Isolation and characterization of antibacterial compounds from a *Garcinia livingstonei* (Clusiaceae) leaf extract

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DECLARATION

I declare that the experimental work described in this thesis was conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. These studies are the results of my own investigation, except where the work of others is acknowledged and has not been submitted to any other University or research institution.

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LIST OF ABBREVATIONS

A549	Type of human lung carcinoma cells
ABTS+	2, 2´-azinobis-(3-ethylbenzothiaoline-6-sulphonic acid)
BEA	Benzene, ethanol, ammonia
CC_{50}	Cytotoxic concentration inhibiting the growth of 50% of the cultured cells
CEF	Chloroform, ethyl acetate, formic acid
CM	Chloroform methanol (9:10)
^{12}CNMR	Carbon Nuclear Magnetic Resonance
COX-2	Cyclooxygenase 2
DMSO d6	deuterated dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EMW	Ethyl acetate, methanol, water
EtOAc	Ethyl acetate
^{1}HNMR	Proton Nuclear Magnetic Resonance
INT	p-Iodonitrotetrazolium violet
JAK	Janus kinase
MeOH	Methanol
MEM	Minimal essential medium
MHZ	MegaHertz
MHB	Muller Hinton broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
R_f	Retention factor
SNP	Single nucleotide polymorphism
STAT 3	signal transducer and activator of transcription 3
TLC	Thin Layer Chromatography
Trolox	6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid
TEAC	Trolox equivalent assay concentration
UV light	Ultraviolet light
WM	Water methanol

ABSTRACT

Although pharmaceutical industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by infectious microorganisms has increased. For a long period of time, plants have been a valuable source of natural products for maintaining human and animal health. The use of plant compounds for pharmaceutical purposes has gradually increased worldwide. This is because there are many bioactive constituents in plants which hinder the growth or kill microbes. Plants could be considered a potential gold mine for therapeutic compounds for the development of new drugs.

In this study, sixteen South African plant species were selected based on their antibacterial activity after a wide screening of leaf extracts of tree species undertaken in the Phytomedicine Programme, University of Pretoria. Literature search excluded eleven plants because of the work already performed on their antibacterial activities, while *Pavetta schumaniana* was found toxic and thus not included in the screening. The remaining four plants namely; *Buxis natalensis, Macaranga capensis, Dracaena mannii* and *Garcinia livingstonei* were screened for antibacterial activity by determining the minimum inhibitory concentrations (MIC) against 4 nosocomial bacterial pathogens *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa*, and also by using bioautography. The extracts of *Macaranga capensis, Garcinia livingstonei, Diospyros rotundifolia* and *Dichrostachys cinerea* had good antibacterial activity with MIC values of 0.03, 0.04, 0.06 and 0.08 mg/ml against different pathogens. The average MIC values of the plant extracts against all the tested pathogens ranged from 0.23-1.77 mg/ml. *S. aureus* was the most susceptible bacterial pathogen with average MIC of 0.36 . The extract of *Diospyros rotundifolia* was the most active with an average MIC against all the organisms of 0.23 mg/ml. The extracts of *Buxus natalensis, Dracaena mannii,* and *Pittosporum viridiflorum, Acacia sieberiana, Erythrina lattissima, Cassine papillosa* and *Pavetta schumanniana* had lower antibacterial activity. *G. livingstonei* was selected for further work on the basis of its good activity.

The bulk acetone extract of *Garcinia livingstonei* (20g) was subjected to solvent-solvent fractionation which yielded seven fractions. Only the chloroform and ethyl acetate fractions showed good bioactivity in the microdilution assay and bioautography. Column chromatography was used to isolate two bioactive biflavonoids from the ethyl acetate fraction. The structures of the two compounds were elucidated using nuclear magnetic resonance (NMR) spectroscopy, and were identified as amentoflavone (**1**) and 4' monomethoxyamentoflavone (**2**). These two compounds have been

previously isolated from plants that belong to the Clusiaceae. The two compounds were isolated in sufficient quantity with a percentage yield of 0.45% for amentoflavone and 0.55% for 4' monomethoxyamentoflavone from 20 g crude acetone extract. The antibacterial activity was determined against four nosocomial bacterial pathogens (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa*). The MIC values ranged from 8-100 µg/ml. Except for *Staphylococcus aureus* which showed resistance to amentoflavone at >100 µg/ml. All the other tested organisms were sensitive to both compounds.

It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. Based on this, the antioxidant activities of the two isolated compounds were tested using the Trolox assay. The two flavones had good antioxidant activity. Amentoflavone had a Trolox equivalent antioxidant capacity (TEAC) of 0.9. The second compound 4' monomethoxyamentoflavone had a TEAC value of 2.2 which is more than double the antioxidant activity of Trolox, a vitamin E analogue.

To assess the safety of the two compounds on cell systems, cytotoxicity was determined using a tetrazolium based colorimetric assay (MTT assay) using Vero monkey kidney cells. The compounds indicated little to low toxicity against the cell line with cytotoxic concentration (CC_{50}) of 386 µg/ml and >600 µg/ml for compound **1** and **2** respectively. Berberine (used as the control toxic substance) had a CC_{50} of 170 µg/ml.

The Ames genotoxicity assay is used to assess the mutagenic potential of drugs, extracts and phytocompounds. The compounds isolated in this study were assayed for genotoxicity using the *Salmonella typhimurium* TA98 strain. Amentoflavone was genotoxic at the concentration of 100 µg/plate, but 4' monomethoxyamentoflavone was inactive at the highest concentration of 400 µg/plate tested.

The results of the antibacterial, antioxidant and cytotoxicity testing were encouraging and indicated the potential usefulness of *Garcinia livingstonei* in traditional medicine and drug discovery. However, the genotoxicity assay revealed potential mutagenic effects of amentoflavone, a compound isolated from the plant. Therefore, it is suggested that application of *Garcinia livingstonei* extracts in the treatment of human and animal ailments be done with caution to avoid mutagenic effects on the treated subjects.

A relatively small change in the structure of the two compounds by replacing an hydroxyl group with a methoxy group had a major effect in increasing antibacterial and antioxidant activity and in decreasing cellular and genotoxicity. This illustrates the potential value of modifying a molecule before its possible therapeutic use.

CONFERENCES AND PROCEEDINGS

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CHAPTER 1

1. INTRODUCTION

Plants have been used worldwide in traditional medicine for the treatment of diseases. It is estimated that even today approximately two-thirds to three-quarters of the world's population rely only on medicinal plants as their primary source of medicines (Suksamrarn *et al.,* 2003). Traditionally used medicinal plants produce a variety of compounds which may have therapeutic properties (Ahmad and Beg, 2000). The substances that can either inhibit the growth of pathogens or kill them and have no or low toxicity to host cells are considered candidates for developing new antimicrobial drugs. Many medicinal plants have provided pharmaceutical companies worldwide with new pharmaceuticals, significantly contributing towards the economic worth of the company.

Currently, the world is faced with a tremendous problem of pathogens with increased antimicrobial resistance due to the abusive and extensive use of antibiotics. Antimicrobial agents are often unaffordable or unavailable in developing countries and the toxicity of some antimicrobial agents is another factor contributing to this problem. Medicinal plants may provide new therapeutic solutions in the form of extracts or compounds which may be active against pathogens. They may also be less costly with lower toxicity. There is therefore justification to study the efficacy and safety of plant extracts.

1.1 LITERATURE REVIEW

1.1.1 Antibacterial drug resistance

Antimicrobial resistance is defined as the ability of a microorganism to withstand a normally active concentration of an antimicrobial agent and this phenomenon is reported frequently (Witte, 1998). The main cause of antimicrobial resistance is inappropriate use. This occurs when antimicrobials are taken for too short a time, at too low a dose, at inadequate potency, or for the wrong disease. Both overuse, such as through the over-prescribing of antimicrobials, which tends to occur in wealthier nations, and under use through lack of access, inadequate dosing, poor adherence, and poor quality drugs, play a role. For example, in some developing countries, antimicrobials can be purchased in single doses without a prescription. Economic hardship means that many patients will stop taking an antimicrobial as soon as they feel better, which may occur before the microbe has been eliminated (WHO, 2008). Antimicrobial resistance has also been postulated to have emerged from the use of antimicrobials in animals and the subsequent transfer of resistance genes and bacteria among animals and animal products and the environment (Nascimento et al., 2000). This is due to feeding animals with growth

promoters thus exposing bacteria to sub-lethal concentrations of drugs over long periods, which would appear conducive to selecting and maintaining resistant organisms (Burt and Reinders, 2003) Mechanisms of antimicrobial resistance have been earlier documented. Tenover (2006) published an excellent review on the mechanisms of antimicrobial resistance in bacteria emphasizing that bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to certain classes of antimicrobial agents. In such cases, all strains of that bacterial species may be resistant to all the members of those antibacterial classes. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent. Several types of antimicrobial resistance are readily spread to a variety of bacterial genera. Firstly, the organism may acquire genes encoding enzymes, such as ß-lactamase, that destroy the antibacterial agent before it can have an effect. Secondly, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Thirdly, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes (Tenover, 2006).

The last events mutation and selection may occur through one of several genetic mechanisms, including transformation, conjugation, or transduction. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to >3 antibacterial drug classes) have become a cause for serious concern, particularly in hospitals and other healthcare institutions where they tend to occur most commonly.

As noted above, susceptible bacteria can acquire resistance to an antimicrobial agent via new mutations (McManus, 1997), and such spontaneous mutations may cause resistance by:

(1) Altering the target protein to which the antibacterial agent binds by modifying or eliminating the binding site (e.g. change in penicillin-binding protein 2b in *pneumococci*, which results in penicillin resistance),

(2) Upregulating the production of enzymes that inactivate the antimicrobial agent (eg, erythromycin ribosomal methylase in staphylococci),

(3) Down regulating or altering an outer membrane protein channel which the drug requires for cell entry (eg, OmpF in *E. coli*), or

(4) Upregulating pumps that expel the drug from the cell (efflux of fluoroquinolones in *S. aureus*) (McManus, 1997).

In all of these cases, strains of bacteria carrying resistance conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution.

Bacteria also develop resistance through the acquisition of new genetic material from other resistant bacteria. This is termed horizontal evolution, and may occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation (McManus, 1997). For each of these processes; transposons may facilitate the transfer and incorporation of the acquired resistance genes into the genome of the host or into plasmids. During conjugation, a Gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium, often via an elongated proteinaceous structure termed a pilus, which joins the two organisms. Conjugation among Gram-positive bacteria is usually initiated by production of sex pheromones by the mating pair, which facilitates the clumping of donor and recipient organisms, allowing the exchange of DNA. During transduction, resistance genes are transferred from one bacterium to another via bacteriophage (bacterial viruses). This is now thought to be a relatively rare event (McManus, 1997). Finally, transformation, which is the process whereby bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis, can move resistance genes into previously susceptible strains.

Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antibacterial agents into their environment. Although a single mutation in a key bacterial gene may only slightly reduce the susceptibility of the host bacteria to that antibacterial agent, it may be just enough to allow its initial survival until it acquires additional mutations or additional genetic information resulting in full-fledged resistance to the antibacterial agent (McManus, 1997). However, in rare cases, a single mutation may be sufficient to confer high-level, clinically significant resistance upon an organism (e.g. high-level rifampin resistance in S *aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni*). Resistance via these mechanisms has led to the emergence of dangerous microbes. Today the only option for control or treatment is the use of conventional antimicrobials which are not effective due to resistance. Therefore, development of new antimicrobials from other sources such as bioactive plant metabolites will help in ameliorating these problems.

1.1.2 Potential of medicinal plants in drug discovery

Natural products, particularly secondary metabolites, have formed the basis of many medicines even though the purposes of these compounds in the plant are very often difficult to explain. It has been suggested that these compounds may be synthesized by the plant as part of the defense system of the plant, e.g. plants are known to produce phytoalexins as a response to attack by bacteria and fungi. Whatever the reasons for the presence of these compounds in nature, they provide invaluable resources that have been used to find and develop new drug molecules (Gurib-Fakim, 2006). Gurib-Fakim (2006) in her review explained that medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health. A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swelling and pain, phenolic compounds that can act as an antioxidant and venotonic, anti-bacterial and antifungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being. To date about 50 drugs have come from tropical plants (Gurib-Fakim, 2006). The existence of undiscovered pharmaceuticals for modern medicine has often been cited as one of the most important reasons for protecting tropical forests, and thus high annual extinction rate in this forests is a matter for concern, to say the least.

Furthermore, three of the major sources of anti-cancer drugs on the market or completing clinical trials were derived from North American plants used medicinally by Native Americans: the Papaw (*Asimina* spp.); the Western Yew Tree (*Taxus brevifolia*), effective against ovarian cancer and the May apple (*Podophyllum peltatum*) used to combat leukemia, lymphoma, lung and testicular cancer (Gurib-Fakim, 2006). A list of some plant-derived drugs is provided in Table 1. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use (Rates, 2000). Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2000). Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum.* More have been added to this list.

Africa, contains about a quarter of the higher plant species of the world (www,aamps.org) including a large medicinal plant flora. Famous African medicinal plants include *Acacia senegal* (Gum Arabic), *Agathosma betulina* (Buchu), *Aloe ferox* (Cape Aloes), *Aloe vera* (North African Origin), *Artemisia afra* (African wormwood), *Aspalathus linearis* (Rooibos tea), *Boswellia sacra* (Frankincense), *Catha edulis* (Khat), *Commiphora myrrha* (Myrrh), *Harpagophytum procumbens* (Devil's Claw), *Hibiscus sabdariffa*

(Hibiscus, Roselle), *Hypoxis hemerocallidea* (African potato) and *Prunus africana* (African Cherry) (Gurib-Fakim, 2006). Madagascar by herself has contributed with *Catharanthus roseus* (Rosy Periwinkle) and has the potential of contributing more in view of the diversity of her flora and fauna (Gurib-Fakim, 2006). The above-mentioned species have been sources of patented phytocompounds which are widely marketed today.

Table 1.1 Plants used in traditional medicine and which have given useful modern drugs (**Adapted from Gurib-Fakim, 2006)**

Botanical names	English name	Indigenous use	Origin	Uses in biomedicine	Biologically active compounds
Adhatoda vesica Nees (Acanthaceae)	-	Antispasmodic, antiseptic, fish poison	India, Sri lanka	Antispasmodic, oxytoxic, cough expectorant	Vasicin (lead molecu for Bromhexin and Ambroxol)
Catharanthus roseus L. (Apocyanaceae)	Periwinkle	Diabetes, fever, arrow poison	Madagascar, Brazil, Peru	Cancer chemotherapy, Muscular relaxation	Vincristine, Vinblasti D-Tubocurarine
Gingko biloba Linne	Gingko	Asthma, antihelmintics (fruits)	Eastern China	Dementia, cerebral deficiencies	Gingkolides
Harpagaphytum procumbens (Burch) de Candolie ex meissner (Pedaliaceae)	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, Rheumatism	Harpogosides, Caffe acid
Piper methysticum L. (Piperaceae)	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant	Kava pyrones
Podophylum peltatum L. (Berberidaceae)	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podophyllatoxin, lignans
Prunus africana Hook. F. (Rosaceae)	African plum	Laxative 'Old man's disease'	Tropical Africa	Prostate hyperplasia	Sitosterol

1.1.3 A perspective of some current drugs from medicinal plants

The search for new drugs from medicinal plants to replace ineffective or expensive drugs currently in use is a continuous process. In a review by Balunas and Kinghorn (2005) a perspective of drugs derived from medicinal plants that have been registered or are undergoing clinical trials have been highlighted. The path to identification of a worthy medicinal plant to isolation of pure effective compound(s) is a lengthy one. Therefore, numerous methods have been utilized to acquire compounds for drug discovery including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry, and molecular modeling (Ley and Baxendale, 2002; Geysen et al., 2003; Lombardino and Lowe, 2004). Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, and

particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs) (Newman et al., 2000, 2003; Butler, 2004).

In 2001 and 2002, approximately one quarter of the bestselling drugs worldwide were natural products or derived from natural products (Butler, 2004). There are also new medicinal plant derived drugs that have been recently introduced to the market. These include:

Arteether (trade name Artemotil) is a potent antimalarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine (TCM) (van Agtmael et al., 1999; Graul, 2001). Other derivatives of artemisinin are in various stages of use or clinical trials as anti-malarial drugs in Europe (van Agtmael et al., 1999).

Galantamine (also known as galanthamine, trade name Reminyl) is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* Losinsk. (Amaryllidaceae) in Russia in the early 1950s (Heinrich and Teoh, 2004; Pirttila et al., 2004). Galantamine is approved for the treatment of Alzheimer's disease, slowing the process of neurological degeneration by inhibiting acetylcholinesterase (AChE) as well as binding to and modulating the nicotinic acetylcholine receptor (nAChR) (Heinrich and Teoh, 2004; Pirttila et al., 2004).

Nitisinone (trade name Orfadin) is a newly released medicinal plant-derived drug that works on the rare inherited disease, tyrosinaemia, demonstrating the usefulness of natural products as lead structures (Frantz and Smith, 2003). Nitisinone is a modification of mesotrione, a herbicide based on the natural product leptospermone, a constituent of *Callistemon citrinus* Stapf. (Myrtaceae) (Hall et al., 2001b; Mitchell et al., 2001). All three of these triketones inhibit the same enzyme, 4-hydroxyphenyl-pyruvate dehydrogenase (HPPD), in both humans and maize (Hall et al., 2001b; Mitchell et al., 2001). Inhibition of the HPPD enzyme in maize acts as a herbicide and results in reduction of plastoquinone and tocopherol biosynthesis, while in humans the HPPD enzyme inhibition prevents tyrosine catabolism and the accumulation of toxic bioproducts in the liver and kidneys (Hall et al., 2001b)

Tiotropium (trade name Spiriva) has recently been released to the United States market for treatment of chronic obstructive pulmonary disease (COPD) (Mundy and Kirkpatrick, 2004; Frantz, 2005). Tiotroprium is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine that has been isolated from *Atropa belladonna* L. (Solanaceae) and other members of the Solanaceae family (Barnes et al., 1995; Dewick, 2002; Mundy and Kirkpatrick, 2004). Tiotropium has shown increased efficacy and longer lasting effects when compared with other available COPD medications (Barnes, 2002; Mundy and Kirkpatrick, 2004).

Modifications of existing natural products exemplify the importance of drug discovery from medicinal plants as NCEs and as possible new drug leads. The drugs discussed below are plant derived drugs

and all are in Phase III clinical trials or registration and are subtle modifications of drugs currently in clinical use (Butler, 2004).

M6G or **morphine-6-glucuronide** is a metabolite of morphine from *Papaver somniferum* L. (Papaveraceae) and will be used as an alternate pain medication with fewer side effects than morphine (Lotsch and Geisslinger, 2001). **Vinflunine** is a modification of vinblastine from *Catharanthus roseus* (L.) G. Don (Apocynaceae) for use as an anticancer agent with improved efficacy (Bonfil et al., 2002; Okouneva et al., 2003). **Exatecan** is an analog of camptothecin from *Camptotheca acuminata* Decne. (Nyssaceae) and is being developed as an anticancer agent (Butler, 2004; Cragg and Newman, 2004). **Calanolide A** is a dipyranocoumarin natural product isolated from *Calophyllum lanigerum* var. *austrocoriaceum* (Whitmore) P.F. Stevens (Clusiaceae), a Malaysian rainforest tree (Kashman et al., 1992; Yang et al., 2001; Yu et al., 2003). Calanolide A is an anti-HIV drug with a unique and specific mechanism of action as a non-nucleoside reverse transcriptase inhibitor (NNRTI) of type-1 HIV and is effective against AZT-resistant strains of HIV (Currens et al., 1996; Buckheit et al., 1999; Yu et al., 2003). Calanolide A is currently undergoing Phase II clinical trials (Creagh et al., 2001).

