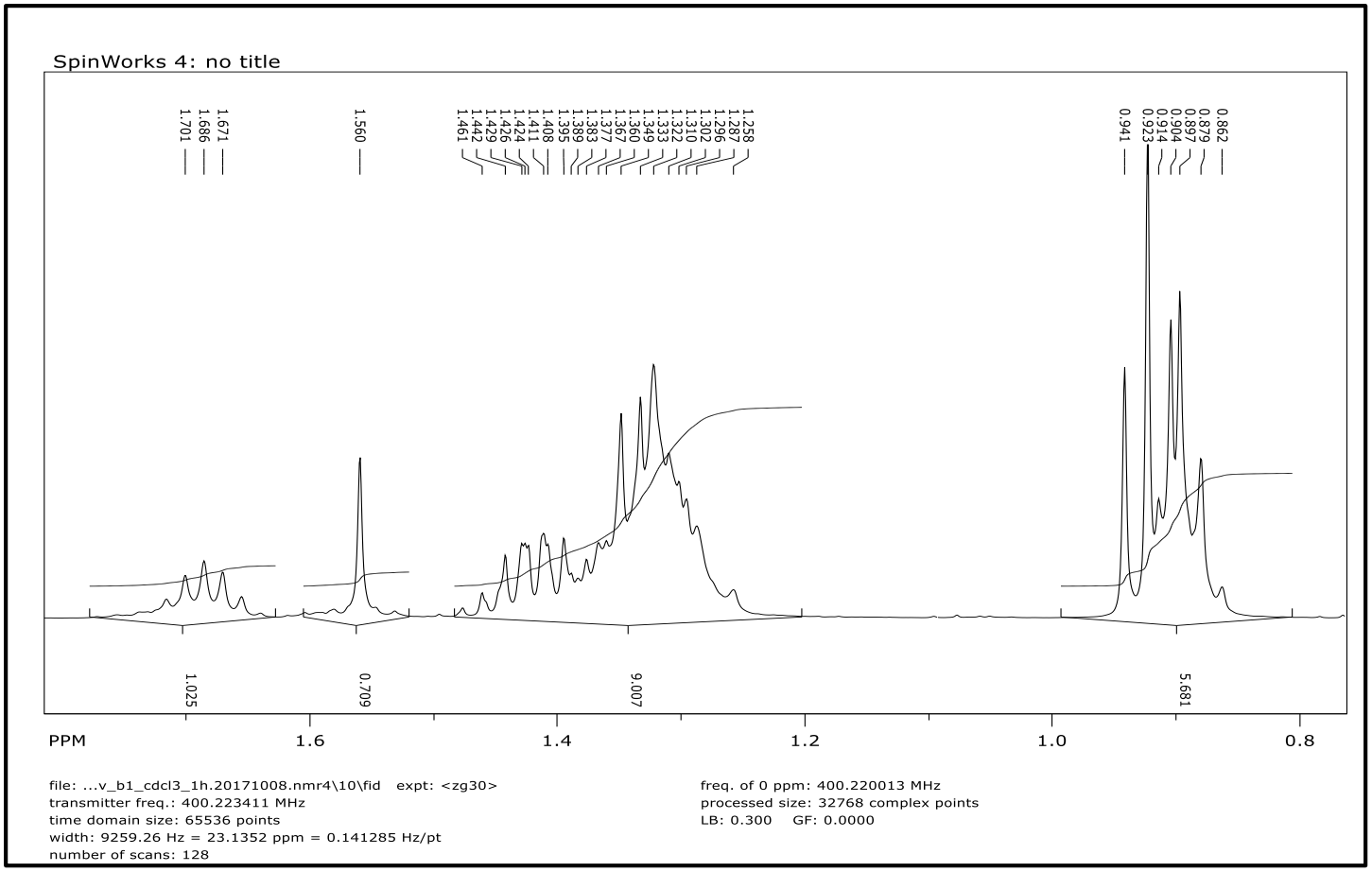

Figure 5.3.3: ^{1}HNMR spectrum (400 MHz) expansion (δ 0.8-1.8) of compound **2** (isolated from the leaves of *Searsia lancea*) run in deuterated chloroform.

5.3.1.3. GC-MS analysis of a bioactive *Searsia lancea* methanol leaf extract sub-fraction.

A sub-fraction [sub-fraction 5 (SFR-5)] obtained from the EtOAc fraction 1 (Figure 5.1) yielded an MIC value of 0.25 mg/ml against both *E. faecalis* and *S. aureus* (Table 5.1). However, only 0.5 mg of SFR-5 were obtained making it difficult to purify the sub-fraction further using preparative TLC. GC-MS analysis (Table 5.2 and Figure 5.4) revealed that the major components of this sub-fraction were 1-tetracosanol (43.98%) and 1-nonadecanol (37.5%). In addition, the sub-fraction also consisted of eicosane (7.67%), 1,7-di-*iso*-propylnaphthalene (4.23%), 1-pentadecanol (3.04%), 2,6-di-*iso*-propylnaphthalene (1.96%) and 1,3-*iso*-propylnaphthalene (1.62%). Although these seven phytocompounds are well-known, the current study revealed their presence in *S. lancea* for the first time. Based on the currently available literature, this is also the first report on the presence of 1,3-*iso*-propylnaphthalene, 1-1,7-di-*iso*-propylnaphthalene, 2,6-di-*iso*-propylnaphthalene, 1-pentadecanol, and 1-tetracosanol in *Searsia* (*Rhus*) species. Eicosane was previously detected in *Rhus vernicifera* (**JAVIDNIA et al., 2008)** and *Rhus succedanea* (**HUIPING, 1987)**, while 1-nonadecanol was found in *Rhus coriaria* (**BAHAR AND ALTUG, 2009)**.

GEHRKE et al. (2013) demonstrated that 1-nonadecanol (Figure 5.5) has potent inhibitory activities against some Gram-positive (*B. subtilis*, *S. aureus*, *Staphylococcus epidermis*, *Streptococcus pyogenes*, *Staphylococcus saprophyticus*) and Gram-negative (*E. coli*, *Shigella sonnei*) bacterial strains, with MIC values ranging between 0.05-0.2mg/ml. The authors also reported that the same compound possesses antifungal properties. Work by **RUKACHAISIRIKUL et al. (2004)** also revealed that 1-nonadecanol has potent anti-tuberculosis activities (MIC = 0.025mg/ml). As demonstrated by 1-nonadecanol, 1-tetracosanol (Figure 5.5) was also shown to have broad-spectrum antibacterial activities (**MAHMOOD et al., 2014)**. Some fungi,

especially actinomycetes, have also been reported to produce 1-tetracosanol (**VENUGOPAL et al., 2014)**, probably as part of their natural defence mechanism.

Another putative antibacterial compound detected in SFR-5 was eicosane (Figure 5.5), which together with 1-nonadecanol and pentadecanol were previously detected in plant extracts with antimicrobial (**SUKATAR et al., 2006; KARABAY-YAVASOGLU et al., 2007; MOHAMMADZADEH et al., 2007; AL-REZA et al., 2009)**, analgesic, anti-inflammatory and antipyretic (**PANTHARI et al., 2012; AL-SNAFI, 2016; SUN et al., 2017)** properties. 1-Nonadecanol and eicosane are both long chain fatty acids whose antibacterial mechanisms are believed to be associated with their strong radical oxygen scavenging properties (**YASA et al., 2009)**.

1-Nonadecanol and 1-tetracosanol are known antibacterial compounds which could have contributed to the antibacterial properties of SFR-5 (Table 5.1). There is, however, a possibility that other phytochemical constituents of SFR-5 such as eicosane worked individually or synergistically to effect the antibacterial activities observed in the present study, an area that warrants further investigation. The antibacterial compounds in this medicinal plant should therefore be isolated and unequivocally identified using spectroscopic and X-ray data analysis.

Table 5.2: GC-MS data of phytocompounds putatively identified in a bioactive *Searsia lancea* leaf ethyl acetate sub-fraction.