Natural products have played an important role as new chemical entities (NCEs)—approximately 28% of NCEs between 1981 and 2002 were natural products or natural product-derived (Newman et al., 2003). Another 20% of NCEs during this time period were considered natural product mimics, meaning that the synthetic compound was derived from the study of natural products (Newman et al., 2003). Combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981–2002. Natural products provide a starting point for new synthetic compounds, with diverse structures and often with multiple stereocenters that can be challenging synthetically (Clardy and Walsh, 2004; Nicolaou and Snyder, 2004; Peterson and Overman, 2004; Koehn and Carter, 2005). Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and number and ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts (Lee and Schneider, 2001; Feher and Schmidt, 2003; Clardy and Walsh, 2004; Piggott and Karuso, 2004; Koehn and Carter, 2005).

Furthermore, since the escalation of interest in combinatorial chemistry and the subsequent realization that these compound libraries may not always be very diverse, many synthetic and medicinal chemists are exploring the creation of natural product and natural-product like libraries that combine the structural features of natural products with the compound-generating potential of combinatorial chemistry (Hall et al., 2001a; Eldridge et al., 2002; Burke et al., 2004; Ganesan, 2004; Tan, 2004).

Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists. Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity

can provide important drug leads. Since the sequencing of the human genome, thousands of new molecular targets have been identified as important in various diseases (Kramer and Cohen, 2004). With the advent of high throughput screening assays directed towards these targets, known compounds from medicinal plants may show promising and possibly selective activity. Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets, as exemplified by indirubin, which selectively inhibits cyclindependent kinases (Hoessel et al., 1999; Eisenbrand et al., 2004) and kamebakaurin, which has been shown to inhibit NF-κB (Hwang et al., 2001; Lee et al., 2002).

Other known compounds have also been shown to act on novel molecular targets, thus reviving interest in members of these frequently isolated plant compound classes. Three examples are cucurbitacin I, obtained from the National Cancer Institute (NCI) Diversity Set of known compounds and found to be highly selective in inhibiting the JAK/STAT3 pathway in tumours with activated STAT3 (Blaskovich et al., 2003), h-lapachone, which selectively kills cancer cells over normal cells through direct checkpoint activation during the cell cycle (Li et al., 2003), and betulinic acid, with selective melanoma cytotoxicity through the activation of p38 (Pisha et al., 1995; Tan et al., 2003; Cichewicz and Kouzi, 2004).

1.1.3.1 Standardized plant extracts used in therapeutics of various ailments

The idea that a whole or partially purified extract of a plant has advantage over a single isolated constituent is not new, but until recently has not been investigated systematically and rationalized. Evidence to support the occurrence of synergism within phytomedicines is accumulating and recently reviewed (Williamson, 2000). Previous results in classical pharmacology, using mixtures of bioactive compounds, have shown that a differentiation between additive and synergistic over additive or potentiating effects is necessary. If two bioactive substances of a mixture have the same pharmacological targets, a pharmacologically synergistic effects may result which can be greater than expected for the individual substance taken together (provided no substance in the mixture exert an antagonizing effect). For several extracts or fractions, synergistic effects could be measured which exceeded the effect of single compounds, or mixtures of them at equivalent concentration (Williamson, 2000). Standardized herbal extracts have been used in treatments of human ailments with success. Herbal extracts from these genera and species are currently used in therapeutics; *Ginkgo, Hypericum, Crataegus*, Echinacea, *Allium sativum*, Valeriana, *Piper methysticum*, Sabal, Urtica, *Vitex agnus-castus*, Harpagophytum, Salix, *Aesculus spp, Silybum marianum*. Extracts of these plant species were found effective compared to isolated constituents. Clinical evidence has shown therapeutic superiority or equivalence of extracts over constituents isolated from them and also for the herbal drug combinations

over only one of the two combinations (Schmidt et al., 2001). Some herbal extracts with therapeutic equivalence with synthetic drugs is shown in Table 1.2.

Table 1.2. Standardized plant extracts with therapeutic equivalence to synthetic drugs (Adapted from Yaniv and Bachrach, 2005)

Herbal extract	Synthesized drug	Indication
Crataegus (Hawthorn)	Capotopril	Heart insufficiency, 1 +II NYHA
Hypericum (St. John's wort)	Imipramine Amitriptyline	Mild and moderate depression
Sabal (saw palmetto)	Proscar (finasteride)	Benign prostatic hyperplasia1 +II
Hedera helix (ivy)	Ambroxol	Chronic bronchitis
Boswellia (incense)	Sulfasalezin	Crohn's disease

From the information provided above it is clear that medicinal plants occupy a central area in the drug discovery process.

1.1.4 Medicinal plant use and phytochemical screening in South Africa

According to Newman et al. (2000), medicinal plants have always had an important place in the therapeutic armoury of mankind. Up to 80% of populations in developing countries are totally dependent on plants for their primary health care. The indigenous people of South Africa have a long history of traditional plant usage for medicinal purposes and cultural practices (Liebenberg, 2004). The Zulu, Xhosa and Sotho people of South Africa use approximately 3 000 plant species for traditional medicinal purposes and of these, some 350 species are the most commonly used and traded medicinal plants (Frusciante et al., 2000).

The use of traditional medicine as an affordable alternative by the majority of South Africans not only highlights the dire need for affordable treatments for several serious diseases, such as AIDS, cancer, diabetes and cardiovascular diseases, but can also serve as a shortcut for the discovery of new drugs from natural sources. The search for antimicrobial agents is actively being pursued due to the high incidence of drug resistance of many bacterial strains. The use of immunosuppressive drugs and the spread of AIDS have resulted in an increased occurrence of opportunistic systemic mycoses. About two thirds of AIDS patients in South Africa use traditional medicine to obtain symptomatic relief and manage opportunistic infections (UNAIDS, 2002).

From the vast source of indigenous knowledge, combined with enormous biodiversity found in South Africa, a large number of species have the potential to be screened for lead active compounds that can be used in pharmacological treatments of these serious diseases. In a review by Fennell et al. (2004),

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summaries of several studies are presented, where selected medicinal plants were screened for antibacterial, antifungal, anthelmintic, anti-amoebic, antischistosomal, antimalarial, anti-inflammatory and antioxidant activities in appropriate *in vitro* tests, and where the active compounds were sometimes isolated. In most of the studies, the results provided a degree of scientific validation for the use of plants in traditional medicine and led to the isolation of a large variety of bioactive compounds with the potential to be used as lead compounds in drug discovery.

The efforts of the Phytomedicine Programme (University of Pretoria, South Africa) have led to the screening, isolation and development of active compounds and extracts some of which were patented. In continuity with their screening work many extracts with, for example, antifungal, antibacterial and anti-babesial activity were evaluated (Masoko et al., 2007; McGaw et al., 2005; Naidoo et al., 2004). Details of these can be found at www.up.ac.za/phyto. Similar efforts exist in many South African Universities' medicinal plants research groups and biomedical research institutions in South Africa.

1.1.5 Economic importance of medicinal plants to South Africa

Documented evidence showed that more than 700 plant species are actively traded for their medicinal uses throughout South Africa (UNIDO, 2002). There are an estimated 27 million consumers of indigenous medicine in the country. Each household spends between 4 to 6 percent of annual income on indigenous medicine and services. Massive demand is generated in terms of number and volume of plant material. Plant products are marketed either self-medicated or as prescription products. These products are traded in residential areas dominated by black consumers or at transport nodes in urban areas (UNIDO, 2002).

Durban is the location of an important market in South Africa, and forms the hub of an active regional trade in indigenous medicine, popularly known as *muthi* plants. One thousand and five hundred tonnes of traditional medicine are sold annually, which are mainly plant parts and products. The industry is worth about US$ 0.22 million per year. Indigenous healers prescribe an annual 4 million products worth US$ 14.58 million (UNIDO, 2002). The most popular medicinal plants traded in this market are *Alepidea amatymbica* Eckl &Zeyh., *Bowiea volubilis* Harv. ex Hook f. *Curtisia dentata* C. A. Smith, *Eucomis autumnalis* (Mill.) Chitt, *Haworthia limifolia* Marloth, *Ocatea bullata* E.Mey, *Scilla natelensis* Planch, *Siphonochilus aethipicus* (Schweinf.) BI. Birtt. and *Warbugia salutaris* (Bertol. f) Chiov.

Similarly at Faraday market situated in central Johannesburg, traders sell 400 tonnes of herbal medicines annually at a turnover of US$ 0.52 million. Most of the material is harvested from the KwaZulu-Natal province and over 4 000 tonnes of medicinal plant material worth US$ 13 million are traded annually. In general the estimated value of medicinal plants traded in South Africa stood at US$ 48.02 million a year, and if all these values are extrapolated, the traditional medicine industry could be

generating up to US$ 220 million revenue. About 20 000 to 30 000 people derive their livelihood from medicinal plant trade. The majority of the people involved are black rural women. Therefore, the medicinal plant industry plays a critical role in empowering a large number of rural women, which is a means of poverty alleviation. Additionally, the revenue generated from medicinal plant markets in these provinces is relatively large.

In a similar vein, a report by Koehn and Carter (2005) has shown that worldwide pharmaceutical research and development (R & D) spending tripled roughly from US$ 10 to US$ 30 billion from 1984-2003. Basic procedures for the validation of drugs from plant origin in developing countries are urgently needed because many people residing in these countries rely on plants for their therapeutics needs. The isolation of bioactive compounds or potentizing of extracts from indigenous medicinal plants may enhance the production of new pharmaceuticals for the treatment of various infectious diseases.

1.1.6 Aims

The aim of this study was to screen selected South African plants for antibacterial activity, isolate and characterize antibacterial compounds and to evaluate the potential value in human and animal health.

1.1.7 Objectives of the study

The objectives of this study were:

1. To screen and evaluate antibacterial activity of leaf extracts of selected South African trees against the four most important nosocomial bacterial species (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Enterococcus faecalis)*.

2. To select the most promising plant species and to isolate the antibacterial compounds present.

3. To elucidate the structure of isolated bioactive compounds.

4. To determine the antioxidant activity of the isolated compounds.

5. To determine the cytotoxicity of the isolated compounds.

6. To determine the genotoxicity of the isolated compounds.

CHAPTER 2

PRELIMINARY SCREENING OF MEDICINAL PLANTS FOR ANTIBACTERIAL ACTIVITY

2.1 INTRODUCTION

There has been a renewed interest in medicinal plants research; and the pharmaceutical industry now considers plants as a viable option for the discovery of new leads. Among the estimated 250,000 plant species on the earth, only a small percentage has been phytochemically investigated; and the fraction subjected to biological or pharmacological screening is even smaller. Moreover, a plant extract may contain several thousand different secondary metabolites but any phytochemical analysis will reveal only a narrow spectrum of its constituents. The plant kingdom thus represents an enormous reservoir of pharmacologically valuable molecules to be discovered (Hostettmann et al., 2000). Searching for new drugs in plants implies screening of the extracts for the presence of novel compounds and an investigation of their biological activities. Suspected novel or bioactive compounds are generally isolated in order to elucidate the structure and to perform further biological and toxicological testing. The path that leads from an intact plant to its pure constituents is long.

However, for investigation of antibacterial activity, the process is relatively simple. For example, a plant extract is prepared to a known concentration and minimum inhibitory concentration (MIC) is determined as described (Eloff, 1998). This will enable the detection of the most promising plant species to continue investigating based on MIC and total activity results. In direct bioautographic methods, a nutrient medium is inoculated with microorganism and sprayed on a thin layer chromatography (TLC) plate containing separated plant extracts. The TLC plate is then incubated for 24 hours and sprayed with tetrazolium chloride salt (INT) and re-incubated for full colour development. An area of inhibition on the TLC is indicated by white spots against pink background (Hamburger and Cordell, 1987). The agar disc diffusion method is another approach used to investigate antimicrobial activity of plant extracts and isolated compounds, but this method has limitations; particularly with regard to dissolution of non-polar compounds into the agar medium.

For this research, sixteen plant species (Table 2.1) were selected based on a wide screening of the antibacterial and antifungal activity of leaves of tree species carried out in the Phytomedicine Programme (see www.up.ac/za) Traditional applications as anti-infective and antidiarrhoeal remedies were also considered. The follwing spp were selected: *Acalypha sonderana, Buxus natalensis, Cassine papillosa, Acacia sieberiana, Dracaena mannii, Dichrostachys cinerea, Diospyros rotundifolia, Erythrina*

lattissima, Garcinia livingstonei, Macaranga capensis, Nacepsia castaneifolia, Pittosforum viridiflorum, Pavetta schumanniana, Rhus leptodycta, Turrea floribudae and *Vittelariopsis marginata.*

2.2 MATERIALS AND METHODS

2.2.1 Collection and preparation of plant material

The plant leaves used for preliminary screening were collected from the Lowveld National Botanical Garden in Nelspruit, Mpumalanga. Plant material was milled to a powder and kept in a cupboard in airtight containers in Phytomedicine laboratory at the University of Pretoria Onderstepoort until used. The plant species used in this study are listed below:

Table 1.3 Sixteen plant species used in preliminary screening for antibacterial activity

Plant species	Families	Traditional uses	References
Acacia sieberiana	Fabaceae	Decoction used as astringent, haemostatic, diarrhoea, opthalmia.	Pujol, 1990
Acalypha sonderana	Euphorbiaceae	Antifungal and antibacterial.	Alade and Irobi, 1993
Buxus natalensis	Buxaceae	Wood for making home pillars	
Cassine papillosa	Celastraceae	Used to clean digestive tract.	Pujol, 1990
Dichrostachys cinerea	Mimosaceae	Diarrhoea, abdominal pain, snake and scorpion bites. Leaves are used for leprosy. Fruits used to treat sores and scabies.	Hutchings et al., 1996
Diospyros rotundifolia	Ebenaceae	Against irritating rash	
Dracaenia mannii	Dracaenaceae	Skin diseases	Okunji,1999
Erythrina lattissima	Papilionaceae	Purgative	AEB Dhlodhlo pers. comm.
Garcinia livingstonei	Clusiaceae	Bark as traditional medicine, powdered root as aphrodisiacs, root decoction taken for abdominal pain during pregnancy, fruits and stem used for coughs, fevers and parasitic diseases.	Cunnigham, 1998,
Macaranga capensis	Euphorbiaceae	Zulu people used the bark of *M. capensis* to treat skin diseases and sunburn	Pujol, 1990
Nacepsia castanafolia	Not found	Not found	Not found
Pavetta schumaniana	Rubiaceae	Toxic to livestock	Kellerman et al., 1988
Pittosporum viridiflorum	Pittosporaceae	Decoction from bark used for febrile complaints,emetics and enemas, for stomach troubles, roots and bark decoction as aphrodisiacs, chest pain and dizziness.	Gelfand et al., 1985
Rhus leptodycta	Anacardiaceae	Anti-inflammatory	
Turrea floribudae	Meliaceae	Emetics, swollen and painful joints	Pujol, 1990
Vittielariopsis marginata	Sapotaceae	Against poisoning, treat indigestion, sexual stimulant.	Pujol, 1990

Source: Hutchings *et al.*, 1996

Of the sixteen species, literature search excluded eleven because of the extensive work done on them including antibacterial activity studies, and one plant (*Pavetta schumanniana)* was highly toxic. The remaining four plant species, i.e. *Buxus natelensis, Dracaena mannii, Macaranga capensis and Garcinia livingstonei* were used for preliminary screening to select one species for further studies.

2.2.2 Extraction

Separate aliquots of 3 g of the powdered leaves of the four selected plant species were extracted with 30 ml acetone. The tubes were shaken on a Labotec shaking machine for an hour and the supernatant was filtered through Whatman No. 1 filter paper into pre-weighed glass vials and placed under a stream of cold air to dryness. After drying, the weight of each extract was determined.

2.2.3 TLC fingerprinting

Plant extracts were resuspended in acetone to give a concentration of 10 mg/ml. Aliquots of 10 µl were loaded onto aluminum-backed thin layer chromatography (TLC) plates (Silica gel 60 F254, Merck) and developed in three mobile systems of differing polarity as described elsewhere (Kotzé and Eloff, 2002). The mobile systems used were as follows:

Benzene:ethanol:ammonia (18:2:0.2) (BEA, non polar)

Chloroform: ethyl acetate: formic acid (10:9:2) (CEF, intermediate polarity)

Ethyl acetate: methanol: water (EMW, 10:1.35:1) (polar).

The developed TLC plates were visualized under UV light at 254 nm and 365 nm to detect UV absorbing or fluorescing bands. The plates were then sprayed with vanillin sprayreagent (0.1g vanillin dissolved in 28 ml methanol, add 1 ml sulphuric acid) and heated at 110ºC to optimal colour development.

2.2.4 Bacterial cultures

Bacterial cultures of *Escherichia coli* (ATCC 27853), *Pseudomonas aeruginosa* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *Enterococcus faecalis* (ATTC 21212) were obtained from the Microbiology laboratory (Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria). The cultures were maintained on Müller Hinton (MH) agar at 4ºC and were inoculated in MH broth at 37ºC and incubated for 18 hours prior to bioautography and microdilution assays.

2.2.5 Bioautography assay of the extracts

TLC plates prepared as described in section 2.2.3, but not sprayed with vanillin spray reagent, were left for four days under an air stream to allow the TLC solvent to evaporate and dry from the plates before being sprayed with an actively growing culture of bacteria. The chromatograms were then incubated for 24 hours at 37°C under 100% relative humidity to allow the microorganism to grow on the plates. After overnight incubation the bioautograms were sprayed with an aqueous solution of 2 mg/ml p-iodonotrotetrazolium violet (INT, Sigma) and incubated for 24 hours for colour development. The clear zones against a red background indicate inhibition of bacterial growth by bioactive compounds in the extract (Hamburguer and Cordell, 1987). A set of TLC plates sprayed with vanillin was used as reference chromatograms for the bioautography plate's areas of inhibition. The Rf values of active zones were correlated with those of bands on the reference chromatograms.

2.2.6 Microdilution assay

The two-fold serial dilution microplate method as described by Eloff (1998) was used to determine the MIC values of plant extracts. Residues of plant extracts were resuspended in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) in triplicate for each experiment were serially diluted two-fold with water in 96-well microtitre plates. A 100 µl aliquot of test bacterial culture was added to each well. Acetone was used as a solvent control and distilled water was used as a negative control. Gentamicin (0.1 mg/ml) was the positive control. As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in distilled water was added to each well and the covered microtitre plates were incubated at 35°C overnight to ensure adequate colour development. The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a formazan product by biologically respiring active organisms (Eloff, 1998).

The MIC values were read after 1 hour and 24 hours after adding INT to the wells. Where bacterial growth is completely inhibited (bactericidal), the solution in the well remains clear after incubation with INT, but inhibition of growth is measured as the smallest first concentration of plant extract that causes a decrease in colour intensity indicated by the formazan salt (inhibitory concentration). The experiment was repeated three times to confirm the results, and three replicates were included in each experiment.

2.2.7 Total activity

Eloff (2000) reported that not only MIC but also the quantity extracted should be taken into account to compare the activity of different plants. This is indeed very important if the extracts are to be used in traditional medicine. Total activity indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit the growth of the tested microorganism. This takes into account the quantity extracted from plant material and is calculated as follows:

Total activity = quantity extracted (mg/g)/ MIC value (mg/ml). The unit of total activity is ml/g. The higher the total activity of a plant extract, the more effective the original plant is (Eloff, 2000). If the total activity is calculated at each step of a bioassay-guided fractionation procedure it is easy to determine if there is a loss of biological activity during isolation, and also synergistic effects can be discovered. This situation is equivalent to the terms efficacy and potency used in pharmacology (Eloff, 2004).

2.3 RESULTS AND DISCUSSION

2.3.1 Quantity extracted

The quantity extracted and the extractive yield from 3 g of each plant species is presented graphically in Figure 2.1 and in Table 2.2 respectively. From the graphical data it can be seen that *Garcinia livingstonei* had the highest percentage extractive yield compared with the investigated other plant species. The lowest extractive yield was obtained with *Buxis natalensis.* Similarly, the quantity extracted as well as total activity for each species is presented in Table 2.2. The quantity extracted in *Garcinia livingstonei* was the highest, 299 g representing 8.97%, while *Buxis natalensis* extracted the lowest, 87 g representing only 2.61%.The results suggest that *Garcinia livingstonei* is a good species to further investigate compared to the other plant species.
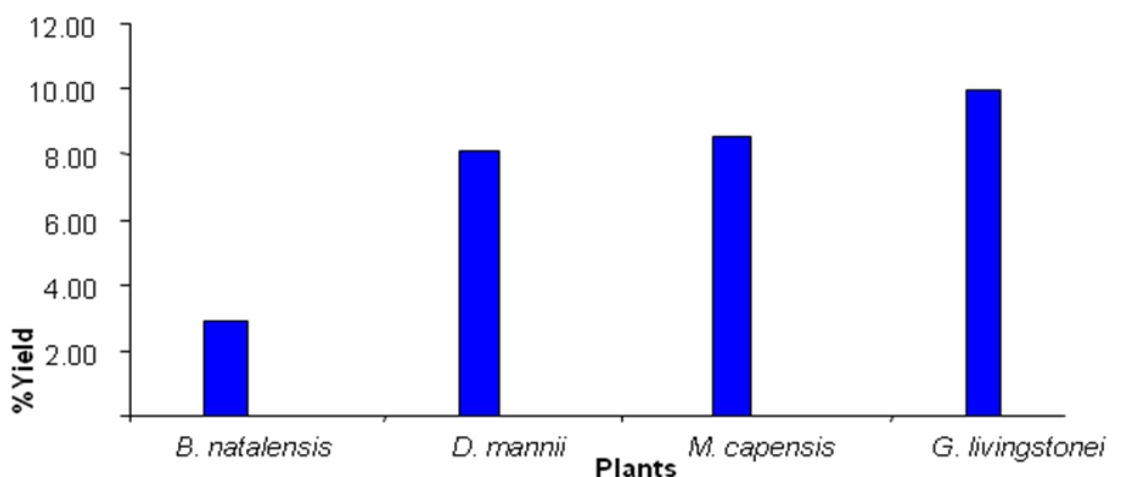
Figure.2.1 Percentage extractive yield from 3 g powdered leaves of four plant species extracted with acetone as solvent

Table 2.1 Total activity of four plant species extracted in acetone against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Enterococcus faecalis*

Plant species	Quantity extracted (mg)	Average MIC (mg/ml)	Total activity (ml/g)
Buxus natalensis	87.00	1.80	48.33
Macaranga capensis	256.00	0.60	426.66
Garcinia livingstonei	299.00	0.53	564.15
Dracaenia mannii	243.00	0.92	264.13

The total activity of *G. livingstonei* was the highest nd it indicated that the active material in one gram of the plant when diluted to 564.15 ml can still inhibit the growth of the organism.

2.3.2 TLC fingerprinting

The results of the TLC fingerprinting are presented in Fig 2.2. Three mobile phase systems, namely benzene/ethanol/ammonia hydroxide (18:2:0.2) [BEA], chloroform/ethyl acetate/formic acid (5:4:1) [CEF] and ethyl acetate/methanol/water (40:5.4:5): [EMW] were used in separating the compounds based on their polarities. Good separation was observed in the CEF mobile phase, followed by EMW and BEA. Therefore CEF was considered the system of choice for separation of compounds for further work in this project.
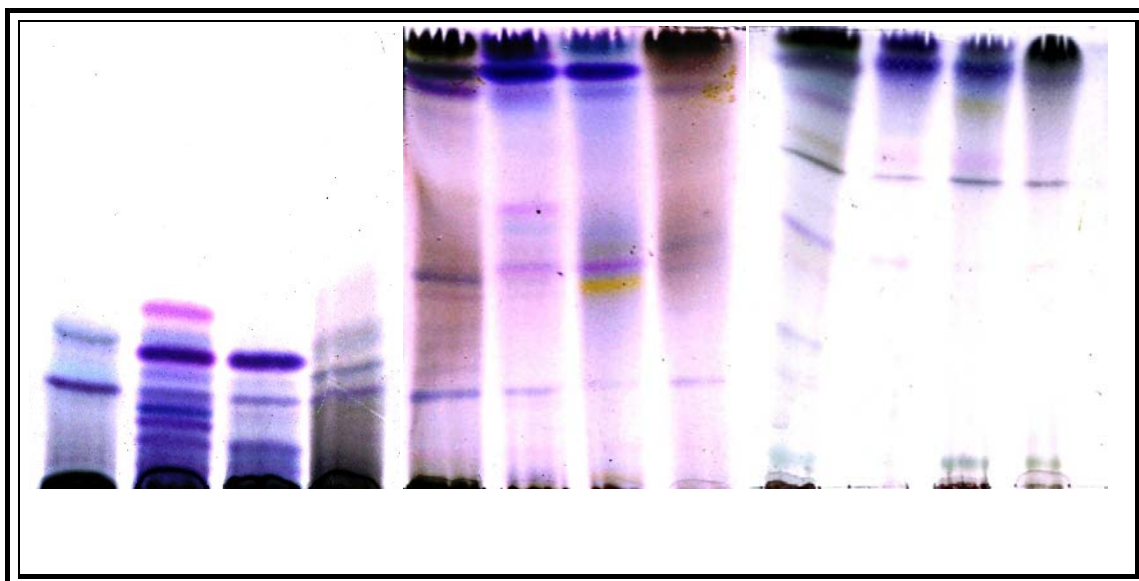
Figure 2.2 TLC chromatograms four plant species (**left to right**) *Buxus natelensis* (**B**), *Macaranga capensis* (**M**), *Garcinia livingstonei* (**G**) and *Dracaena mannii* (**D**) extracted with acetone and developed in benzene/ethanol/ammonia hydroxide (18:2:0.2) [BEA], chloroform/ethyl acetate/formic acid (5:4:1) [CEF] and ethyl acetate/methanol/water (40:5.4:5): [EMW] (**left to right**), sprayed with vanillin sulphuric acid in methanol.

2.3.3 Bioautography

Bioautography is a technique used to detect bioactive compounds based on their localization on developed TLC plates which have been sprayed with bacteria and a detection reagent such as INT. From the results obtained so far, inhibition of bacterial growth was evident in extracts of *Garcinia livingstonei* against all the tested microorganisms (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis*). However, other plants like *Macaranga capensis* showed activity zones only against *Pseudomonas aeruginosa, Enterococcus* faecalis and *Staphylococcus aureus.* Compounds in other plants species like *Buxis natelensis* and *Dracaena mannii* were not active and did not show inhibitory activity against the tested organisms (Fig 2.3). It is interesting to note that the bands which appeared yellow on the TLC fingerprints of *Garcinia livingstonei* are the bioactive compounds in this plant following comparison of the Rf values
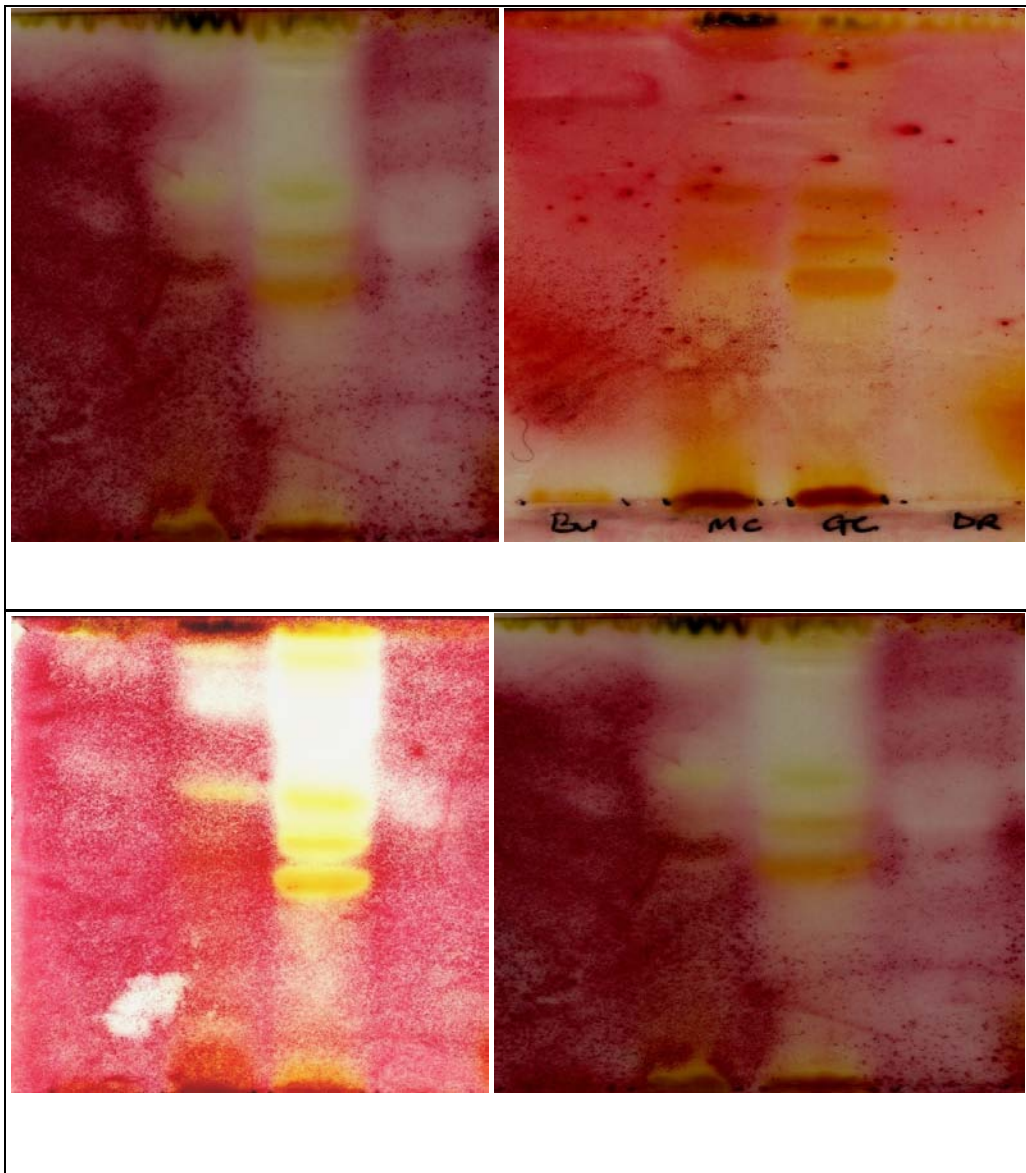
Figure 2.3. Bioautograms of *Buxus natelensis* (**B**), *Macaranga capensis* (**M**), *Garcinia livingstonei* (**G**) and *Dracaena mannii* (**D**) developed in chloroform/ethyl acetate/formic acid (5:4:1) [CEF] and sprayed with actively growing cultures of [A] *Escherichia coli,* [B] *Pseudomonas aeruginosa,* [C] *Staphylococcus aureus* and [D} *Enterococcus faecalis,* INT (*p*-iodonitrotetrazolium violet) solution was sprayed 24 hours after incubation. Clear/yellow zones on chromatograms indicate bacterial growth inhibition.

2.3.4 Microdilution assay

The MIC values of the four plant extracts against the four test bacteria after 1 hour and 24 hours of incubation are shown in Table 3.1. The results showed that all tested organisms were susceptible to the plant extracts in varying concentrations. Although only acetone as a solvent was used in the extraction of plant materials, the choice of this solvent was informed by its low toxicity to the tested pathogens among other criteria (Kotze and Eloff, 2002).

Based on susceptibility of the tested organisms to various extracts, the most susceptible organism was *Enterococcus faecalis* with an average MIC value of 0.35 mg/ml, followed by *Staphylococcus aureus* with average MIC value of 0.5 mg/ml, *Escherichia coli* 0.64mg/ml and *Pseudomonas aeruginosa* 1.25 mg/ml after 1 hour of incubation. Increases in MIC values were observed in *Enterococcus faecalis* and *Escherichia coli* from 0.35 to 0.75 and 0.64 to 2.5 mg/ml respectively after 24 hours of incubation. The MIC values in *Staphylococcus aureus* and *Pseudomonas aeruginosa* did not increase from the previous values even after 24 hours of incubation. Therefore, the results imply that MIC values of 0.5 and 1.25 mg/ml observed against *Escherichia coli and Pseudomonas aeruginosa* respectively which did not increase after 24 hours of incubation were likely bactericidal. In contrast, the MIC values of 0.35 and 0.64 mg/ml recorded against *Enterococcus faecalis* and *Escherichia coli* which increased to 0.75 and 2.5 mg/ml might reflect bacteriostatic activity. *Garcinia livingstonei* had the lowest average MIC of 0.53 mg/ml of all the plant extracts tested (Table 2.2) and a high total activity of 564.15 ml/g (Table 2.1). This is the measure of effectiveness and quantity of antimicrobial compounds present in a plant extract (Eloff, 2004). These results have placed *Garcinia livingstonei* as the most active plant to work with compared to the other species tested.

Table 2.1 Average MIC values in mg/ml of four plant species against four nosocomial bacteria (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Enterococcus faecalis)*

Microorganisms	Time (h)	1	2	3	4	Average	Gentamicin (µg/ml)
Escherichia coli	1	1.25	0.63	**0.06**	**0.06**	**0.50**	0.06
	24	2.5	2.5	2.5	2.5	**2.5**	0.03
Staphylococcus aureus	1	1.25	**0.03**	**0.07**	0.63	**0.5**	0.05
	24	1.25	0.16	0.16	0.31	**0.47**	2.08
Pseudomonas aeruginosa	1	1.25	0.63	0.63	1.25	**0.94**	0.63
	24	2.5	0.63	0.63	1.25	**1.25**	0.63
Enterococcus faecalis	1	0.63	**0.09**	**0.04**	0.63	**0.35**	0.16
	24	2.5	0.16	0.16	0.16		
	Average	1.64	0.60	0.53	0.85		

1=*Buxus natalensis*, 2= *Macaranga capensis*, 3= *Garcina livingstonei*, 4= *Dracaena mannii*

2.4 CONCLUSION

Only acetone was used as the initial extracting solvent for the extraction of plant constituents because of its relative non toxicity to the test organisms used, and because it extracts a range of polar and non-polar compounds. This solvent extracted many compounds from the plant extracts in this study as shown by TLC. The bioautography analysis, the MIC values and the total activity indicated that there are bioactive compounds present in *Garcinia livingstonei* that can be isolated and identified.

All the extracts were active on the organisms tested after 1 hour in *in vitro* microdilution assay with MICs as low as 0.03 mg/ml. In the case of *E. coli* the activity was only bacteriostatic.

Only *Macaranga capensis* and *Garcinia livingstonei* showed activity in bioautography. The activity of *Garcinia livingstonei* was more pronounced than *Macaranga capensis*. There was little correlation between the MIC values and bioautography in some plant species. This may be due to the escape of volatile compounds from chromatograms during removal of the solvent, and/or decomposition of unstable compounds. Based on the results *Garcinia livingstonei* was selected for further investigation.

CHAPTER 3

FRACTIONATION AND ISOLATION BIOACTIVE COMPOUNDS FROM *GARCINIA* LEAF EXTRACTS

3.1 INTRODUCTION

Garcinia is a large genus of polygamous (male and female flowers on the same plant) trees or shrubs, distributed in tropical Asia, Africa and Polynesia belonging to the family Clusiaceae. There are two genera in this family, *Hypericum* and *Garcinia* (Hutchings et al., 1996). *Garcinia* is a rich source of bioactive molecules including xanthones, flavonoids, benzophenones, lactones and phenolic acids (Selvi et al, 2003). For the last several years, small and complex molecules have been isolated from various species of *Garcinia*, including xanthones and xanthone derivatives (Bennet and Lee, 1989; Minami et al., 1994). A polyisoprenylated benzophenone, garcinol, is present in members of the Guttiferae (Krishnamurthy et al., 1981; Gustafson et al., 1992; Williams et al., 2003). (–)-Hydroxycitric acid is found in the fruit rinds of *G. cambogia*, *G. indica* (Jayaprakasha and Sakariah, 1998, 2002) and *G. cowa* (Jena et al., 2002).

Garcinia is the largest genus of the Clusiaceae family with about 400 species widely distributed in tropical Asia, Africa, New Caledonia and Polynesia (Waterman, 1986). *Garcinia* species are known to be rich in oxygenated and prenylated phenol derivatives (Peres et al., 2000; Bennett and Lee, 1989), some of them exhibiting biological activities such as antifungal (Sordat-Diserens et al., 1991), anti-inflammatory (Gopalakrishnan et al., 1980), antioxidant (Hay et al., 2004) and antitrypanosomal (Abe et al., 2003).The leaves and seeds of other species like *Garcinia dulci* have been traditionally used against lymphatitis, parotitis, struma and other disease conditions (Kasahara and Henmi, 1986). HIV-inhibitory prenylated benzophenone, guttiferone A (Gustafson et al., 1992) has been isolated from *Garcinia livingstonei.*

Garcinia livingstonei (African mangosteen) is a small to medium-sized tree that produces edible fruits and grows at low altitudes (Zakaria et al., 2006). It is found, particularly in South Africa, in riverine fringes and in open woodland. Extracts of the leaves and flowers are reported to exhibit antibiotic properties (Diserens et al., 1992). In other parts of East Africa like Tanzania, the plant thrives in riverine forests and in open woodland at low altitudes (0-800 m), often under larger trees in this region (Jones et al., 1996). The fruit of *G. livingstonei* are edible (Mbuya et al., 1994).