Chemical name	Similarity %	Area %	Retention time	Molecular formulae	Molecular weight
1-Tetracosanol	96	43.98	19.46	$C_{24}H_{50}O$	354
1-Nonadecanol	95	37.5	15.91	$C_{19}H_{40}O$	284
Eicosane	95	7.67	15.99	$C_{20}H_{42}$	282
1,7- Di-*iso*-propylnaphthalene	93	4.23	15.16	$C_{16}H_{20}$	212
1-Pentadecanol	92	3.04	13.74	$C_{15}H_{32}O$	228
2,6- Di-*iso*-propylnaphthalene	87	1.96	15.22	$C_{16}H_{20}$	212
1,3-Di-*iso*-propylnaphthalene	91	1.62	14.66	$C_{16}H_{20}$	212

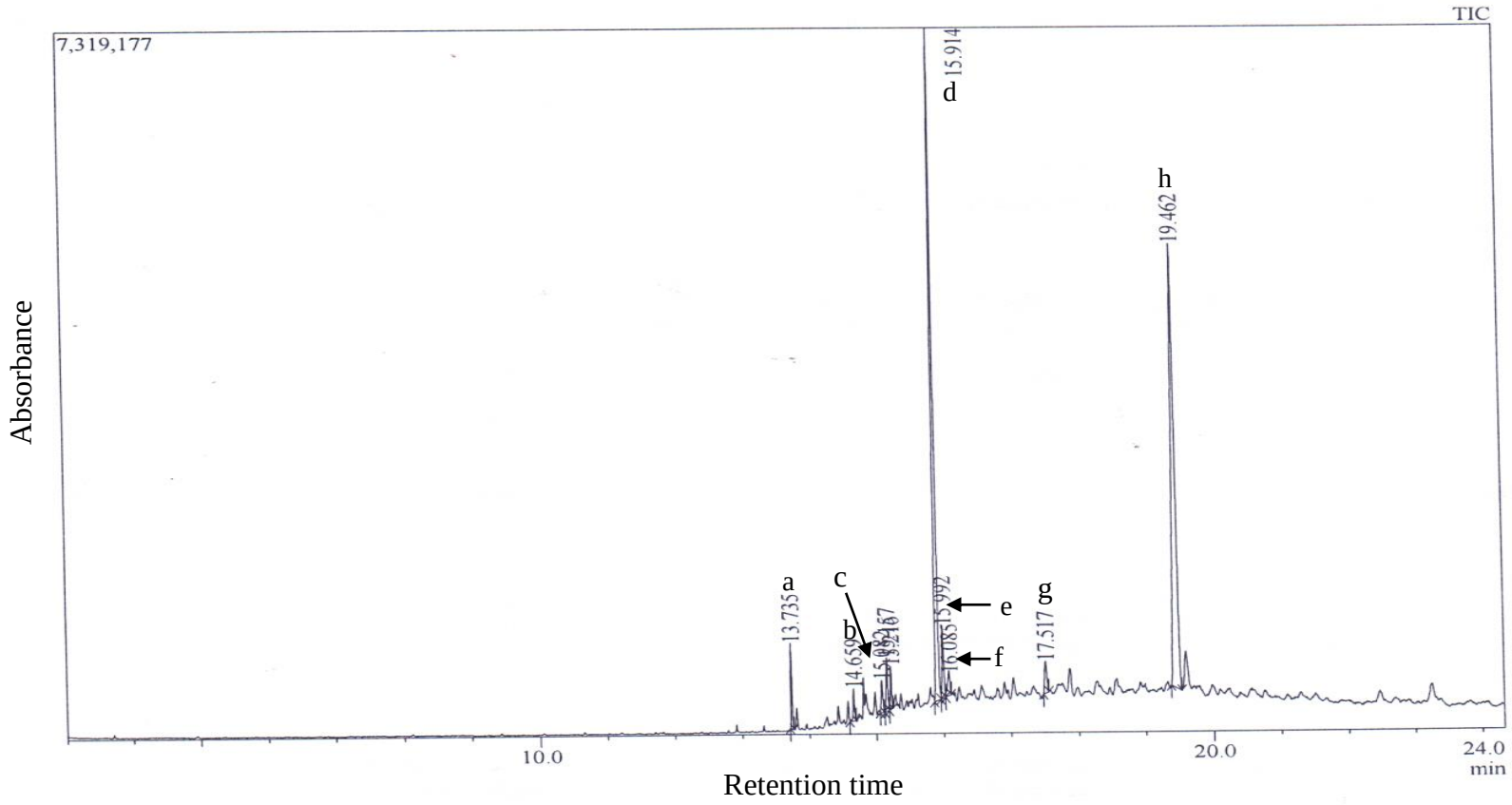

Figure 5.4: GC-MS chromatogram of a *Searsia lancea* leaf ethyl acetate sub-fraction. a = 1-Pentadecanol, b = 1,3-Di-*iso*-propylnaphthale, c = 1,7-Di-i*so*-propylnaphthale, d = 1-Nonadecanol, e = Eicosane, f = Eicosane, g = Eicosane, h = 1-Tetracosane.

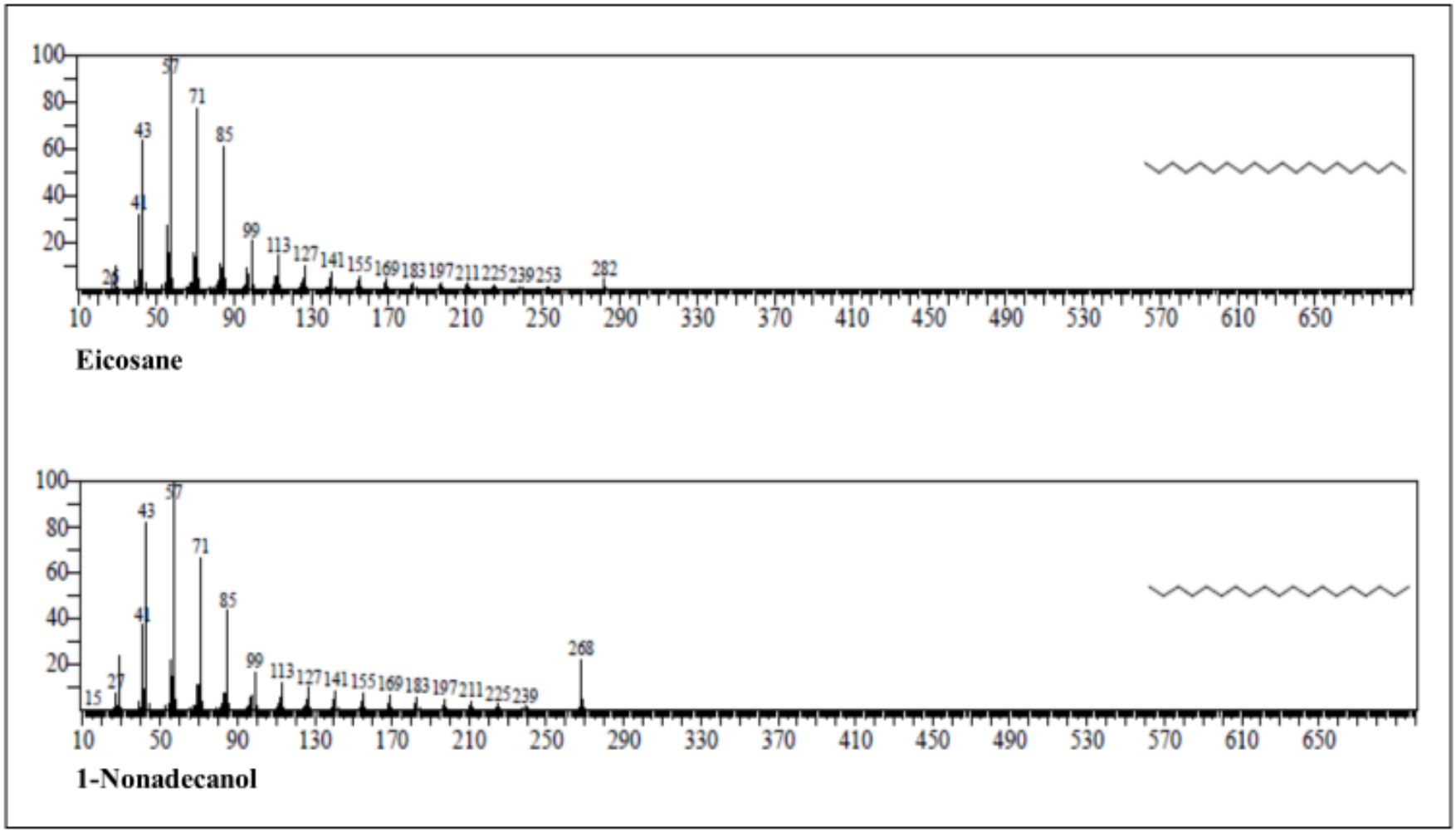

Figure 5.5: Mass spectra and chemical structures of antibacterial compounds (eicosane and 1-nonadecanol) putatively identified by GC-MS in a *Searsia lancea* leaf ethyl acetate sub-fraction.