Since we have shown that *G. livingstonei* gave low inhibiting activity on four tested bacterial pathogens and high total activity as well as high percentage extractive yield observed in the preliminary screening (Chapter 2), therefore we selected it for further study. Bioautograms of *G. livingstonei* extract also showed zones of growth inhibition on the plates against the four organisms tested. These results led to

the selection of this species for further antibacterial research work. Photographic images of *G. livingstonei* are depicted in Fig.3.1

In order to discover new bioactive compounds from plant sources which could become new leads or new drugs, extracts should be simultaneously evaluated by chemical screening and by using various biological or pharmacological targets. The standard procedure of searching for active plant metabolites involves biological screening followed by activity guided fractionation. Simple and inexpensive bioassays have been introduced in phytochemical laboratories for rapid screening of crude plant extracts. Bioassays also serve as a guide during the isolation process. Thus, all fractions are biologically evaluated and those continuing to exhibit activity are carried through further isolation and purification until pure active principles are obtained. In this way, different properties and effectiveness against different types of ailments, including microbial afflictions and parasitic diseases, can be investigated. One major drawback of the bioassay-guided fractionation strategy is the frequent isolation of previously known metabolites. The chemical screening of crude extracts therefore constitutes an efficient complementary approach allowing localization and targeted isolation of new types of constituents with potential activities (Hostettmann and Marston, 2002). Sometimes new activities are discovered for known compounds so useful information is still obtained.
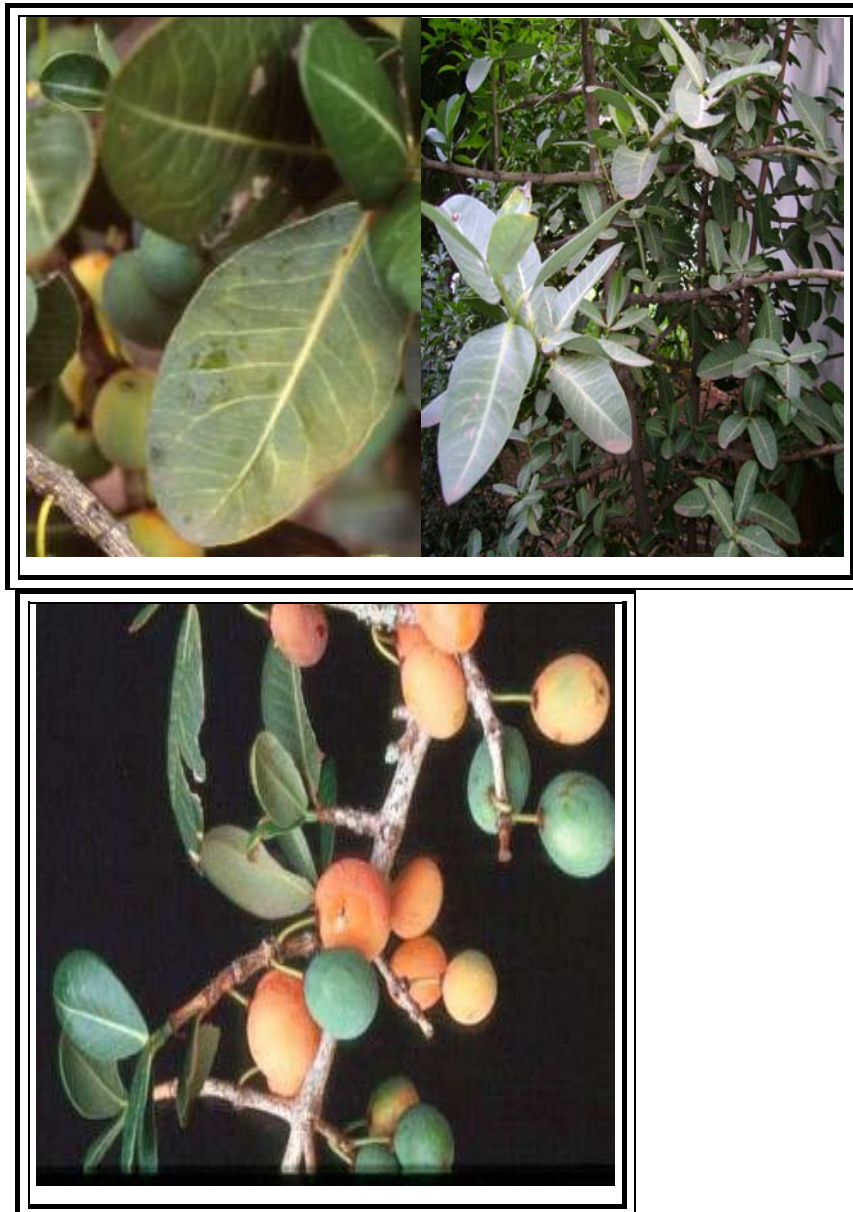
Figure.3.1 Broad leaves of old *Garcinia livingstonei* plant (**A**), broad leaves of a young *Garcinia livingstonei* plant photographed from the University of Pretoria Botanical Garden in Hatfield, Pretoria (**B**) and edible fruits of *Garcinia livingstonei* (**C**). Plates **A** and **C** were downloaded from www.up.ac.za/botanical

3.2 MATERIALS AND METHODS

3.2.1 Plant collection

The plant leaves were collected from the University of Pretoria Botanical Garden in Hatfield in June 2008. *Garcinia livingstonei was* identified by the garden manager, Ms Lorraine Middleton of the Department of Botany, University of Pretoria. The plant material was dried in the shade at room temperature to avoid photo-oxidation of metabolites from direct sunlight. Dried plant material was ground to a very fine powder using a Jankel and Kunkel Model A10 Miller and stored in a dark place at room temperature until required.

3.2.2 Bulk extraction of plant material

Approximately 300 grams of powdered plant material was exhaustively extracted with 3 litres of acetone. The process was repeated three times, using a fresh aliquot of the same acetone each time. The mixture was shaken vigorously for six hours on a Labotec shaking machine to facilitate the extraction process. The supernatant was filtered through cotton wool and Whatmann No. 1 filter paper using a Buchner funnel. The extract was concentrated to minimum volume using a Büchi rotavapor R-114 (Labotec) at 45°C. The reduced extract was transferred to a pre-weighed glass container and placed under a stream of air at room temperature to dryness. The quantity extracted was determined.

3.2.3 Solvent-solvent fractionation of dried plant extract

Twenty grams of the dried acetone extract was subjected to solvent-solvent fractionation as described by Suffness and Douros (1979) and adapted by Eloff (1998). The technique and schematic presentation of the procedure is provided in Fig 3.2. The solvent-solvent fractionation afforded seven fractions. There were five extraction steps in solvent-solvent fractionation process. Each step of the fractionation was carried out once, the steps are described.

Step 1: Chloroform-water extraction: in this step chloroform was mixed with water at a ratio of 1:1, as such 100:100 of chloroform-water was used to dissolve the extract in separating funnel and mixed well by shaking slightly. No partition was observed, then another 100:100 was added without partition, finally 50 ml of chloroform was added to increase the polarity and partition was achieved with water fraction on top and chloroform at the bottom. The chloroform layer was collected in a preweighed bottle.

Step 2: Water and butanol extraction: The water fraction which contained 250 ml water was further separated by adding equal volume of n-butanol to yield a water and a n-butanol fraction

Step 3: Hexane extraction: The chloroform fraction was reduced to minimum volume in a rotary evaporator. The reduced volume was dissolved in 1:1 mixture of hexane and 10% water in methanol, thus 100: 90:10 making up to 100:100. No partition was achieved and the polarity was raised a little with 180:20 so that a hexane and a 10% water/methanol fraction were obtained.

Step 4: Carbon tetrachloride extraction: The 10% MeOH-fraction was further diluted to 10% water in methanol collected in separatory funnel. This was further diluted to a 20% water in methanol by adding 0.125 ml of water to every ml of the 10% water in methanol the volume of water added was 37.5 ml equal parts of carbon tetrachloride was added to obtained a carbon tetrachloride fraction and water in methanol fraction.

Step 5: Chloroform and 35% water in methanol extraction: The 20% water in methanol resulting from carbon tetrachloride extraction was further diluted to 35% water in methanol fraction by adding 0.2308 ml of water to every 20% water in methanol. In this case 235.3 ml of water was added and extracted by adding chloroform and 35% water in methanol fraction.

3.2.4 Preparation of fractions for TLC fingerprinting and bioautography
The dried fractions were dissolved to a concentration of 10 mg/ml in acetone and TLC fingerprinting was done as described in section 2.2.3. The mobile system used was chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (intermediate polarity) because it was observed to be the best mobile system during the screening phase. Bioautography was carried out as explained in section 2.2.5.

3.2.5 Microdilution assay
The microdilution assay of Eloff (1998) was used to determine the MIC values of *Garcinia livingstonei* against four bacterial organisms. This method was earlier described in section 2.2.6. Gentamicin was used as positive control, and the organisms used were listed in section 2.2.4

3.2.6 Column chromatography of the active fractions
Silica gel 60 (400 g) (Merck) was mixed with chloroform to form a slurry and packed to a height of 37 cm in a 5 cm diameter glass column. Ethyl-acetate fraction of acetone extract (20 g) was dissolved in a small volume of acetone, mixed with 25 g of silica gel 60 (Merck), allowed to dry under a stream of cold air and loaded on top of the packed column. Initially the column was eluted with 98% chloroform in 2% methanol (CM) and subsequently, the polarity of the eluting solvent was sequentially increased.

Essentially, a volume of 1 000 ml of 98% CM was initially used, followed by the same volume of each of the following solvent mixtures: 96% chloroform in 4% methanol, 94% chloroform in 6% methanol, 90% chloroform in 10% methanol, and finally the column was eluted with 100% chloroform. The fractions were labeled 1 to 40. Fractions of approximately 20 ml each were collected in test tubes; the volume of solvent was reduced to a minimum by evaporation at room temperature and transferred to preweighed glass vials to dry completely under a stream of cold air

3.2.7 Thin layer chromatography of the column fractions

After column chromatography the collected fractions were loaded onto 10×20 cm TLC plates. Two plates each were developed in CEF and CM (9:1). One set of TLC plates eluted with each of the two solvent systems was sprayed with vanillin-sulphuric acid spray reagent and served as reference chromatograms. Fractions with similar Rf values were pooled together and dried at room temperature. Thus, fractions **2** and **3** were pooled together, and fractions **4** to **9** all were dried at room temperature

3.2.8 Purification of column fractions

From the TLC plates in section 4.2.1.3 any fraction(s) that showed indications of impurities were washed with hexane to obtain pure compounds.

3.2.9 Isolation of pure compounds

Fractions **2** and **3** were pooled together and considered as compound **1**, fractions **4** to **9** as compound **2** respectively. The two compounds were obtained in pure form. This was confirmed by TLC analysis using various solvent systems. About 10-20mg of each sample was sent for NMR analysis using Variant Unit Innova 300 MHz system (Oxford instruments) and Brüker DRX instrument at the Medical University of South Africa (MEDUNSA).

Figure 3.2 Schematic representation for solvent-solvent fractionation of *Garcinia livingstonei* acetone leaf extracts (Suffness and Douros, 1979, adapted by Eloff, 1998).

3.3 RESULTS AND DISCUSSION

3.3.1 Quantity of *Garcinia livingstonei* extracted using bulk exhaustive extraction

Garcinia livingstonei leaves (300g) extracted with acetone yielded 20 g of the dried extract. The percentage extraction yield was 6.7%.

3.3.2 Solvent-solvent fractionation yield

A result of solvent-solvent fraction is shown in Table 3.1. The technique yielded seven fractions. Hexane extracted the highest quantity of plant material (7.71 g) and lowest quantity of plant material was extracted in ethyl-acetate (0.41 g) fractions. In total 16.71 g was extracted from initial weight of 20 g. Therefore, about 3.29 g was lost from the total weight of 20 g dried extract during the procedure.

Table 3.1 Quantity of the fractions extracted from solvent-solvent fractionation of acetone extract of *Garcinia livingstonei* leaves

Fractions	Quantity extracted (g)
Hexane	7.71
Carbon tetrachloride	2.04
Chloroform	0.92
Ethyl acetate	0.41
Butanol	1.42
Aqueous methanol	1.47
Water	2.74
Total	16.71

3.3.3 TLC fingerprints and bioautograms

TLC fingerprints of *Garcinia livingstonei* fractions after bulk extraction and solvent-solvent fractionation are shown (Fig. 4.2). The separation in CEF indicates a number of separated non- polar compounds. Some of the extracted compounds did not move on the TLC plate and have remained at the base of the TLC plates in both CEF and EMW (Fig 3.3), indicating that they were polar and unable to develop in the selected solvent system. The separation of the fractions in the CEF and polar EMW systems was good, and it is easy to obtain a reasonable indication of the number of extracted compounds. Solvent-solvent fractionation was a good technique to use when initially separating the components of the crude plant

extract of *Garcinia livingstonei* because compounds appeared to separate relatively well into the different extractants, with more polar compounds in the butanol fraction, and more non-polar compounds mainly in the hexane fraction. Liquid-liquid extraction enables you to get large amount of material although it is more time demanding and laborious than other techniques like high pressure liquid chromatography coupled with ultra violet photodiode (HPLC-UV) and liquid chromatography coupled mass spectrometry (LC-MS). Chloroform and ethyl acetate extracted the most bioactive compounds (Fig. 3.4); these compounds also reacted well with the vanillin-sulphuric acid spray reagent used on the chromatograms. The Rf values of the two compounds are 0.92 and 0.88 for (**1**) and (**2**) respectively.
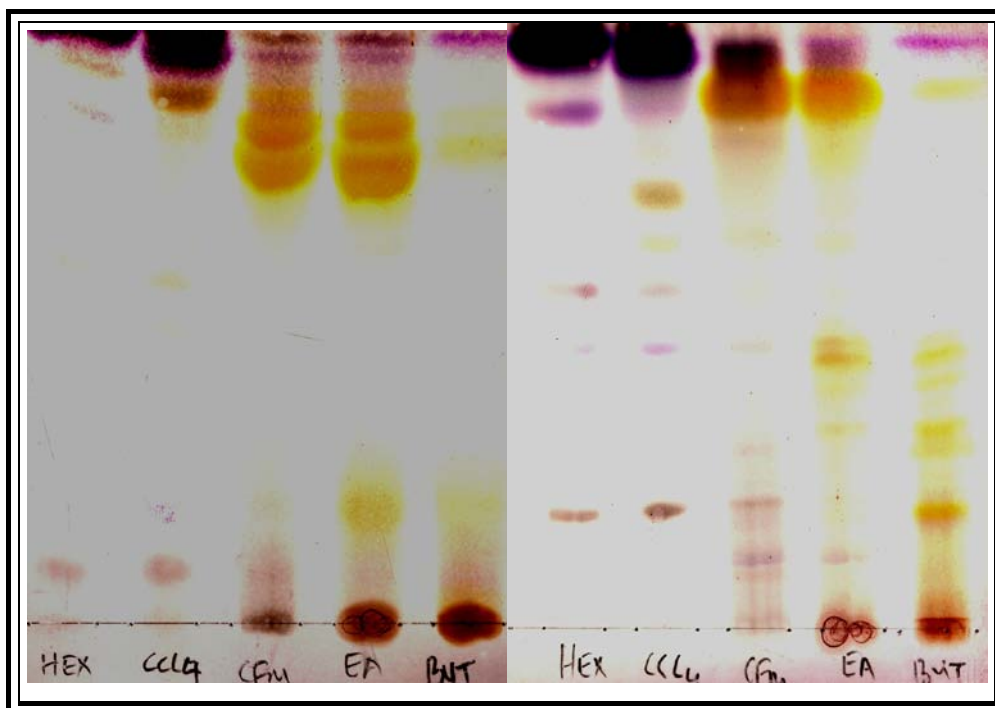
Figure 3.3 TLC fingerprints of *Garcinia livingstonei* fractions Hexane (**HX**), Carbon tetrachloride (**CT**), Chloroform (**CF**), Ethyl acetate (**EA**) and Butanol (**BT**) developed in chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (**A**) and ethyl acetate/methanol/water (40:5.4:5): [EMW] (**B**) sprayed with 0.1% vanillin sulphuric acid
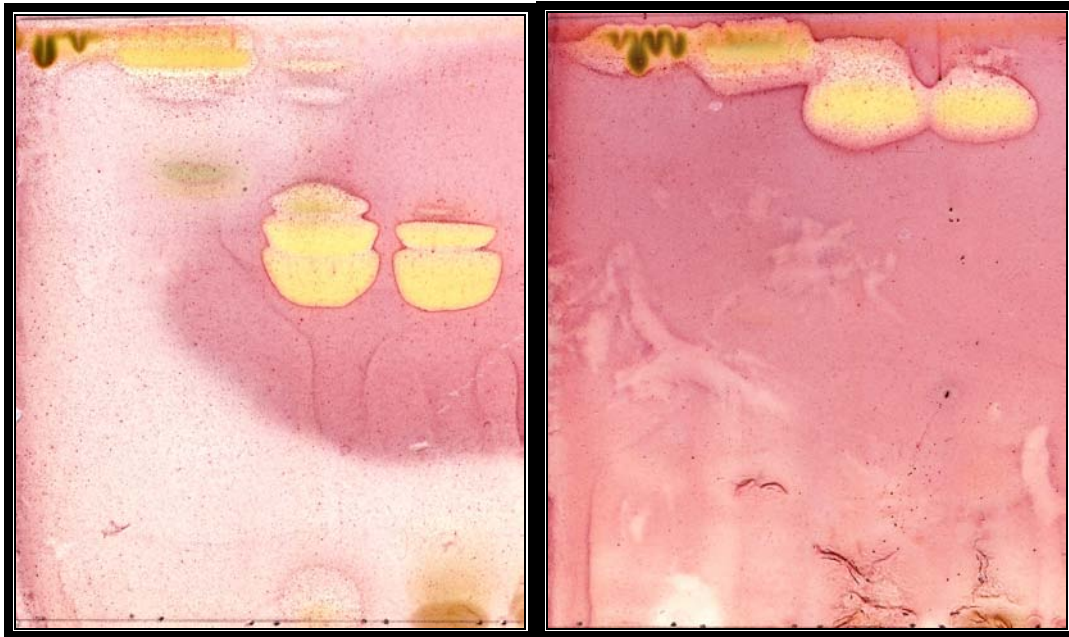
Figure 3.4 Bioautograms of *Garcinia livingstonei* fractions, hexane (**HX**), carbon tetrachloride (**CT**), butanol (**BT**) chloroform (**CF**) and ethyl acetate (**EA**) and developed in chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (**A**) and ethyl acetate/methanol/water (40:5.4:5): [EMW] (**B**) and sprayed with actively growing culture of *Escherichia coli. p*-iodonitrotetrazolium violet (INT) solution was sprayed 24 hours after incubation. Yellow zones on chromatograms indicate bacterial growth inhibition.
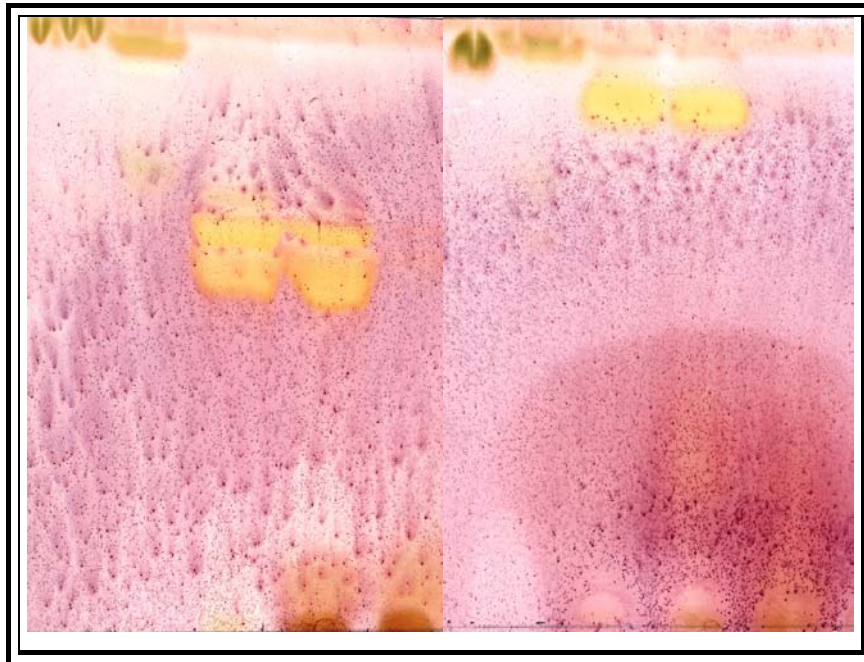
Figure 3.5 Bioautograms of *Garcinia livingstonei* fractions, hexane (**HX**), carbon tetrachloride (**CT**), chloroform (**CF**), ethyl acetate (**EA**) and butanol (**BT**) developed in chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (**A**) and hexane (**HX**),carbon tetrachloride (**CT**), chloroform (**CF**) and ethyl acetate (**EA**) developed in ethyl acetate/methanol/water (40:5.4:5): [EMW] (**B**) and sprayed with actively growing culture of *Staphylococcus aureus. p*-iodonitrotetrazolium violet (INT) solution was sprayed 24 hours after incubation. Yellow zones on chromatograms indicate bacterial growth inhibition.