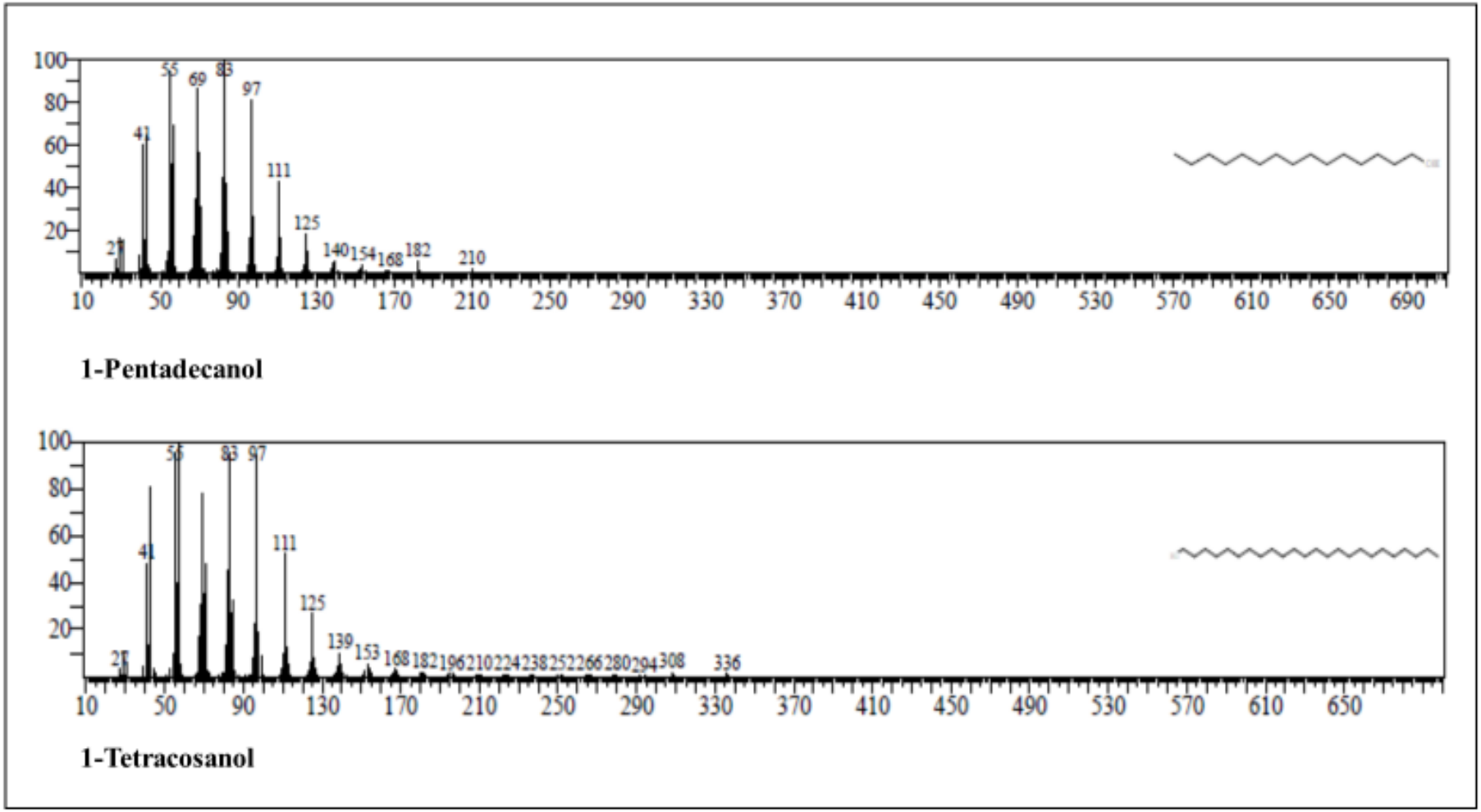

Figure 5.5: Continued (Mass spectra and chemical structures of 1-pentadecanol and 1-tetracosanol).

5.3.2. Identification of putative antibacterial compounds in *Protea caffra*

P. caffra twigs exhibited broad-spectrum antibacterial activities (Table 3.1-3.2, **Chapter 3**) and this novel finding stimulated interest in determining their phytochemical profiles. Acetone, cold ethyl acetate and hot ethyl acetate were used to fractionate the phytocompounds in a methanolic extract of *P. caffra* twigs. The MIC values of the resultant four sub-fractions are presented in Table 5.3.

All sub-fractions, except the acetone fraction, demonstrated noteworthy antibacterial activities (MIC < 1mg/ml) against some of the test bacterial strains. The best antibacterial property was exhibited by the cold ethyl acetate fraction which was active against both Gram-negative (*E. coli, K. pneumoniae*) and Gram-positive (*E. faecalis, S. aureus*) bacterial strains, with MIC values ranging between 0.078 and 0.6 mg/ml. However, the methanol sub-fraction was active against only one test bacterial strain, *S. aureus* (MIC = 0.6). None of the fractions were active against *N. gonorrhoeae* and the three drug-resistant bacterial strains evaluated.

According to GC-MS, the *P. caffra* twig MeOH extract consisted of 15 compounds (Table 5.4). The GC-MS chromatograms of the sub-fractions evaluated are presented in Figures 5.6-5.9. No peaks were obtained from the methanol sub-fraction (Figure 5.9) suggesting that perhaps the temperatures used during GC-MS analysis could have been too low for the analyte to volatilize.

Table 5.3: Minimum inhibitory concentration (MIC, mg/ml) values of fractions obtained from a methanol extract of *Protea caffra* twigs.

	MIC (mg/ml)							
Fraction	**Ec**	**Ec D**	**Ef**	**Kp**	**Kp D**	**Sa**	**Sa D**	**Ng**
Acetone	>2.5	>2.5	>2.5	>2.5	>2.5	1.5	>2.5	>2.5
Cold ethyl acetate	**0.6**	>2.5	**0.078**	**0.3**	>2.5	**0.15**	>2.5	>2.5
Hot ethyl acetate	>2.5	>2.5	**0.3**	>2.5	>2.5	**0.6**	>2.5	>2.5
MeOH	>2.5	>2.5	>2.5	> 2.5	>2.5	**0.6**	>2.5	>2.5
Neomycin[a] (µg/ml)	>100	>100	5.25	>100	>100	3.9	25	-
Ciprofloxacin[a] (µg/ml)	-	-	-	-	-	-	-	7

[a] = positive control, Ec D = Multidrug-resistant *Escherichia coli*, Ef = *Enterococcus faecalis*, Kp = *Klebsiella pneumoniae*, Kp D = Multidrug-resistant *Klebsiella pneumoniae*, Sa = *Staphylococcus aureus*, Sa D = Penicillin resistant *Staphylococcus aureus*, Ng = *Neisseria gonorrhoeae*, MeOH = Methanol, - = Not tested.

*Values in bold are considered noteworthy.

The major components of the cold ethyl acetate sub-fraction were polygalitol (34.76%), phenol, 4-(1,1,3,3-tetramethylbutyl) (9.8%), Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclol) (8.2%), 1-adamantanecarboxylic acid (8.07%) and carbamic acid (7.03%), which together accounted for approximately 60% of the total compounds found within that sub-fraction (Table 5.4). Some phytochemical constituents within this sub-fraction namely 1-adamantanecarboxylic acid, 1-heptacosanol and 1-nonadecanol (Figures 5.5 and 10) are known biologically active phytocompounds which most likely contributed to the antibacterial property of *P. caffra* twigs (Table 3.1-3.2).

1-Adamantyl heterocycle is often incorporated into anti-infectious molecules to improve their efficacy (**HASSAN et al., 2010)**. Several potent antimicrobial and antiviral agents such as Rimantadine (**MANCHAND et al., 1990)**, Oxadiazole (**EL-EMAM et al., 2004)**, Isoxazole (**MAKAROVA et al., 2002)** and Thiadiazole (**KRITSANIDA et al., 2002)** are all 1-adamantanyl derivatives. 1-Heptacosanol, another compound detected in the cold ethyl acetate fraction, is a fatty alcohol present in plants (**KOAY et al., 2013)**, marine algae (**MURUGAN AND IYER, 2014)** and cuttlefish, *Sepiella inermi*s (**RAVICHANDIRAN et al., 2013)**. This compound has potent antibacterial (**SHARMA AND MENGHANI, 2017)** and antifungal (**SUDIRGA AND GINANTRA, 2017)** properties. It also has several potential therapeutic applications given that it was detected in medicinal plant extracts with antioxidant (**MURUGAN AND IYER, 2014; AL-ABD et al., 2015)**, nematocidal (**SULTANA et al., 2010)** and antidiabetic (**UNNIKRISHNAN et al., 2014)** properties. Unlike 1-nonadecanol which is a known antibacterial phyto-compound (**YASA et al., 2009)**, polygalitol has not yet been shown to have antibacterial properties, but, has been detected in plant extracts with potent antibacterial activities (**ALAGAMMAL et al., 2012)**, suggesting the compound could have some antimicrobial properties.

Table 5.4: GC-MS data of phytocompounds putatively identified in different sub-fractions of methanolic extracts of *Protea caffra* twigs.

Sub-fraction	Chemical name	Similarity %	Area %	Retention time	Molecular formulae	Molecular weight
Cold ethyl acetate	Polygalitol	95	34.76	13.35	$C_6H_{12}O_5$	164
	Phenol, 4-(1,1,3,3-tetramethylbutyl)-	89	9.8	15.38	$C_{14}H_{22}O$	206
	Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclol)	77	8.2	14.94	$C_{14}H_{22}O$	206
	1-Adamantanecarboxylic acid, 2-propenyl	89	8.07	15.03	$C_{14}H_{20}O_2$	220
	Carbamic acid, N-[1,1-bis(trifluoromethyl)ethyl	90	7.03	14.85	$C_{19}H_{25}F_6NO_2$	413
	Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-	80	6.23	15.27	$C_{15}H_{24}O$	220
	Hexestrol, O-acetyl-	88	3.57	15.16	$C_{20}H_{24}O_3$	312
	1-Heptacosanol	94	2.86	19.41	$C_{27}H_{56}0$	396
	1,2-Bis(p-acetoxyphenyl)ethanedione	77	2.1	14.74	$C_{18}H_{14}0_6$	326
	1,3-Benzenediol, 4-propyl-	83	1.83	13.73	$C_9H_{12}0_2$	152
	Phthalic acid, butyl tridecyl ester	75	1.74	18.53	$C_{22}H_{28}O_4$	356
	1-Nonadecanol	93	1.60	15.89	$C_{19}H_{40}O$	284
	Phenol 2,4-bis(1,1-dimethylethyl)	94	1.16	12.84	$C_{14}H_{22}O$	206
Hot ethyl acetate	1-Heptacosanol	90	70.57	14.16	$C_{27}H_{56}0$	396
	1,3,5-Benzenetriol	95	15.42	13.55	$C_6H_60_3$	126
	Polygalitol	72	7.31	12.99	$C_6H_{12}O_5$	164
	1,3-Benzenediol, 4-propyl-	70	3.93	13.73	$C_9H_{12}O_2$	152
	ß–Δ-Glucopyranose, 1,6-anhydro-	70	2.78	11.60	$C_6H_{10}O_5$	162
	1-Nonadecanol	93	1.6	15.89	$C_{19}H_{40}O$	284
Acetone	Oxalyl acid	93	51.12	3.23	$C_2C_{I2}O_2$	126
	Polygalitol	95	48.88	13.22	$C_6H_{12}O_5$	164

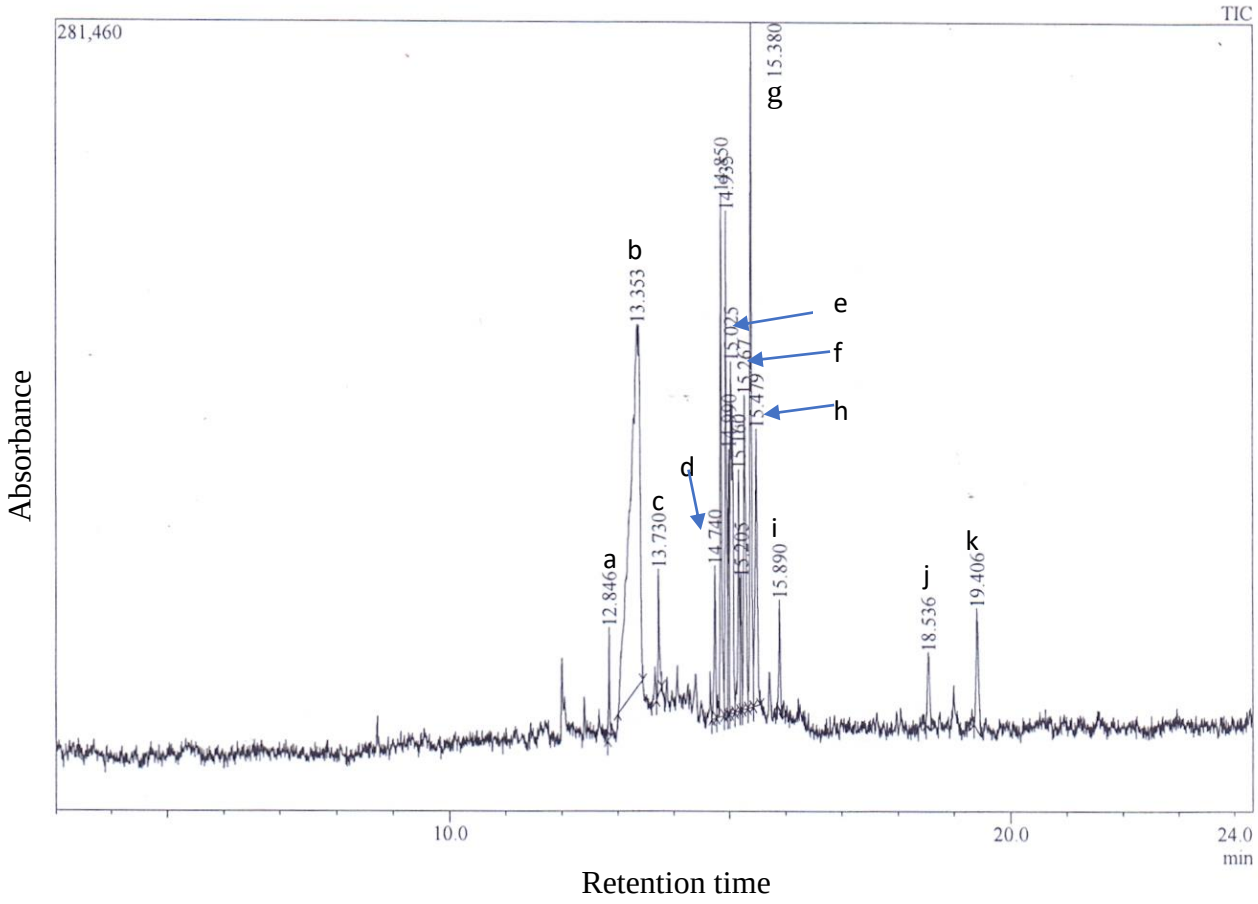

Figure 5.6: GC-MS chromatogram of a cold ethyl acetate sub-fraction obtained from methanolic extracts of *Protea caffra* twigs. a = Phenol 2,4-bis(1,1-dimethylethyl); b = Polygalitol; c = 1,3-Benzenediol, 4-propyl-; d = 1,2-Bis(p-acetoxyphenyl) ethanedione; e = 1-Adamantanecarboxylic acid, 2-propenyl; f = Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-; g = Phenol, 4-(1,1,3,3-tetramethylbutyl)-; h = Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-; i = 1-Nonadecanol; j = Phthalic acid, butyl tridecyl ester; K = 1-Heptacosanol.