3.4 Minimum inhibitory concentrations of the fractions

The MICs of the fractions of *Garcinia livingstonei* is provided in Table 3.2. The MIC ranged from 0.095 to 1.875 mg/ml. The lowest MICs were observed in chloroform, carbon tetrachloride, ethyl-acetate and hexane fractions and the, highest MIC was recorded in aqueous methanol fraction. In terms of microbial susceptibility to the test extract, *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus* were the most susceptible organisms. The least susceptible organism was *Pseudomonas aeruginosa*.

Table 3.2 Average MIC values of the fractions of *Garcinia livingstonei* leaf extracts tested against four bacteria

	MIC of the fractions (mg/ml)					
Bacteria	HX	ET	CF	CT	BT	AM
E. coli	0.23	0.175	**0.095**	**0.115**	0.78	1.875
S. aureus	0.23	**0.195**	0.31	0.355	1.25	1.875
E. faecalis	nt	0.99	0.195	**0.115**	nt	nt
P. aeruginosa	2.5	1.405	0.095	**0.115**	0.78	1.875

HX = Hexane, Et = Ethyl-acetate, CF = Chloroform, CT = Carbon tetrachloride, BT = Butanol, AM = Aqueous methanol

nt = not tested

3.3.5 TLC fingerprints of fractions and isolated compounds

The TLC fingerprints of the fractions and isolated compounds are shown on Figure 3.6 and 3.7 respectively. It appears that the solvent system was not equilibrated during the chromatography leading to a strong smile effect on the chromatogram (Figure 3.6.)

From the chromatograms, fractions **2** and **3** contained a compound with the same Rf value and thus were pooled together, and the same was done for fractions **4-9**. But, before pooling fractions 2 and **3** together fraction **2** was washed with hexane to remove traces of impurities. The pooling of these fractions together based on their R_f values and polarities led to the final isolation of two pure compounds (named compound **1** and **2**).

The chromatograms of the two isolated compounds developed in chloroform: methanol (9:1) is shown in Figure 3.7.The R_f values of the two isolated compounds are close together. Based on the vanillin spray reagent colour, they may have a closely related basic skeleton
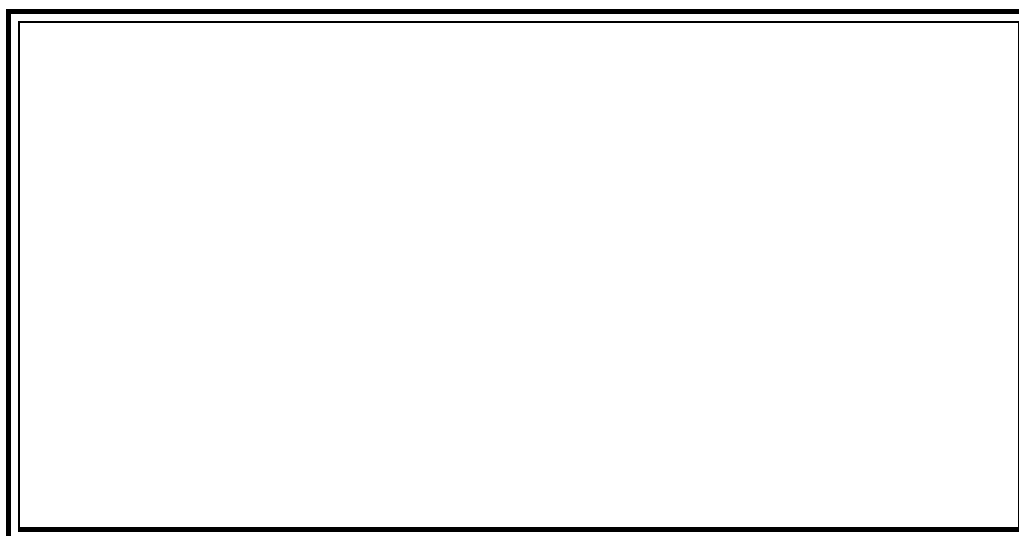
Figure 3.6 TLC chromatogram showing nine fractions (**2-9**) obtained from open column chromatography of Ethyl acetate fraction of the acetone extract of *Garcinia livingstonei* leaves. The plate was developed in Chloroform methanol (9:1) solvent system and sprayed with vanillin sulphuric acid.
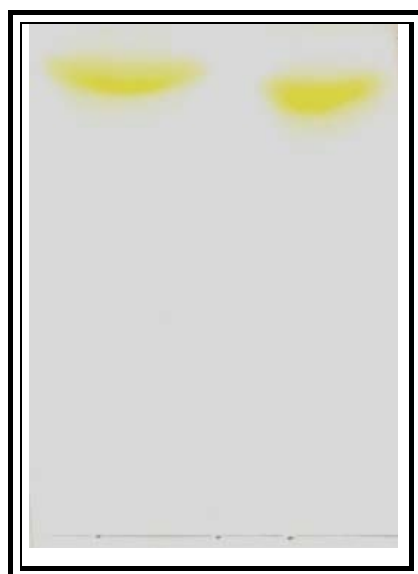
Figure 3.7 TLC chromatogram showing isolated compounds **1 (Left)** and **2 (right)** obtained from open column chromatography of ethyl acetate fraction of *Garcinia livingstonei* leaf extract. The plate was developed in CEF mobile system and sprayed with vanillin sulphuric acid.

3.3.6 Rf values of isolated compounds

 The Rf values of the sub-fractions obtained from open column chromatography (Figure 3.6) and the two isolated compounds (Figure 3.7) are depicted in Table 3.3. Although the Rf values of sub-fractions did not differ to a greater extent as such the sub-fraction with close Rf values where pooled together and dried at room temperature. The results indicated that the two isolated compounds (**1** and **2**) which were obtained by pooling sub-fractions with close Rf values led to the isolation of two pure compounds with similar Rf values 0.99 (**compound 1**) and 0.88 (**compound** 2) respectively Table 3.3. These Rf values may suggest a closer relationship between the two compounds, even though MS and NMR should be performed to be sure.

Table 3.3 Rf values of isolated compounds and compounds in the fractions obtained from open column chromatography. Compound **1** and **2** were developed in CEF while Fractions **2-9** were developed in chloroform methanol (9:1) mobile phase.

Isolated compounds and fractions	Rf values
Compound 1	0.88
Compound 2	0.92
Fraction 2	0.44
Fraction 3	0.36
Fraction 4	0.28
Fraction 5	0.14
Fraction 6	0.13
Fraction 7	0.13
Fraction 8	0.15
Fraction 9	0.17

3.3.7. CONCLUSION

The fractions had a good activity with MIC values ranging from 0.095-2.5 mg/ml (Table 3.2). The activities observed in the fractions correspond with the activity of the crude acetone extracts recorded at the preliminary screening of *Garcinia livingstonei.* All fractions showed low MICs on the four organisms tested except aqueous methanol fraction which had high MIC of 1.875 mg/ml on all the organisms tested. The chloroform fraction had good activity on at least three of the four organisms tested. Two organisms namely *Staphylococcus aureus* and *Pseudomonas aeruginosa* were less sensitive to hexane and butanol fractions, the MIC values were 1.25 and 2.5 respectively. The most active fractions; ethyl-acetate and chloroform had good activity on bioautography and microdilution assay against both Gram positive and Gram negative bacteria with the exception of *Pseudomonas aeruginosa* that was less sensitive to ethyl acetate fraction.

During the preliminary screening procedure, all fractions had antibacterial activity, indicating that plant constituents active against at least three organisms were extracted in those fractions. The chloroform and ethyl acetate fractions had good activity both the microdilution assay and bioautography so these were selected for further isolation work due to the ease of localization of the active constituents on the bioautograms.

Solvent-solvent fractionation and column chromatography were employed to isolate two pure compounds (compounds **1** and **2**). Two solvent systems were used, namely CEF and CM. The two compounds isolated had similar Rf values.The isolated compounds were polar in nature. The MIC of these compounds will be evaluated in the next chapter.

CHAPTER 4

STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

4.1 INTRODUCTION

The process of preclinical drug discovery consists of two steps; finding initial hits (binding ligands to a medicinal relevant target, usually a protein) and lead optimization (Heller and Kessler, 2001). Ever since nuclear magnetic resonance spectroscopy (NMR) became popular in analytical studies, its capabilities and applications have continued to evolve. Originally designed as a way to verify the structure of relatively small compounds, the technology of NMR has exploded and become a valuable means for studying protein and other chemical structures. NMR has proved to be a valuable tool in pharmaceutical research, as it has entered a new arena of drug discovery and structural genomics. NMR can provide information on the three-dimensional structures of small molecules in solution, high-molecular-weight complexes, and the details of enzyme mechanisms that can be used to aid in drug design. In the present scenario, the availability of high magnetic fields; improved software, high resolution probes, and electronics, more versatile pulse programmers, and most importantly the development of 2D, 3D and 4D NMR, have revolutionized the field of drug discovery and development (Neeraj Umpumanyu et al., 2007).

4.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

In the past few years, applying innovative approaches, much progress has been made in improving NMR as a powerful tool for industrial drug research. Different software packages have been developed for complete spectral assignment of all peaks. The introduction of pre-amplifier and radio frequency coils of the probe heads to about 20 K enhances high resolution NMR and increases the signal to noise ratio about four fold. New hardware setups for data acquisition enable a "Just-in-time" preparation of the samples, including the transfer to the magnet, locking and shimming. Numerous new pulse sequences, improve previous technology in terms of sensitivity, resolution, selectivity, speed and efficiency. Several "new" NMR parameters such as residual dipolar coupling (RDC) (Prestegard, 2000) cross-correlated relaxation, "unusual" Non-Karplus-type coupling constants, or scalar couplings across hydrogen bonds provide more and more information about the interacting molecules.

A recent review by Yang (2006) reported NMR as the most powerful spectroscopic tool for obtaining structural details of complex organic compounds. The structural assignment of all isomers is possible by

considering chemical shifts, coupling constants, and integration ratios of the NMR data. It is also needed for unambiguous structure determination, especially for the stereospecific identification of unknown active compounds that may be of interest for the development of pharmaceuticals and functional foods. All compounds having NMR measurable nuclei can be detected including 1H and 13C, which are major structural elements of organic compounds.

Even though the technique is powerful, NMR spectroscopic analysis requires time-consuming isolation and purification steps in order to acquire NMR spectra on individual components. One of the setbacks of NMR technique is its low sensitivity and its inability to detect some NMR "silent" functional groups having poor or non-existent magnetic properties, such as SO4 and NO2 (Giinther, 1995), and hence it is not useful for structure determination of such metabolites (Yang, 2006). Relatively high concentrations of analytes are required to achieve sufficient detection sensitivity (Yang, 2006).This technique is extensively used in biomedical, pharmaceutical, environmental, food and natural products analysis, as well as for the identification of drug metabolites (Albert, 2004; Jaroszewski, 2005).

4.2 MATERIALS AND METHODS

4.2.1 Sample preparation for NMR analysis

The Varian Unit Innova 300 MHz NMR system (Oxford instruments) and Brüker DRX – 400 instruments were used for 13C and 1H NMR. The compounds isolated from *Garcinia livingstonei* (Chapter 4) were dried, weighed (10-20 mg) and dissolved in c. 2 ml of deuterated dimethylsuphoxide (DMSO-d_6) (Merck) because the isolated compounds were soluble in dimethylsuphoxide. The solutions were then pipetted into NMR tubes (commonly of 5 mm diameter to a depth of 2-3 cm) using clean Pasteur pipettes and taken to the Department of Botany, University of Pretoria for proton (1H) and carbon 13 (13C) spectroscopy.

4.3 RESULTS AND DISCUSSION

4.3.1 Identification of the isolated compounds

4.3.1.1 Compound 1

Compound **1** was isolated as yellow amorphous solid. The NMR spectra and spectral data analysis of this compound is presented in Appendix 1a-e and Appendix Table 1. The structure of the compound is shown in figure 4.1. These are consistent with two flavone units linked through the C-3 of a flavone ring to the C-8 of the second flavone. This class of compound consists of three ring systems A, B, and C.

(Fig 4.1.) Ring B which has a hydroxy substitution at C-4 often gives a typical 4 peak pattern of two doublets with a characteristic coupling constant, this pattern was clearly shown in C-3, C-5, C-2 and C-6 with resonance at 6.84 ppm and 7.66 ppm respectively and a coupling constant of 8.8 ppm. The chemical shift of the protons at C -3 and C -5 are shielded by the C-4 oxygen substitution (electron withdrawing effect due to the electronegative chemical environment), hence the chemical shift of C-3, C-5 were observed at 6.84 ppm while C-2, C-6 were observed at 7.66 ppm.

The protons at H-6 and H-8 in ring A consist of 2 doublets with signal at 6.24 ppm and 6.51 ppm respectively with a coupling constant of 1.8 HZ; this is due to the meta-coupling of these two related protons. The H-6 doublet always occurs at a higher field (lower chemical shift) than H-8 due to the different inductive effect in their chemical environment. This is often characteristic of flavones which contain the 5, 7 dihydroxy substitution as observed in compound A. Compound A was isolated in its aglycone form as the absence of glycoside derivative is confirmed by the complete absence of glucosyl signals in the chemical shift range of 4–5 ppm. Although H-3 have related chemical shift to H-6 and H-8, signal due to H-3 is readily distinguished from the H-6 and H-8 signal because it occurred as a singlet at 6.74 ppm compared to the signal from H-6 and H-8 which consistently occur as doublet. This flavonoid type is clearly differentiated from the monohydroxylated form because the C-6 proton in ring A of the monohydroxylated form always occurred as a singlet instead of the doublet observed in the isolated compound. The protons in the A-ring often appear more downfield than the ring protons and it shows some characteristic signal based on the substitution pattern of the ring that makes its identification confirmatory.

The occurrence of the signals of H-2′ and H-6′ at 8.12 ppm and 8.04ppm was affected by the nature of substitution on the C-ring. The observed resonances differentiated the isolated compound from other classes of flavonoids with different oxidation level on the C-ring such as chalcones, aurones, dihydroflavonols, isoflavones and flavonols. While the coupling constant of these classes of flavonoids is similar for the C-2 and C-6, their chemical shift signals differ by the substitution pattern on the C-ring. Different spectral patterns are observed where there are dual substitutions at the 3rd and 4th position on the C-ring. The observation of the characteristic H-2, H-6, H-3, H-5 doublet signals clearly differentiate the isolated compounds from the doubly substituted flavonoids at the 3rd and 4th position which often give multiple signals. The H-3 proton of compound A gave a very clear singlet at 6.72ppm due to the absence of neighbouring protons. The clear singlet distinguished it from the doublet signals from H-2, H-6, H-3 and H-5 signals at similar resonance. This is also differentiated from the isoflavones class with C-2 substitution which occurred further downfield because it's in a beta position to the ketone group at the fourth position of carbon; it is also differentiated from the chalcones which exist as a doublet due to the

double bond between C-2 and C-3 while the flavanones are known for their overlapping quartets due to the coupling of protons on their C-2 and C-3 with single bond.

The signals for the C-6 and C-8 in the ^{13}C spectra with a 5, 7, dihydroxy substitution were unambiguously differentiated by the multiplicity of the coupled protons on them as discussed above. The resonances for the two carbons were observed at 99.7 ppm and 94.7 ppm respectively (C-6 always at a lower field and higher chemical shift than C-8 because of their chemical environment). The signal for I-C-6 and II-C-6 appeared at 99.7 ppm, while II-C-8 shifted significantly downfield (higher chemical shift) due to the substitution effect of the biflavonoid linkage. The ketone signals on I-C-4 and II-C-4 were clearly observed at 182 and 182.8 ppm respectively. The spectrum interval between 116 and 131 consists of signals due to the carbon atoms of the aromatic ring systems of the two B rings. The signal for the carbon atoms for I-C-6, I-C-8, II-C-6 and II-C-8 in the spectrum were identified readily to be at 99 ppm, 94.2 ppm, 99.12 ppm and 103.4 ppm respectively. The carbon spectrum also confirmed the absence of the glycosidic form of the isolated compound. Correlation of the ^{1}H and ^{13}C NMR signals with previously isolated biflavonoids clearly indicated compound **1** to be amentoflavone (Pelter et al., 1971, Markham et al., 1987; 1990). The data of the current isolation of amentoflavone from *G. livingstonei* coincides with several reports (Lin et al 1997, Cholba et al., 1991; Mora et al., 1990; Sanz et al., 1990).
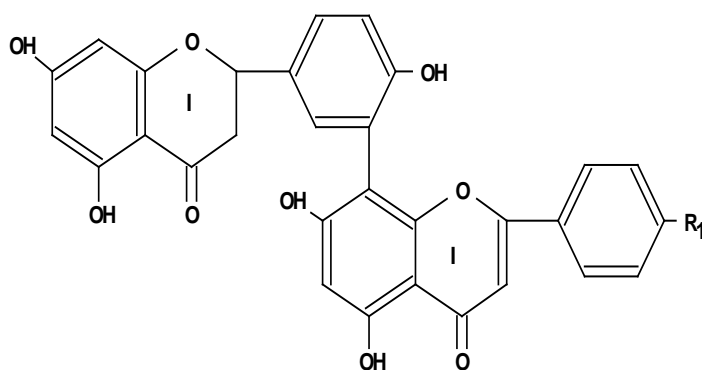
R1=OH

Figure 4.1 Structure of the isolated compound (1) amentoflavone

4.3.1.2 Compound 2

Compound **2** was isolated as a yellow solid which showed a similar spectral pattern to compound **1** as shown by all the proton and carbon spectra for the two compounds. The only major difference was the observation of a methoxy signal in both proton and carbon spectra in compound **2**. There was 3.802 ppm in ^{1}H NMR and 55.86 ppm signal in its ^{13}C NMR spectrum. Correlation of the obtained data with a previously isolated methoxy derivative of amentoflavone confirmed compound **2** to be 4′-monomethoxylamentoflavone. Similar compound was isolated in *Podocalyx loranthoides* (Suarez et al., 2003). The NMR spectra and spectral data analysis of this compound is presented in Appendix 2a-e and Appendix Table 2. The structure of the compound is shown in figure 4.2.
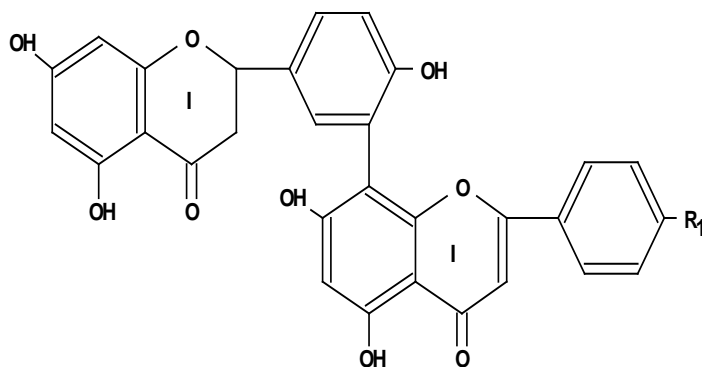
R1=OMe

Figure 4.2 Structure of isolated compound (2) 4' monomethoxyamentoflavone

The isolated flavonoids amentoflavone and 4' monomethoxyamentoflavone have previously been isolated from *Podocalyx loranthoides* (Suaˊrez *et al.*, 2003)..Amentoflavone was isolated from, *Rhus succedanea* (Lin *et al.*, 1997), *Selaginella tamariscina* (Woo *et al.*, 2005) and *Byrsonima crassa* (Cardoso *et al.*, 2006).