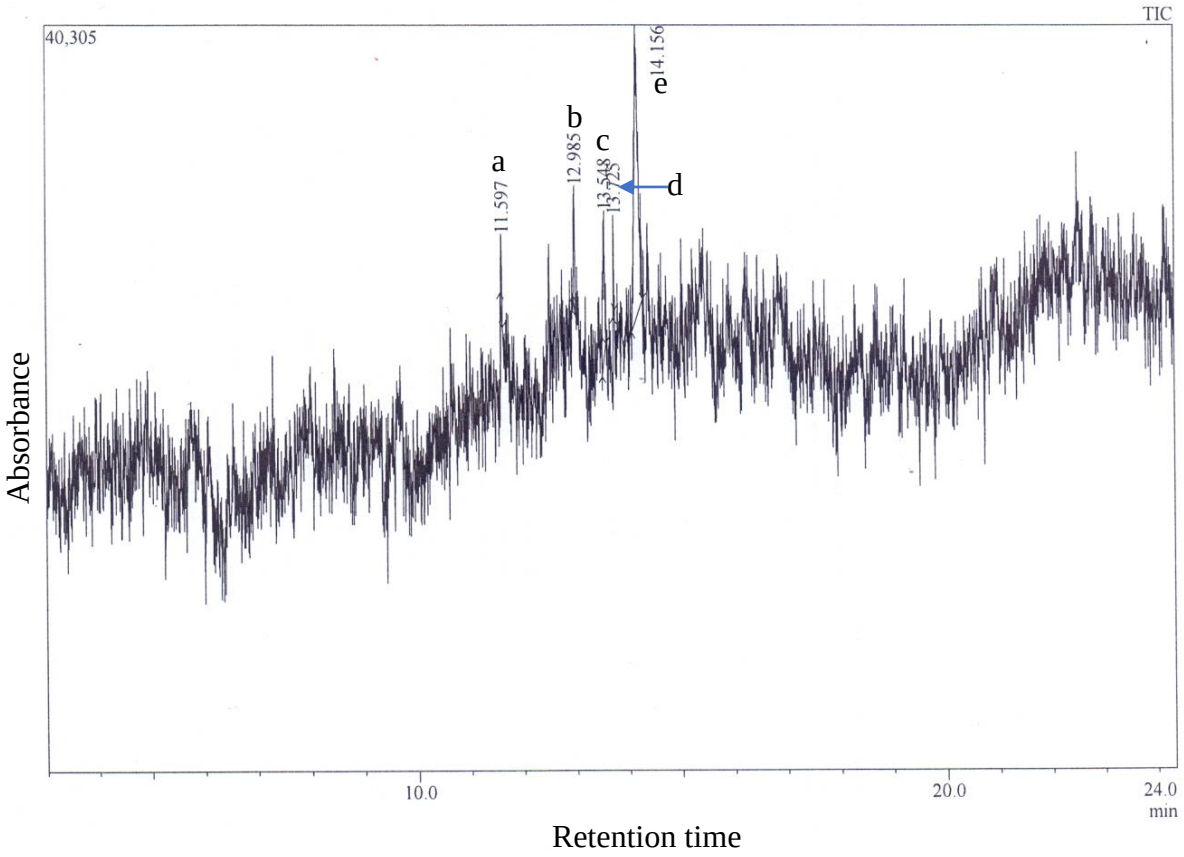

Figure 5.7. GC-MS chromatogram of a hot ethyl acetate sub-fraction obtained from methanol extracts of *Protea caffra* twigs. a = ß–Δ-Glucopyranose, 1,6-anhydro-, 1,6-anhydro-; b = Polygalitol; c = 1,3,5-Benzenediol, 4-propyl-; d = 1,3-Benzenediol, 4-propyl-; e = 1-Heptacosanol.

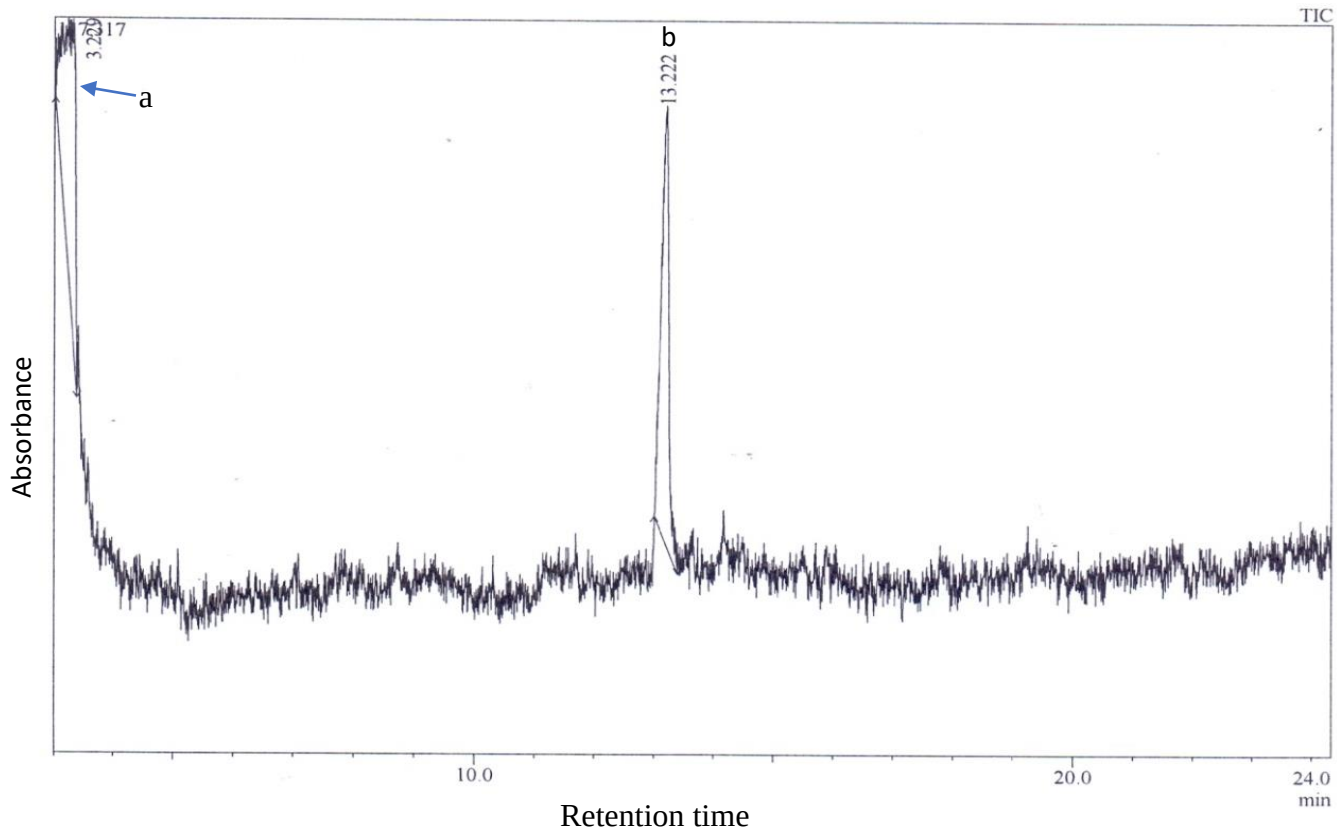

Figure 5.8: GC-MS chromatogram of an acetone sub-fraction obtained from methanolic extracts of *Protea caffra* twigs. a = Oxalyl acid; b = Polygalitol.

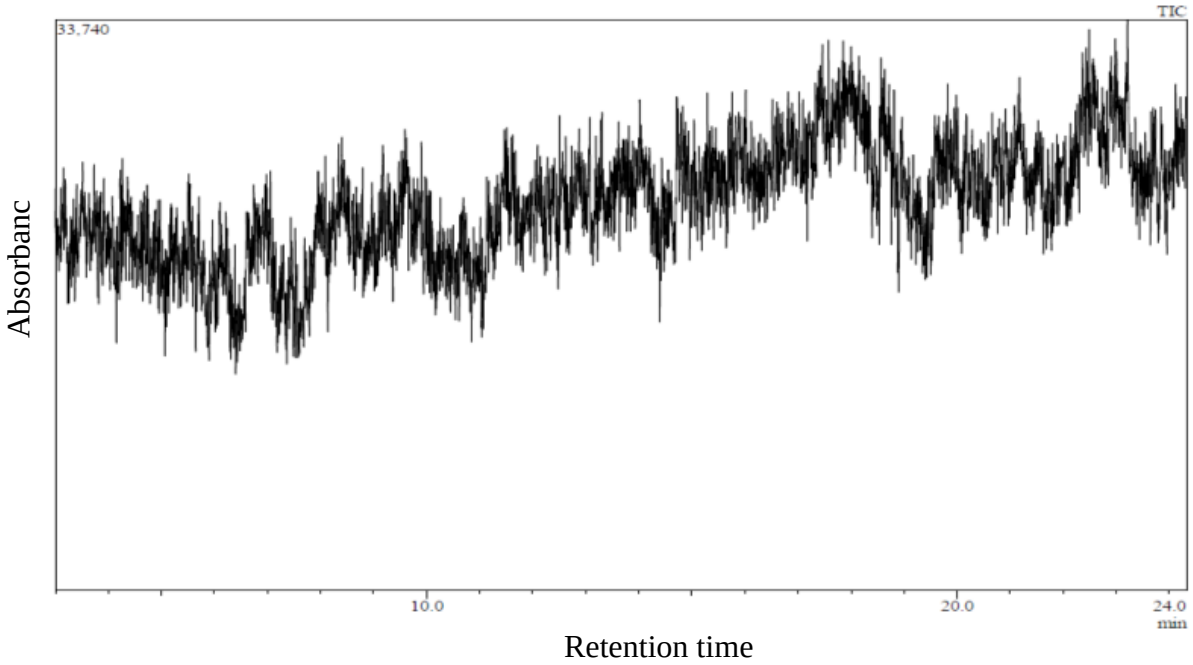

Figure 5.9: GC-MS chromatogram of a methanol sub-fraction obtained from methanol extracts of *Protea caffra* twigs.

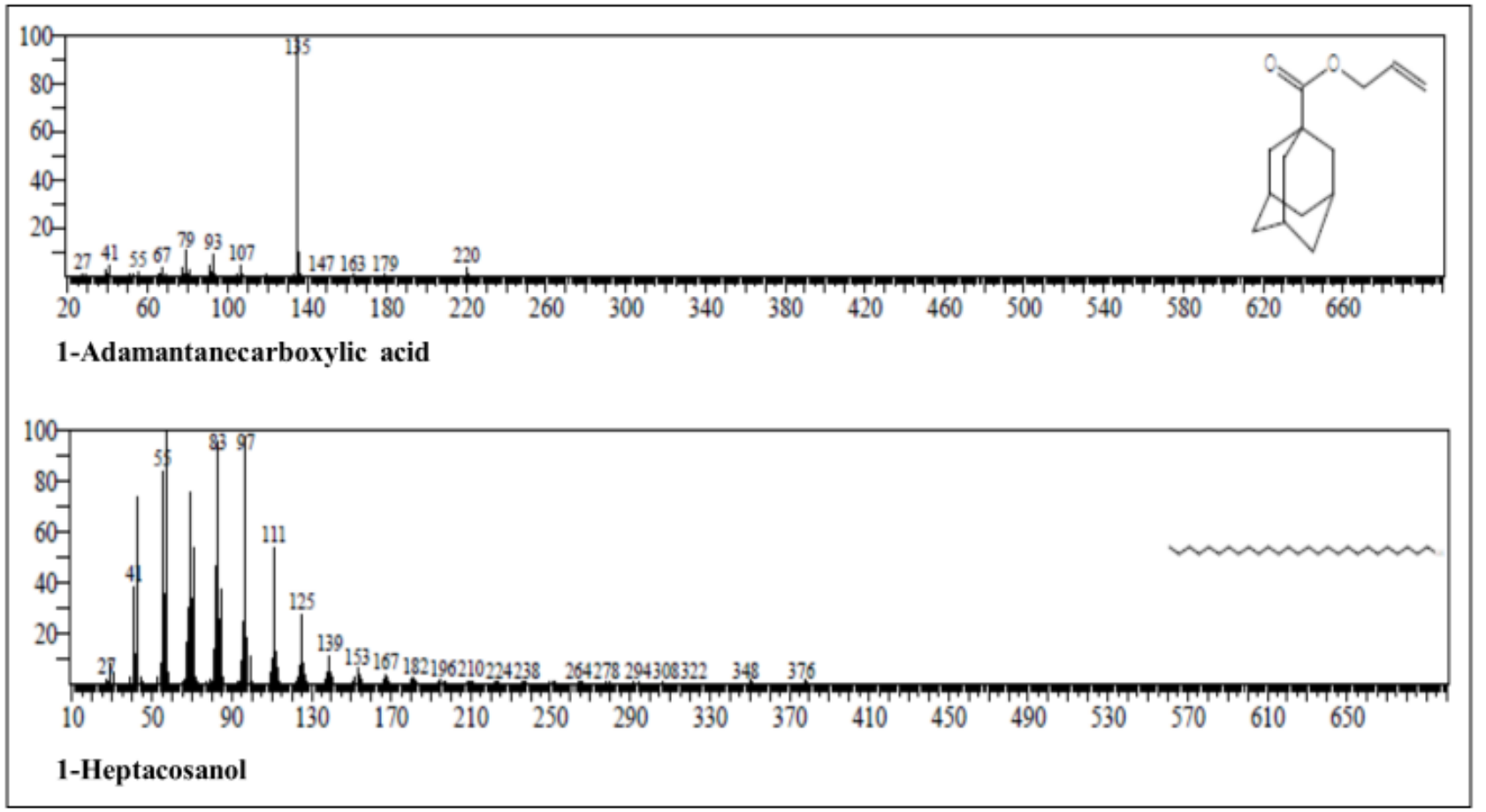

Figure 5.10: Mass spectra and chemical structures of antibacterial compounds (1-adamantanecarboxylic acid and 1-heptacosanol) putatively identified by GC-MS in methanolic *Protea caffra* extract sub-fractions.