4.4 CONCLUSION

The structures of the two compounds were elucidated using nuclear magnetic resonance (NMR) spectroscopy techniques. The two compounds amentoflavone (**1**) and 4' Monomethylamentoflavone (**2**) are biflavonoids. These two compounds have the same parent structure. Compound **2** containing methoxy group and compound 1 a basic hydroxyl group.

The family Clusiaceae to which *Garcinia* belongs contains a large number of bioactive compounds. The genus *Garcinia* is a rich source of bioactive molecules including xanthones, flavonoids, benzophenones, lactones and phenolic acids (Selvi et al, 2003). Isolation of biflavonoids from the leaves of *Garcinia livingstonei* was not surprising as these constituents have been previously isolated from other species in the same genus and family, either from acetone or methanol extracts. Amentoflavone (**1**) has earlier been isolated from *Garcinia livingstonei* (Pelter et al 1971) and 4' monomethoxyamentoflavone (**2**) was first isolated and characterized from *Podocalyx loranthoides* (Suarez et al., 2003). This is the first isolation and characterization of 4' monomethoxyamentoflavone from *Garcinia livingstonei.*

Bioassay guided isolation has proved to be an important technique in the isolation of bioactive constituents. Chemical characterization provides the identity of the compounds. In the next chapter the antibacterial and antioxidant activities of the isolated compounds as well as their safety based on genotoxicity and cytotoxicity will be discussed.

CHAPTER 5

IN VITRO ANTIBACTERIAL ANTIOXIDANT, CYTOTOXICITY AND GENOTOXIC ACTIVITIES OF THE ISOLATED COMPOUNDS

5.1 INTRODUCTION

No published reports on the antibacterial activity of the two biflavonoids isolated from different plants was found. However, information exists on other compounds isolated from *Garcinia* species. Garcinol which has been isolated from *Garcinia bancana* had MIC value of 16 µg/ml. Similarly moreollic acid and morelic acid, isolated from fruits of *Garcinia hanburyi* both had good activity on methicillin resistant *Staphylococcus aureus* (MRSA) with a MIC value of 25 µg/ml. The xanthone nigrolineaxanthone isolated from *Garcinia nigrolineata* was very active on MRSA with a MIC of 2 µg/ml. 8-Desoxygartanin and ananixanthone isolated from the same plant gave MIC values of 16 and 32 µg/ml respectively (Rukachaisirikul *et al.*, 2005). In the same vein, xanthochymol isolated from *Garcinia dulci* inhibited the growth of penicillin sensitive strain ATCC 25923 and MRSA SKI *Staphylococcus aureus* at a concentration of 8 µg/ml, whereas rhamnazin from the same plant showed higher MIC of >128 µg/ml (Deachathai *et al.*, 2006). It appears there is no information on the antibacterial activity of amentoflavone from *Garcinia livingstonei* and those isolated from other species. The antibacterial activity of 4' monomethoxyamentoflavone isolated from *Podocalyx loranthoides* has not beeen determined.

Pharmacologically, amentoflavone *from Selaginella tamariscina* inhibits the induction of nitric oxide synthase by inhibiting NF-kB activation in macrophages. It also mediates down regulation of COX-2 expression in A549 cells (Woo *et al.*, 2005), and is an anti-inflammatory compound. Similarly, amentoflavone from *Selaginella tamariscina* was reported to give neuroprotection against diverse experimental cytotoxic insults on neurons (Woo *et al.*, 2005). 4' monomethoxyamentoflavone and amentoflavone are relatively non-toxic (Kang *et al.*, 2005) although amentoflavone has been reported as a mutagenic compound (Cardoso *et al.*, 2006). These results agreed with our present findings.

It appears there is no information on the antibacterial activity, antioxidant activity or safety of amentoflavone or 4' monomethoxyamentoflavone. These aspects were investigated in this chapter.

Initial screening of potential antibacterial compounds from plants substances usually starts with crude extracts (Afolayan and Meyer, 1997; Rojas et al.,1992).The most commonly used screens to determine antimicrobial susceptibility are the broth dilution assay (Ayafor et al., 1994) and the disk or agar well diffusion. There are many difficulties with agar diffusion techniques and a serial microplate dilution method using tetrazolium violet as indicator of growth is used very widely for determining MICs of bacteria and fungi (Eloff, 1998a). Adaptations such as agar overlay method (Mayr-Harting et al., 1972) may also be used. In some cases, inoculated plates or tubes are exposed to UV light (Taylor et al., 1996) to screen for the presence of light sensitizing photochemicals. Other variations of these methods are also used. For instance to test the effects of extract on invasive *Shigella* species, non cytotoxic concentrations of the extracts can be added to Vero cell cultures exposed to a *Shigella* inoculum (Vijaya et al, 1995). The decrease in cytopathic effect in the presence of the plant extract or isolated compound is then measured. After initial screening of phytochemicals, more detailed studies of their antibiotic effects should be conducted. At this stage, more specicific media can be used and MICs can be effectively compared to those of a wide range of currently used antibiotics (Cowan, 1999).

It has been long recognized that many naturally occurring substances in higher plants have antioxidant activity. There has been increased interest in oxygen containing free-radicals in biological systems and their implied roles as causative agents in the aetiology of a variety of chronic disorders. The screening of plant extracts using the DPPH free radical method has proved to be effective for the selection of those extracts/compounds having antioxidant activity. These extracts may be rich in radical scavengers, such as flavonoids, known as antioxidants (Mensor et al, 2001). Antioxidant activity of extracts or compounds can also be measured by the decolorization assay which measures antioxidant activity in relation to Trolox, a water soluble vitamin E analogue (Re *et al.*, 1999). The method involves prior generation of the radical monocation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS $^+$).The blue/green chromophore ABTS$\cdot$ $^+$ is produced through the reaction between ABTS and potassium sulphate (Re *et al.*, 1999). The addition of antioxidants to the free radical reduces it to a colourless ABTS, a reaction that depends on the concentration of the antioxidant and the duration of the reaction. The extent of decolorization as percentage inhibition of the free radical is then calculated relative to the reactivity of Trolox under the same conditions (Re *et al.*, 1999). One difficulty in assessing antioxidant activity is the option of which method to use. Different methods seem to give different values and the methods have their advantages and disadvantages. The TEAC has the major advantage that it is applicable to both aqueous and lipophilic systems (Re *et al*, 1999).

Southern Africa has over 25 000 species of higher plants, mostly endemic, of which about 3 000 are used in traditional medicine (Van Wyk and Gericke, 2000). Recently there has been considerable

interest in mutagenicity (Elgorashi et al., 2002, 2003; Marques et al., 2003) and antimutagenicity (Ferrer et al., 2001; Negi et al., 2003; Verschaeve et al., 2004) of medicinal plants used by traditional herbalists. Frequently used plants in traditional medicine are assumed safe, due to their long-term use (Elgorashi et al., 2002) and are considered to have no side effects because they are 'natural' (Popat et al., 2001). This perception is not scientifically based and it is important to determine toxicology of plant extracts, especially those that are used frequently over long periods.

The Ames test (Maron and Ames, 1983) was used in this study as it is widely used in the determination of possible gene mutations caused by extracts. A positive response in any single bacterial strain either with or without metabolic activation is sufficient to designate a substance as a mutagen (Zeiger, 2001). The Ames test is reliable, quick and easy. This is used to screen for possible carcinogens, however, a positive result does not necessary indicate the substance as being a carcinogen. Also, if a substance screened does not suggest a mutagenic response it does not necessary confirm that it is not mutagenic or not carcinogenic. It confirms that the substance is not mutagenic to the particular bacterial strain used and for the genetic endpoint tested. To test for carcinogenicity a 2 year carcinogenicity test would have to be performed by testing the effect of the mutagenic sample in mice and rats (male and female) (Zeiger, 2001). The same can be said for antimutagenic screening.

5.2 MATERIALS AND METHODS

5.2.1 Microdilution assay
Minimum inhibitory concentrations of the isolated compounds were determined as described previously in section 2.2.6 (Eloff, 1998a). Bacterial cultures were maintained as described in 2.2.4.

5.2.2 Antioxidant assay
The antioxidant activities of the isolated compounds were determined using the Trolox and DPPH assays. The assays are described below.

5.2.2.1 Preparation of ABTS

The ABTS was prepared as described (Re et al, 1999). This involved mixing 192 mg of ABTS with 50 ml of water made a 7 mM stock solution of ABTS. The ABTS free radical was produced by reacting ABTS stock solution with 33 mg (2.45 mM) of potassium sulphate (final concentration). The solution was prepared 12 - 16 hrs before use and stored at 4 ^{0}C, until required.

5.2.2.2 Trolox assay: Experimental procedure

Different concentrations of isolated compounds **1, 2** and fraction extracts or Trolox were prepared by serially diluting 1 mg/ml of each sample. The prepared ABTS $^{+}$ solution was diluted with ethanol to an absorbency of 0.7 ± 0.02 at 734 nm (ethanol used as blank) after which 1.0 ml was added to 10 μL of the 1 mg/ml solution of Trolox. The absorbance reading was taken after 6 min of reaction time. This was repeated for the remaining concentrations of Trolox, compounds **1** and **2** and the fraction extracts. All determinations were carried out in triplicate according to Re et al. (1999).

5.2.3 Genototoxicity assay

The potential mutagenic effects of the isolated compounds **1** and **2** were detected using the Ames test. The Ames assay was performed with *Salmonella typhimurium* strain TA 98 using the plate incorporation procedure described by Maron and Ames (1983) and adapted by Elgorashi et al. (2003). One hundred μl of bacterial stock was incubated in 20 ml of Oxoid Nutrient Broth for 16 h at 37°C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (isolated compounds, solvent control or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 h at 37°C. After incubation, the number of revertant colonies (mutants) was counted. All cultures were made in triplicate (except the solvent control where five replicates were made) for each assay. The positive control used was 4- nitroquinoline 1-oxide (4-NQO) at a concentration of 0.1 mg/plate. The isolated compounds were tested at various concentrations of 400, 200, 100, 10 and 1 μg/plate respectively.

5.2.4 Tetrozolium-based colorimetric assay (MTT)

The cytotoxicity of the isolated compounds was determined as described by Mosmann, (1982) and McGaw et al (2007). The isolated compounds were tested for cytotoxicity against Vero monkey kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria).The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell

suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. Plates were incubated overnight at 37ºC in a 5% CO2 incubator and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the isolated compounds (20 mg/ml) were prepared by dissolving them in DMSO. Serial 10-fold dilutions of isolated compounds were prepared in growth medium and added to the cells. The viable cell growth after 120 hours incubation with isolated compounds was determined using tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay, sigma) described by Mosmann (1983). Briefly after incubation, 30 µl of MTT (5 mg/ml in phosphate buffered solution, PBS) was added to each well and the plates were incubated for a further 4 hours. The medium was aspirated from the wells and 50 µl DMSO added to each well to solubilize the formazan produced by mitochondrial activity. The absorbance was measured on a Titertek Multiscan MCC/340 microplate reader at 540 nm test wave length of 690 nm. Berberine chloride (sigma) was used as positive control. The intensity of colour is directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated three times.

5.3 RESULTS AND DISCUSSION

5.3.1 Minimum inhibitory concentration of the isolated compounds

The minimum inhibitory concentration values of the isolated compounds against four bacterial species are shown in Table 5.1. All the microorganisms were sensitive against the two compounds at the concentration tested, and the MIC values ranged from 8– 15 µg/ml. It is interesting to note that the activities of the two compounds were similar since they exhibited similar MICs against the test organisms (Table 5.1). The MIC values of compound 2 against *E. coli* and *E. faecalis* were comparable to that shown by gentamicin.

The two isolated compounds are biflavonoids. Biflavonoids have been observed to possess both biological and pharmacological activities (Kim et al 2007). In this study, *Staphylococcus aureus* was less sensitive to amentoflavone (**1**) with a higher MIC of 100 µg/ml compared to the lower MIC range of 8 - 60 µg/ml observed for both the two compounds against the other three organisms. The reason for this difference could be related to enhanced uptake by the bacteria due to the methoxy group replacing a hydroxyl group (Kim et al 2007)..

Lin et al (2005) showed that structural characteristics were related to activity. In his case methylation of the hydroxyl groups of the biflavonoids resulted in diminished activity. This structure-activity study demonstrates that hydroxyl groups and at least one flavone unit in the biflavonoids are required for

activity and active compounds become inactive when hydroxyl groups are methylated. But, in this study compound **2** (4' monomethoxyamentoflavone) which is a methylated derivative of compound **1** (amentoflavone) had a much higher antibacterial activity against *Staphylococcus aureus* with an MIC value of 40 μg/ml compared with compound **1** (amentoflavone) which had an MIC of 100 μg/ml. These results did not agree with earlier reports (Lin et al., 2005) Table 6.1. Methoxyamentoflavone was also more active against all the other microorganisms used in or tests.

Therefore, the mere presence of a hydroxyl group in amentoflavone may not fully explain its inactivity on *Staphylococcus aureus*, as observed in this study. This is because the methoxyamentoflavone exhibited good antibacterial activity also against the other three organisms tested. The most sensitive organism was *Enterococcus faecalis* with an MIC value of 8 μg/ml then *Escherichia coli* and *Pseudomonas aeruginosa* with MIC values of 40 μg/ml and 60 μg/ml respectively.

Compounds Isolated from other *Garcinia* species have given moderate activities against methicillin resistant *Staphylococcus aureus* (MRSA) (Sukponduma, 2005; Rukhachasirukhul et al 2005; Koguem, 2005). Compounds isolated from *Garcinia dulci* have shown good bioactivities against a penicillin sensitive strain, ATCC 25923, and a methicillin-resistant strain SK1 (MRSA SK1) of *Staphylococcus aureus* with MIC values of 8 μg/ml with only one compound having weak activity giving MIC values greater than128 μg/ml. This is in accordance with the results obtained against *Staphylococcus aureus* for compound **1** (amentoflavone) in this work (Table 5.1).

Table 5.1 Average Minimum inhibitory concentration values of crude extract and the isolated compounds (µg/ml) against four bacteria.

Microorganisms	Crude extract (µg /ml)	1	2	Gentamicin (µg/ml)
Escherichia coli	1570	40	8	2
Staphylococcus aureus	500	40	40	10
Pseudomonas aeruginosa	1250	100	60	20
Enterococcus faecalis	550	60	8	2

1= amentoflavone, **2**= 4' monomethoxyamentoflavone.

5.3.2 Trolox assay of isolated compounds

The Trolox assay is widely applied to assess the total amount of radicals that can be scavenged by an antioxidant, which is the antioxidant capacity. It is a tool for tracking down the presence of unknown antioxidants in complex mixtures. For this application the assay has been used with success (van Overveld et al., 2000). However, variable results were found with this assay.

The results of the Trolox assay are shown in Figures 5.1 and 5.2 for the two isolated compounds **1** and **2** respectively. The Trolox standard line was plotted with percentage inhibition of the ABTS+ radical against logarithmic concentration of the trolox.

The Trolox curve of compound **1** (amentoflavone) is presented in Figure 5.1. The Trolox curve of this compound had a gradient of 2.916 and a percentage fit of 96.6%, but the TEAC value was 0.9 indicating a similar antioxidant activity to that of Trolox.

The Trolox curve of compound **2** (4' monomethoxyamentoflavone) Figure 5.2 has a gradient of 46.79 and a percentage fit of 82.7%. With a TEAC value of 2.2, this compound has twice the antioxidant activity of the vitamin E analogue. A similar result was obtained by Zishiri (2004), where he reported a TEAC value of 2.3 in an extract labeled 1a in *Combretum woodii*. Therefore, compound **2** (4' monomethoxyamentoflavone) is a good antioxidant compound. *Garcinia* species are a source of free radical inhibitors as several antioxidant compounds have been isolated from *Garcinia vieillardii* (Hay et al., 2004), *Garcinia brazilliense (Martins 2008),* from the crude extracts *Garcinia virgata* (Merza et al., 2004), *Garcinia dulci* (Deachathai et al., 2006) respectively.
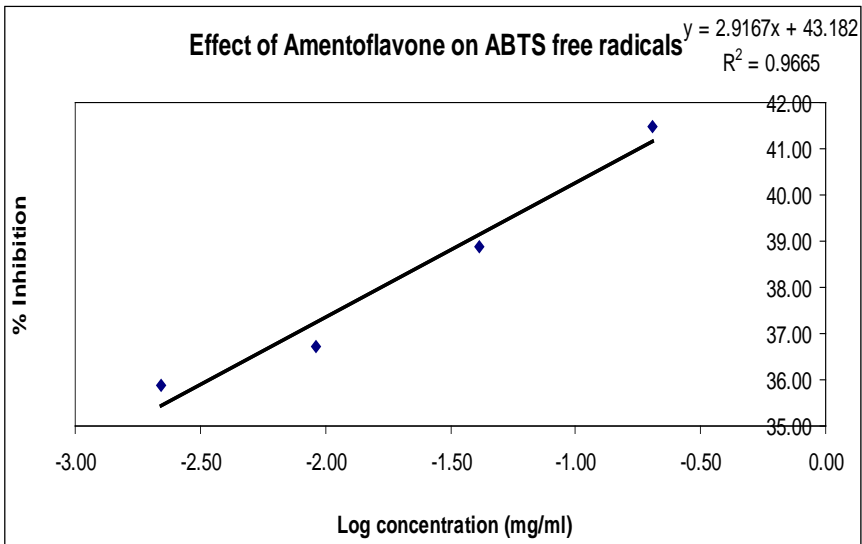
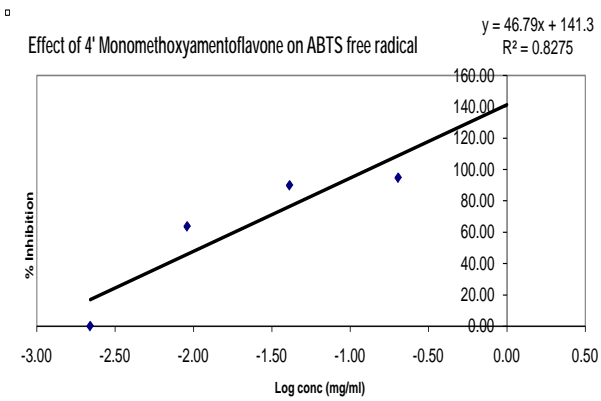**Figure 5.1** Standard curve of percentage inhibition of ABTS+ free radical by amentoflavone (**Compound 1)**

Figure 5.2 Standard curve of percentage inhibition of ABTS+ free radical by monomethoxyamentoflavone (**Compound 2)**

5.3.4 Ames genotoxicity assay

The Ames test is very useful for detecting mutagens under laboratory conditions. It was specifically designed to detect chemically induced mutagenesis. In fact, it is commonly used in an initial screening to determine the mutagenic potential of new chemicals and drugs (Mortelmans and Zeiger, 2000).