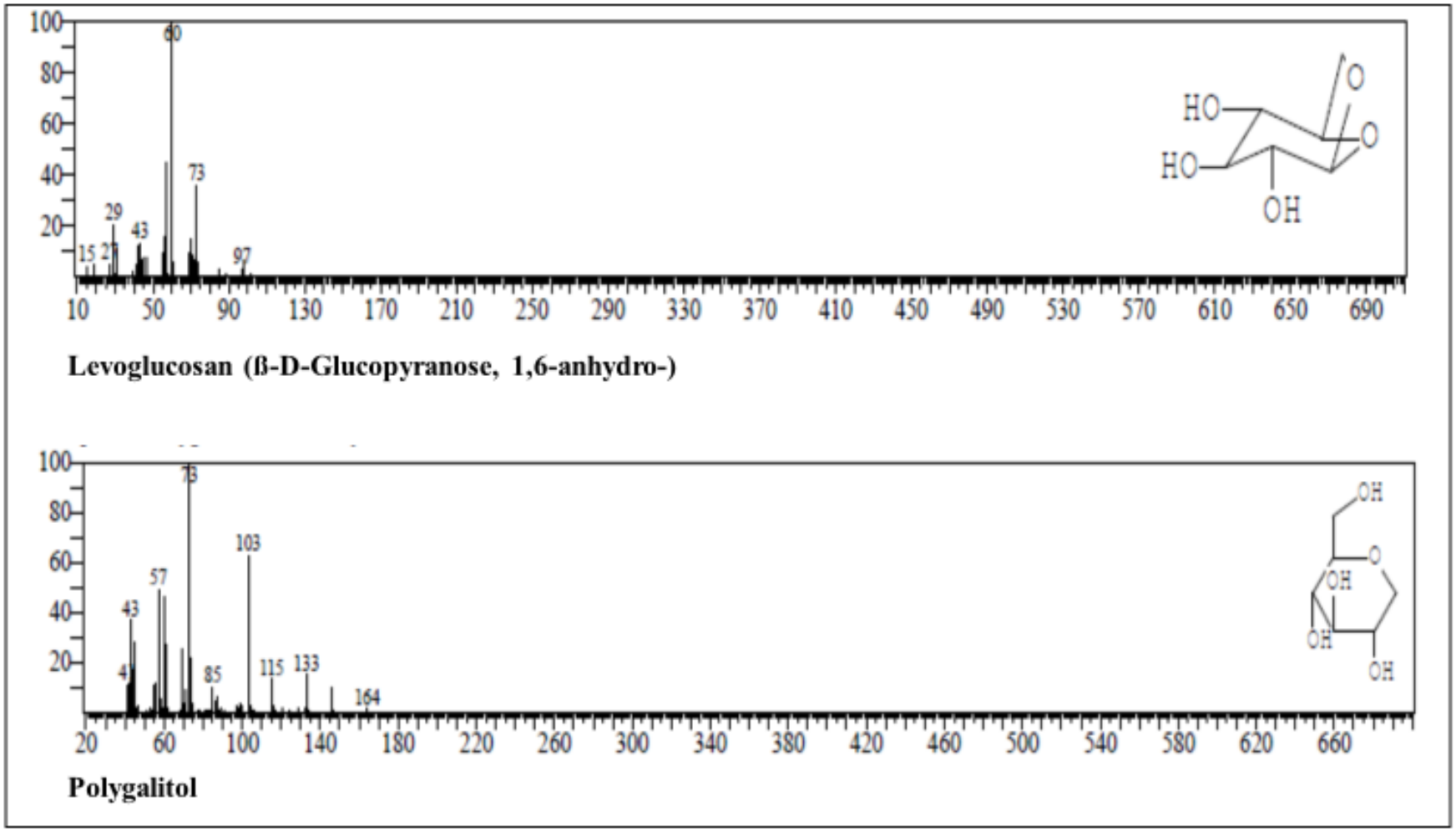

Figure 5.10: Continued (levoglucosan, polygalitol).

The hot ethyl acetate fraction consisted of 1-heptacosanol (70.57%), 1,3,5-benzenetriol (15.42), and polygalitol (7.31%) as its major components (Table 5.4). As already alluded to, 1-heptacosanol possesses potent antibacterial properties and since it was the major phytochemical constituent in this fraction, it was probably the one that inhibited the growth of both *E. faecalis* and *S. aureus* in the present investigation (Table 5.3). Polygalitol and other phytocompounds detected in this sub-fraction could also have contributed to the observed antibacterial activities. It was of great interest to note that *P. caffra* produces levoglucosan (Figure 5.10), an important source of C1-C10 and C1-C13 carbon skeletons used to produce the antibiotics erythromycin A and B (**KOCHETKOV et al., 1989; SVIRIDOV et al., 1990)**.

As shown in Table 5.4, only two compounds were identified in the acetone sub-fraction namely, oxalyl chloride (51.12%) and polygalitol (48.88%). Oxalyl chloride is a synthetic compound used in the oxidative processes involved in manufacturing antibiotics, pesticides, herbicides and other organic products (**ABUBAKER AND WANDRUSZKA, 1991; MAO et al., 2004)**. There are no indications in the current literature suggesting that this compound is produced by plants and that it is biologically active. Oxalyl chloride was therefore probably incorporated into *P. caffra* tissues from an external source. Further investigation would reveal which of the two compounds present in this fraction was responsible for the weak antibacterial activity observed against *S. aureus* (MIC $\geq$ 1.25 mg/ml).

5.4. Conclusions

The unprecedented evolution and global spread of drug-resistant bacterial strains pose a serious threat to global public health. New effective antibiotics are therefore urgently needed to prevent an era of untreatable bacterial infections. Due to their intricate chemical diversity, plants are potential rich sources of therapeutic drugs. The two compounds (compound **1** and compound **2**) isolated from the leaves of *S. lancea* in the present study exhibited promising inhibitory activities against *E. faecalis* and *S. aureus* (MIC range: 0.016-0.25 mg/ml). These findings substantiate the traditional use of *S. lancea* to manage fevers and diarrhoea. However, both compounds were isolated in low quantities (<1.5 mg) and hence could not as yet be unequivocally identified through 1-D and 2-D NMR spectroscopic data analysis. The study, however, provides important leads for future investigations which could be strengthened by the analysis of larger plant mass. If the isolated antibacterial compounds are novel, their structures could be used as models upon which new antibacterial agents could be designed. Due to its efficacy (0.016 mg/ml), compound **1** could be used directly as an active ingredient in some anti-infection pharmaceuticals. It is, however, imperative that the mode of action, safety, pharmacodynamic and pharmacokinetic potentials of both compounds be fully investigated to ascertain their clinical potentials.

GC-MS analysis revealed the presence of two antibacterial compounds (1-nonadecanol and 1-tetracosanol) in *S. lancea* leaves, which further justified the traditional use of this plant to treat bacterial infections. However, further studies are warranted to confirm the identity of these compounds using IR, NMR and X-ray data analysis. In addition, the pharmacological and toxicological properties of these compounds need to be thoroughly investigated.

Based on GC-MS, the *P. caffra* twigs methanol extract evaluated, consisted of 15 volatile compounds. Two of the detected compounds, 1-adamantanecarboxylic acid and levoglucosan, are often incorporated into antimicrobial moieties either as carbon skeletons or to improve the efficacy of the parent drug. The study also revealed that *P. caffra* is a potential source of two known antibacterial compounds namely 1-heptacosanol and 1-nonadecanol, and another compound, polygalitol, believed to possess antibacterial properties. Further studies are, however, warranted to determine the specific bioactive molecules responsible for the antibacterial properties of *P. caffra* twigs observed in the present investigation.

CHAPTER 6: GENERAL CONCLUSIONS

In light of the current widespread evolution of drug-resistant microbial strains, recent estimates predicts that by the year 2050, the leading cause of death globally would be infections caused by drug-resistant microbial mutants (**O'NEILL, 2014)**. The current state of affairs dictates an urgent need to develop new effective antibiotics, as well as to restore the efficacy of ineffective antibiotics. Natural products and their derivatives have attracted so much attention as potential sources of drug leads that could be effective in combating drug-resistance microbial strains. The current study was aimed mainly at identifying the principal antibacterial compounds in South African medicinal plants with potent and extended-spectrum antibacterial properties.

The seven medicinal plants (*Bolusanthus speciosus, Cucumis myriocarpus, Ekebergia capensis, Prunus africana, Protea caffra, Searsia lancea* and *Solanum panduriforme*) evaluated were selected based on their ethnomedicinal uses, pharmacological potentials and plant material availability. Aqueous and organic solvent extracts from these plants were subjected to antibacterial susceptibility testing, mutagenicity evaluations and phytochemical profiling to identify plants with the best pharmacological and phytochemical properties to consider for further phytochemical analysis. Subsequently, bioactivity-guided isolation techniques were used to isolate antibacterial compounds from the leaves of *S. lancea.* Spectroscopic data analysis revealed the chemical properties of the isolated compounds. The phytochemical constituents of an active *S. lancea* leaf ethyl acetate sub-fraction and that of a methanolic extract of *P. caffra* twigs were putatively identified using GC-MS.

The study revealed for the first time the antigonococcal properties of *P. africana* and *S. lancea* leaves. If traditional healers/herbalists could include these two plants in managing

gonorrhoea, that would not only help delay the evolution of antimicrobial-resistant gonococcal strains, but will also help conserve our biodiversity by preventing the overharvesting of the few medicinal plants currently being used to treat this venereal disease. The antibacterial compounds in these medicinal plants should, however, be identified and assessed for their potential use in mono- and combination therapies. This might in the long run help alleviate the global burden of gonorrhoea, which in more recent years has been linked with increased Human immuno-deficient virus (HIV) infection rates.

The current study also reaffirmed that there is a high-risk of obtaining pseudo-negative results when plant extracts are screened for antibacterial activities using the disk-diffusion technique. The microdilution bioassay was found to be much more sensitive in screening test plant extracts for antibacterial properties than the disk-diffusion assay.

The DCM bark extract from *B. speciosus* yielded the overall best antibacterial activity (MIC = 0.039 mg/ml against penicillin-resistant *S. aureus*) and demonstrated broad-spectrum antibacterial activities. *P. caffra* seeds (DCM extract) and twigs (MeOH extract), as well as *S. lancea* leaves (MeOH extract) also demonstrated broad-spectrum antibacterial activities. Interestingly, *B. speciosus*, *P. africana* and *S. lancea* exhibited promising activities against Gram-negative, MDR bacterial strains (*E. coli* and *K. pneumoniae*). By inference, clinically relevant broad-spectrum and potent antibacterial compounds effective against both drug-sensitive and multi-drug resistant (MDR) bacterial strains could potentially be obtained from medicinal plants.

There are growing concerns that the continued irrational use of antibiotics might cause several MDR bacterial strains to acquire advanced resistance and ultimately evolve into mutants

resistant to all known classes of antibiotics, a phenomenon known as extensive drug-resistance. To curb the development of advanced drug-resistance, concerted efforts should be made to discover antimicrobial agents effective against MDR bacterial strains from medicinal plants such as *B. speciosus*, *P. africana* and *S. lancea*. Given that these three plants belong to different taxonomic groups, chances are that their bioactive compounds are structurally diverse and each of them has a unique antibacterial mode of action. Future studies should therefore not only focus on identifying the antibacterial compounds in each of these medicinal plants, but consideration should also be given to elucidating their antibacterial mechanisms. Given that plants have an unlimited potential to produce chemically diverse secondary metabolites (**GURIB-FAKIM**, **2006)**, there are reasonable prospects of discovering novel classes of antibacterial agents from these natural resources.

Apart from discovering new antibacterial agents and novel classes of antibiotics from plants, the problem of antimicrobial-resistance could also be successfully tackled by discovering compounds that can reverse the effects of drug-resistance. In a bid to find these compounds, a preliminary study was conducted to assess the antibacterial synergistic interactions between different plant extracts, as well as between the plant extracts and antibiotics against MDR *E. coli* and MDR *K. pneumoniae*. A comparison of the checkerboard and time-kill results indicated that the former overestimated the degree to which chloramphenicol (antibiotic) synergistically interacted with three different plant extracts (*B. speciosus* MeOH bark extract, *P. africana* DCM and MeOH leaf extracts) against MDR *E. coli*. It is therefore recommended that all synergistic interactions detected within combinations of antibiotics and plant extracts by the checkerboard bioassay be re-affirmed using the more sensitive time-kill bioassays, as is customary in most clinical trials.

The chemo-diversity present in the plant kingdom provides exciting prospects of discovering antibiotic-resistance modifying phytocompounds and as such, searching for these compounds should become one of the top research priorities, especially in many African countries with a rich biodiversity. Scientific evaluations of synergistic combinations utilised in traditional medicine could open new avenues for novel pharmaceutical drug discovery. Using these natural products in combination therapies to potentiate the efficacy of some currently available antibiotics will undoubtedly prolong the era of successful antibiotic therapy. Additionally, this approach seems more financially appealing compared to developing new synthetic drugs that will have to undergo lengthy clinical trials prior to approval.

The test plant organs contained varying concentrations of total phenolics, condensed tannins, flavonoids, hydroxybenzoic and hydroxycinnamic acids. The extended-spectrum activities demonstrated by some extracts from *P. africana* leaves, *P. caffra* twigs and *S. lancea* leaves could be attributed to higher levels of phenolic compounds within these plant organs. However, even though the bark of *B. speciosus* demonstrated the best antibacterial activity in the current study, they contained very low quantities of almost all phenolic compounds assessed. It was interesting also to note that some plant organs such as the leaf, bark and flower of *P. caffra* and the leaf of *E. capensis* contained relatively higher levels of different phenolic compounds but did not yield noteworthy antibacterial activities (MIC > 1 mg/ml). Empirically, both the quantity and quality of phenolic compounds present in medicinal plant extracts seemed equally important in determining antibacterial efficacy and spectrum. Even though these two parameters are crucial, the possibility remains that different phenolic compounds synergised each other to effect the observed antibacterial activities, an area that future studies could possibly explore. It is also recommended that plant organs that did not demonstrate good antibacterial activities (MIC > 1 mg/ml) but contained higher levels of phenolic compounds

should be screened for other relevant pharmacological activities such as antioxidant, antiviral, antifungal, anti-inflammatory, antimutagenic and anticancer properties.

Based on the Ames test, all medicinal plant extracts that demonstrated noteworthy antibacterial activities in the present study were non-mutagenic against two *S. typhimurium* tester strains, TA98 and TA102 (without S9 metabolic activation). While this is an encouraging finding, further *in vivo* and *in vitro* toxicological studies are necessary before these medicinal plants are regarded entirely safe to use in both conventional and folk medicine.

The two compounds isolated from the leaves of *S. lancea* exhibited noteworthy inhibitory effects on *E. faecalis* and *S. aureus* (MIC: 0.016-0.25 mg/ml). However, since both compounds were obtained in low quantities (< 1.5 mg) their identity was not confirmed and it was also not possible to screen them for other pharmacological activities. Apart from structural elucidation and identifying them, it is imperative that their antibacterial mechanisms, pharmacodynamic and pharmacokinetic properties be determined to ascertain their clinical relevance.

GC-MS revealed the presence of 1-tetracosanol, 1-nonadecanol, eicosane, 1,7-di-*iso*-propylnaphthalene 1-pentadecanol, 2,6-di-*iso*-propylnaphthalene 1,3-*iso*-propylnaphthalene in *S. lancea* leaves. 1-Nonadecanol and 1-tetracosanol are known antibacterial compounds, which together with eicosane have been detected in several essential oils with antibacterial properties. The study therefore provides further evidence suggesting that *S. lancea* is a potential source of essential oils with therapeutic advantages. However, the antibacterial compounds in this medicinal plant still need to be unequivocally identified through spectroscopic data analysis. Furthermore, the toxic potentials of each of these compounds need to be thoroughly assessed.

GC-MS analysis also revealed the presence of 1-adamantanecarboxylic acid, 1-heptacosanol, levoglucosan, 1-nonadecanol and polygalitol in *P. caffra*. This is the first report on the presence of these pharmacologically important phytocompounds in *P. caffra*, an ornamental plant rarely used in folk medicine. Based on these findings, it is recommended that the other members of *Protea* species (> 300) be explored as potential sources of anti-infection and other therapeutic molecules.

Overall, the findings from this study underscore the important role that medicinal plants could play, if fully exploited, in addressing the triple-challenge of drug-resistance, looming pharmaceutical drug shortages and increasing human mortality rates.

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