The isolated compounds were tested for their genotoxic properties using the Ames test as described by Maron and Ames (1983). Only limited quantities of the isolated compounds (**1** and **2**) were available so, the Ames (plate incorporation) test was for this reason only performed on *Salmonella typhimurium* strain TA98 according to the well-known protocol of Maron and Ames (1983). The strain TA98 allows detection of frame shift mutations. Verschaeve et al. (2004) reported that this strain detects most mutagenic compounds so it is the most appropriate strain to be used in case of shortage of sample, and this also led to the selection of strainTA98 in this work. Five replicate plates were used for negative (solvent) controls whereas triplicate plates were incubated for cultures exposed to the compounds. Negative and positive control cultures gave numbers of revertant per plate that were within the normal limits in accordance with literature data (Mortelmans and Zeiger, 2000). A compound is considered mutagenic if the number of revertants per plate (mean of 5 or 3 replicate plates as indicated (Table 6.2) is at least doubled over the spontaneous revertant frequency.

Amentoflavone had potential mutagenic effects at the concentrations from 100-400 µg/plate. There seemed to be a dose dependent effect as shown by an increase in the number of revertant colonies with the increase in concentration of the mutagenic compound. In contrast with amentoflavone the methoxy derivative 4' monomethoxyamentoflavone was inactive (Table 5.2). Mutagenic activity of amentoflavone has been reported earlier (Cardoso et al., 2006). Structure-mutagenicity relationship was studied and reported by Jaen et al. (1993). In their report, they showed that structural requirements for mutagenicity include an amine group and a fully aromatic tricyclic structure. The compounds isolated in this work conform to aromatic tricyclic structure; the two only differed in presence of hydroxyl and methoxy group(s) in compound **1** and **2** respectively. The presence of a methoxy group might have accounted for the inactivity of compound **2** (4' monomethoxyamentoflavone) observed in the Ames genotoxicity assay in this work.

The results of the genotoxicity assay showed the presence of genotoxic elements in *Garcinia livingstonei*. Therefore, based on our results, the use of this plant extract in traditional medicine for the treatment of infections and diarrhoea should be done with caution to avoid mutagenic effects in humans and animals.

Table 5.2 Mean number of revertants per plate in *S. typhimurium* strain TA98 exposed to isolated compounds **1** and **2**

Concentration (µg/plate)					
Compound	400	200	100	10	1
1	135±3.6	76.3±3.5	56±7	30±2.6	32±7.6
2	-	-	20.6±1	18.7±1.5	19.3±1.5
+ve control	371±35.7				
-ve control	24.8±.3.4				
4-NQO(0.1mg/plate)					

Compound **1** (amentoflavone), Compound **2** (4' monomethoxyamentoflavone), **4-NQO**=4- nitroquinoline 1-oxide, **+ve** = positive, **-ve** = negative

5.3.5 Tetrozolium-based colorimetric MTT assay of isolated compounds

In vitro cytotoxicity assays are useful for screening purposes to define cellular toxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisenbrand et al., 2002). The method of Mosmann (1983) was used. This is based on the principle that a mitochondrial dehydrogenase enzyme from viable cells will cleave the tetrazolium ring of pale yellow MTT and form dark blue formazan crystals which are largely impermeable to the cell membrane, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of formazan product created which is read spectrophotometrically.

The cytotoxic concentrations of the compounds inhibiting the growth of 50% of the cells (CC_{50}) were calculated according to the formula of Hussain et al (1993). The formula for the calculation of CC_{50} is provided below as follows:

C> CC_{50} - {[(C > CC_{50} - C < CC_{50}) * (Abs > CC_{50})]/ (Abs > CC_{50} - Abs <CC_{50})}

Where **Abs CC_{50}** is the average of the absorbance of the control's cells absorbance divided by 2

The calculated CC_{50} values of the two compounds were 381 µg/ml and >600 µg/ml for compounds **1** and **2**, respectively. In comparison with the calculated CC_{50} of berberine, the positive control, which gave a CC_{50} of 170 µg/ml, these compounds are relatively non-toxic at the concentrations tested.

The therapeutic index calculates the safety factor between the concentration that would kill the pathogens and the concentration that is toxic to cells. For amentoflavone this value was between c. 4 and 10 for the four bacterial pathogens and for 4' monomethoxyamentoflavone the value was between >10 to >75 for the four bacterial pathogens. This indicates the relative safety of using these compounds in therapy.

There are several reports on the inactivity of amentoflavone in cytotoxicity assays. Woo et al (2005) have measured cytotoxicity of amentoflavone on RAW 264.7 cells by MTT assay. They found that cell viability was not significantly affected by amentoflavone up to 100 µM. Equally, Silva et al (1994) reported no cytotoxic activity of amentoflavone isolated from *Selaginella willdenowii* and the ability of amentoflavone to significantly reduce the SNP-induced cell death. Amentoflavone also significantly reduced Ab25–35-induced cytotoxicity with higher protective effects. Amentoflavone also reduced etoposide-induced cell death (Kang et al., 2005).

Similarly, Kuo et al (2008) evaluated several diterpenoids, biflavonoids, aromatics, and several monoflavonoids for cytotoxicity against human KB, Hela, Hepa, DLD, and A-549 tumor cell lines. In their experiments amentoflavone had no cytotoxicity.

4' Monomethoxyamentoflavone (**2**) was non-toxic at the highest concentration tested, which agrees with Lin et al (1994) who reported inactivity of two other derivatives of amentoflavone 7, 7"-di-O-methylamentoflavone and 7, 4', 7", 4"-tetra-O-methylamentoflavone against L929 murine cells. Studies on structure-cytotoxicity relationships have shown that the presence of two methoxyl groups, as in 4', 7"-di-O-methylamentoflavone enhanced the cytotoxicity of the compound on Co12 and U373 cell lines (Silva et al., 1994). This was not the case in this study. It is possible that different cell types could have different responses to isolated compounds. However, there is only one methoxyl group attached to compound (**2**) isolated in this study. Structure–activity relationships of biflavonoids are important in determining cytotoxicity, and methoxy as well as hydroxy groups in biflavonoids and monoflavonoids respectively, play a crucial role in mediating cytotoxic activity. The two compounds amentoflavone and 4' monomethoxyamentoflavone were observed to be non-toxic to Vero monkey kidney cells at the concentrations tested.

5.4 CONCLUSION

Garcinia livingstonei contains antimicrobial compounds that possess activity against different bacteria. The bacteria tested were sensitive to the isolated compounds at various concentrations tested. *Staphylococcus aureus* showed less sensitivity to amentoflavone (**1**) with MIC value of ≥100 µg/ml than to monomethoxyamentoflavone (2). The MIC ranges of the two isolated compounds were 8-100 µg/ml. The antioxidant capacity of the two compounds was evaluated using the Trolox assay. The antioxidant status of amentoflavone (**1**) was equivalent to vitamin E analogue. 4' monomethoxyamentoflavone (**2**) was found to have twice antioxidant capacity than the vitamin E analogue (Trolox).

The results of the MTT assay showed the compounds to be safe on mammalian cellular toxicity assay using Vero cells. However, when the genotoxic potential of the two based compounds was assessed; amentoflavone(**1**) had genotoxic properties against using strain TA98 whereas 4' monomethoxyamentoflavone (**2**) was inactive. The results suggest application of *Garcinia livingstonei* crude extract in traditional medicine should be done with caution so as to avoid mutagenic effects in humans and animals.

CHAPTER 6
GENERAL CONCLUSION

The aim of this study was to screen several South African tree leave for isolation and characterization of antibacterial and antioxidant compounds and to determine the cytotoxicity and genotoxicity of the isolated compounds from the most promising plant species. During the preliminary screening sixteen plants were randomly selected based on their ethnomedical usage. Of these 16, 11 were excluded because of extensive work performed on them. Thus, only four plants were screened for their biological activities on four nosocomial bacteria. The four plants are; *Buxus* natalensis, *Macaranga capensis*, *Garcinia* livingstonei and *Dracaena mannii* had good MICs. The remaining plants were excluded because of active work done on them including antibacterial activity studies as reported in various literatures; *Pavetta* was highly toxic. *Garcinia livingstonei* was selected as the most promising species to work with chiefly because of its very low average minimum inhibitory concentration (0.09 mg/ml) and high total activity compared with the rest of the species. Also, clear zones of inhibition on bioautograms of extracts of *Garcinia livingstonei* makes it easy to isolate the bioactive compounds. Even though some other species had low minimum inhibitory concentrations, absence of zones of inhibition on the bioautograms excluded those plants from further studies. The reason why there was absence of activity in bioautography of the excluded plants might be due to the fact that the compounds were volatile and escaped from the TLC after loading.

For the bulk extraction, 300 g of *Garcinia livingstonei* leaves were extracted with acetone which yielded 20 g of dried black extract. The percentage extractive yield was 6.7%. The dried extract was subjected to solvent-solvent fractionation which afforded seven fractions. The activities of these fractions were tested on four bacterial organisms; chloroform and ethyl acetate fractions were the most active with clear zones of inhibition against *Escherichia coli* and *Staphylococcus aureus.*

Column chromatography led to the isolation of amentoflavone and 4' monomethoxyamentoflavone. There were indications of the presence of many other compounds based on the separation on the TLC plates, but the other compounds were not active against the test bacteria in the bioautography assay. Compounds **1** and **2** were isolated in pure form and in sufficient quantity for all the conducted biological assays. The quantity of compound **1** was 89 mg and that of compound **2** was 110 mg, and were all active on the four bacterial organisms tested at average MIC range of 8-100 µg/ml, methoxyamentoflavone being more active than amentoflavone. Of the four organisms tested, *Staphylococcus aureus* was the least sensitive to compound **1** with an MIC value of 100 µg/ml, whereas

Enterococcus faecalis was the most sensitive organism with the lowest MIC values of 8 µg/ml for methoxyamentoflavone. These compounds could be possible leads for the development of antibacterial pharmaceuticals if different derivatives are syntesized. The major difference in toxicity results from a hydroxyl with a methoxy moiety, the methoxy giving higher antibacterial activity in our case.

The two compounds were tested for antioxidant activity, and amentoflavone had an antioxidant capacity close to that of Trolox with a TEAC value of 0.9. However, 4' monomethoxyamentoflavone) possessed antioxidant activity twice that of the vitamin E analogue (Trolox) with a TEAC value of 2.2. Therefore, the two compounds and especially monomethoxyamentoflavone are potential antioxidant compounds.

The mutagenic potential of the two compounds was assessed by the Ames test against *Salmonella* strain TA98. Amentoflavone had some genotoxic effect on the tested strain with double the number of induced revertant colonies compared with the blank (negative control) at the concentration of 100 µg/plate. However, 4' monomethoxyamentoflavone was inactive in this assay.

On all accounts 4' monomethoxyamentoflavone proved to be more optimal than amentoflavone in all its biologic activities. It had higher antibacterial activity, higher antioxidant activity coupled with a much lower cellular and genotoxicity.

These results indicate the potential value of relatively minor structural changes for the usefulness and application of chemical compounds.

CHAPTER 7
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APPENDIXES: SPECTRA AND SPECTRAL DATA ANALYSIS FOR THE ISOLATED COMPOUNDS

**Appendix Table 1:** ^{1}H NMR and ^{13}C NMR for isolated compound (**1**) amentoflavone

| Carbon position	Chemical shift			
	^{1}H		^{13}C	
	I	II	I	II
2	-	-	161.39	164.56
3	6.72	6.66	103.4	102.9
4	-	-	182	182.8
5	-	-	161.4	160
6	6.24	6.46	99	99.12
7	-	-	162.5	161.9
8	6.51		94.2	103.4
9	-	-	158.1	156
10	-	-	104.6	104.6
1	-	-	120.5	122.3
2	8.12	7.66	128.0	128.4
3	-	6.84	122.3	116
4	8.04	-	159.8	116.1
5	-	6.84	116.7	116.1
6	8.04	7.66	131.8	128
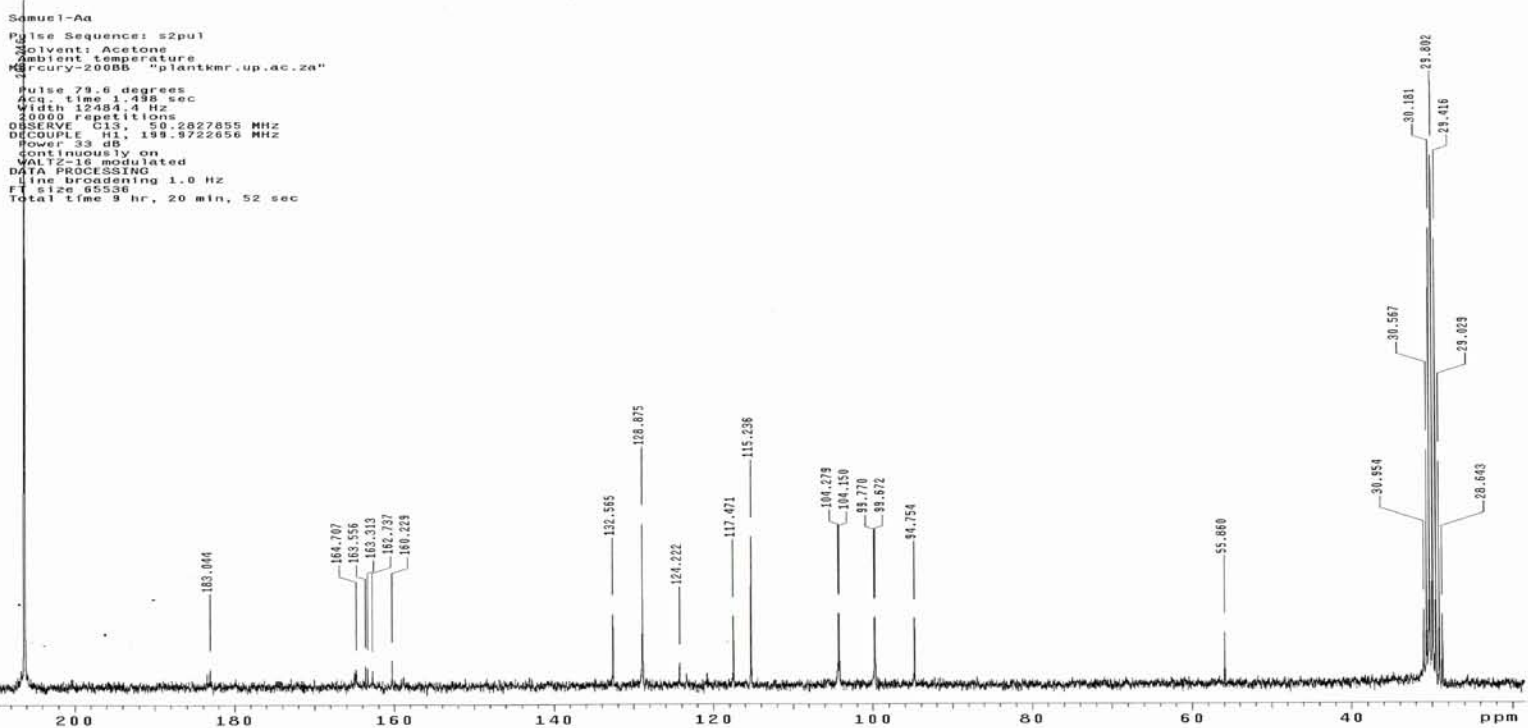
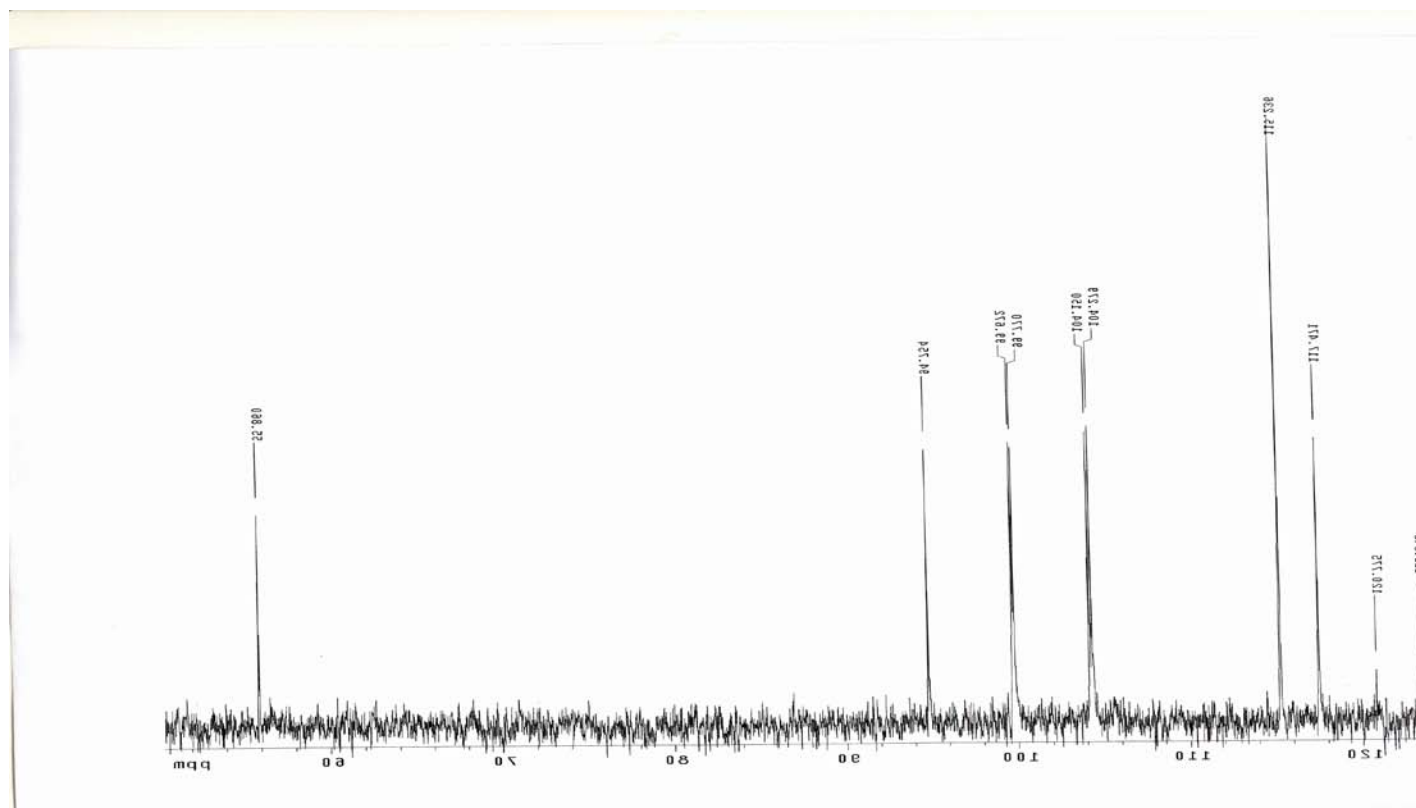Appendix 2a: ^{1}H NMR spectrum of compound 1 (amentoflavone)
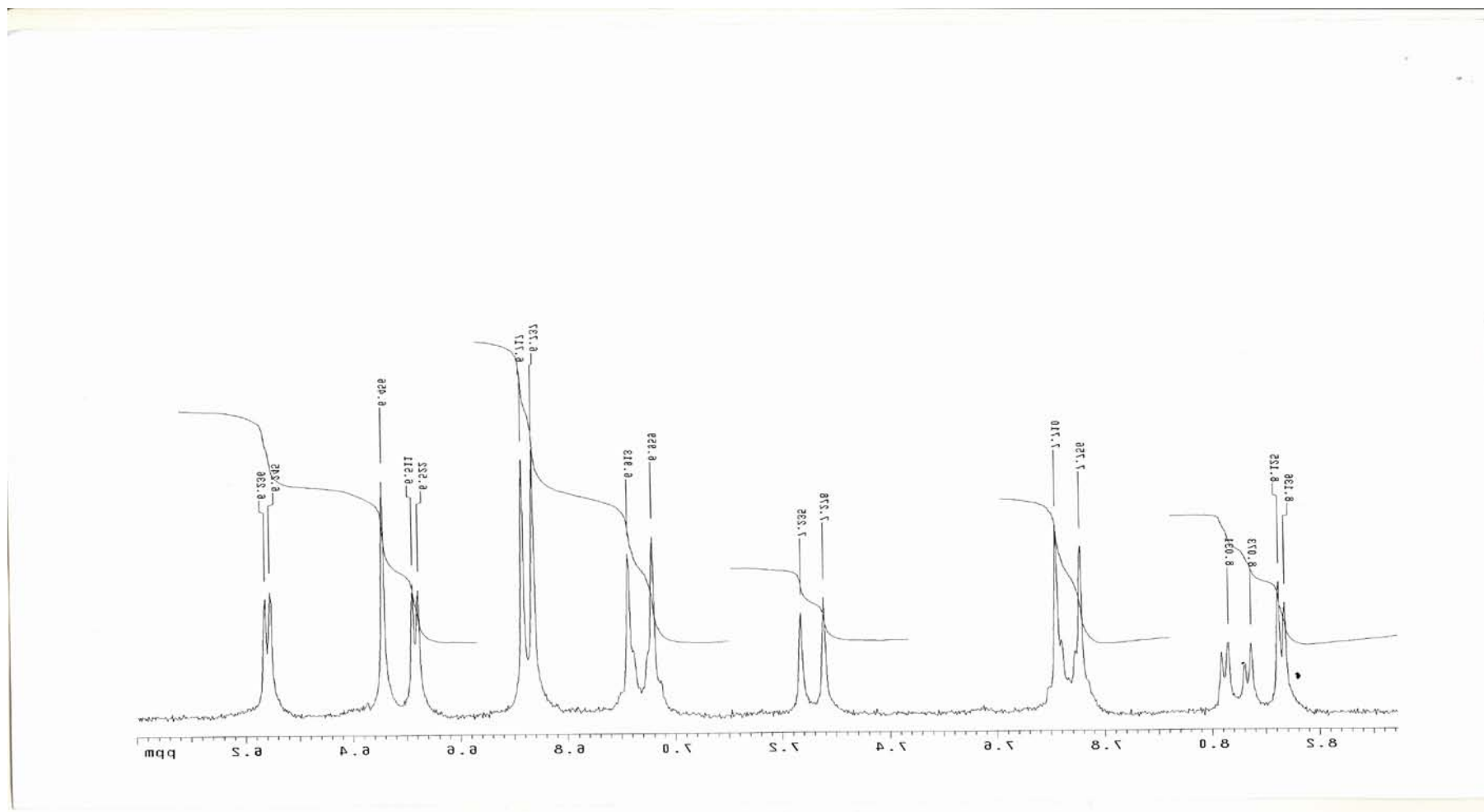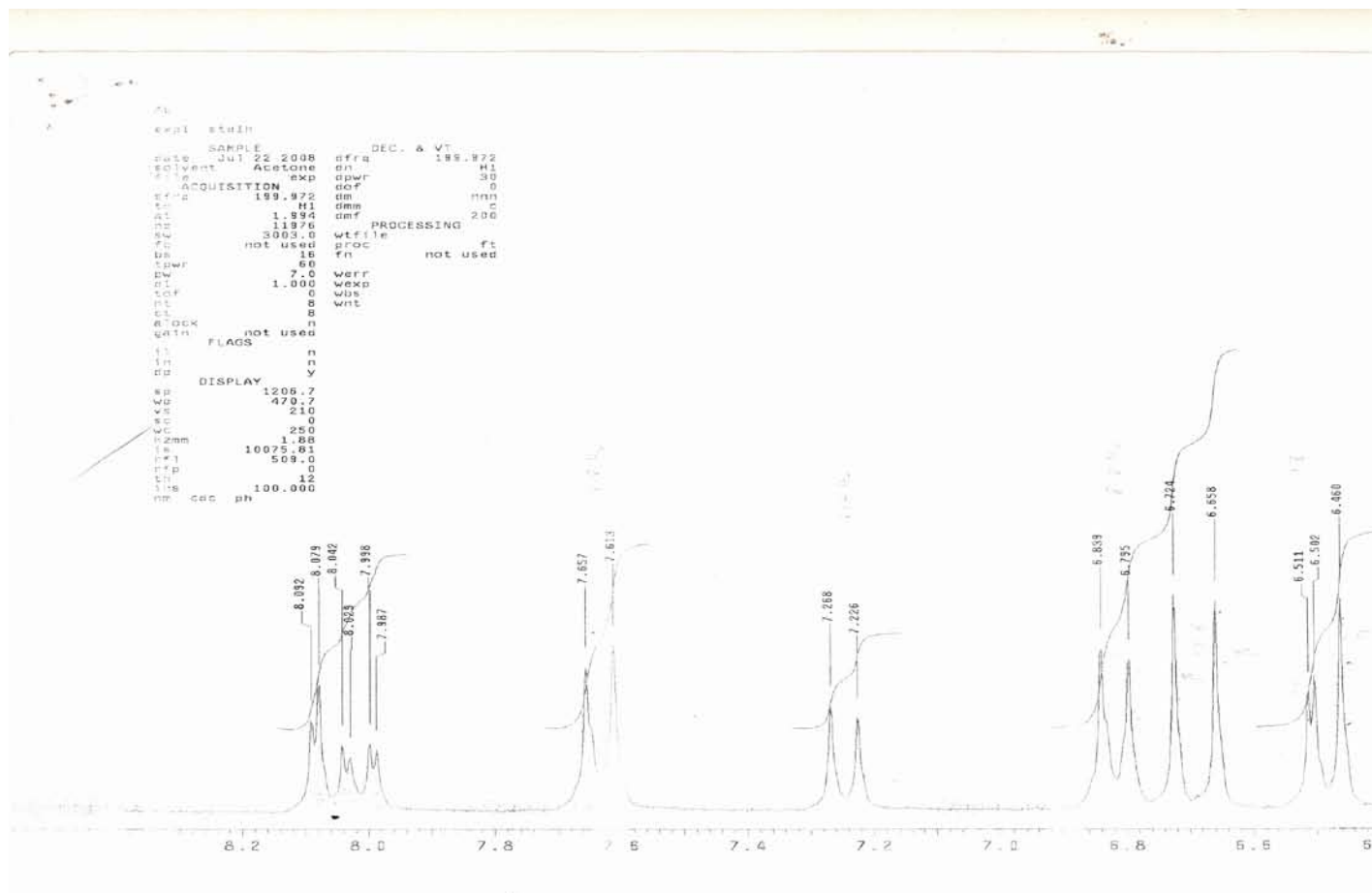
Appendix 2b: ¹H NMR spectrum of compound 1 (amentoflavone)
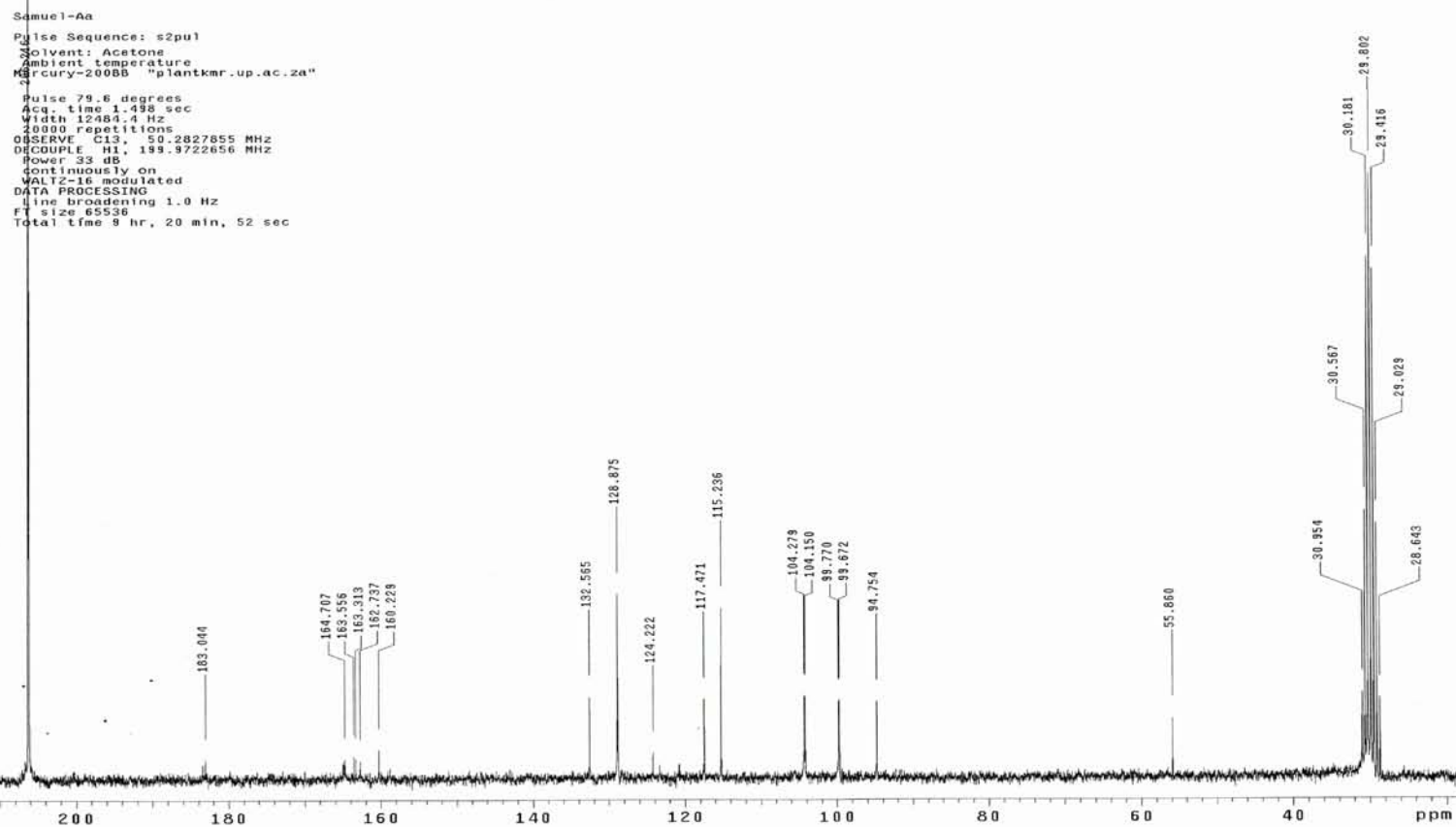
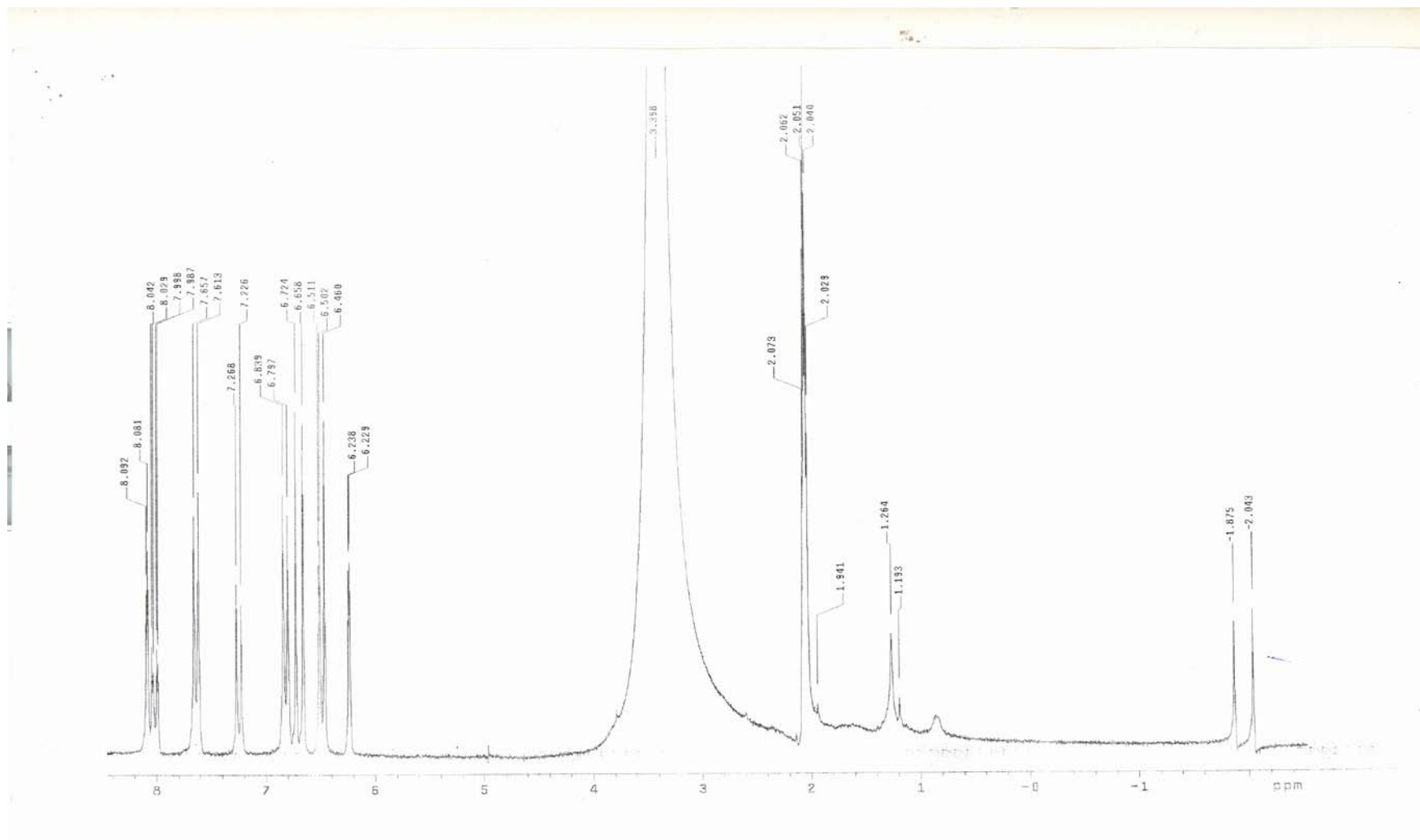Appendix 2c: ^{13}C NMR spectrum of compound 1 (amentoflavone)
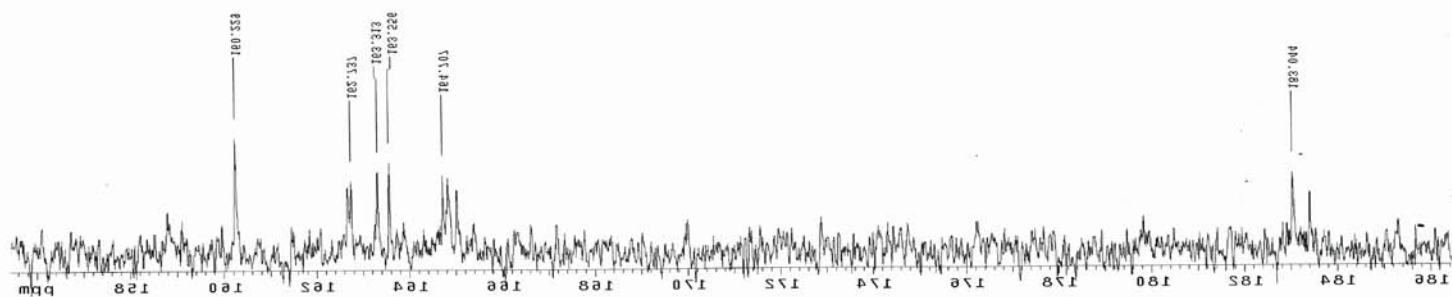
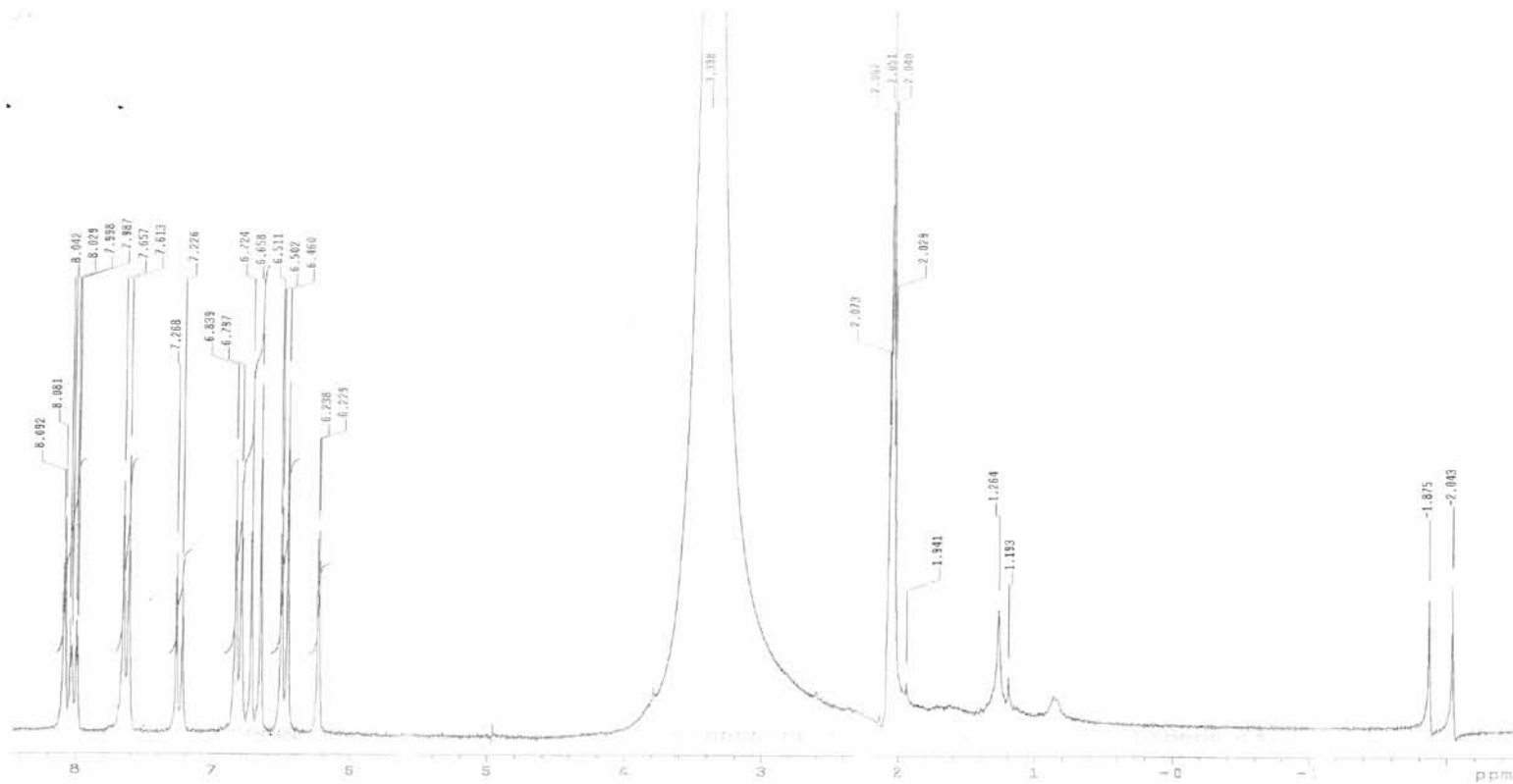Appendix 2d: ^{13}C NMR spectrum of compound 1 (amentoflavone)

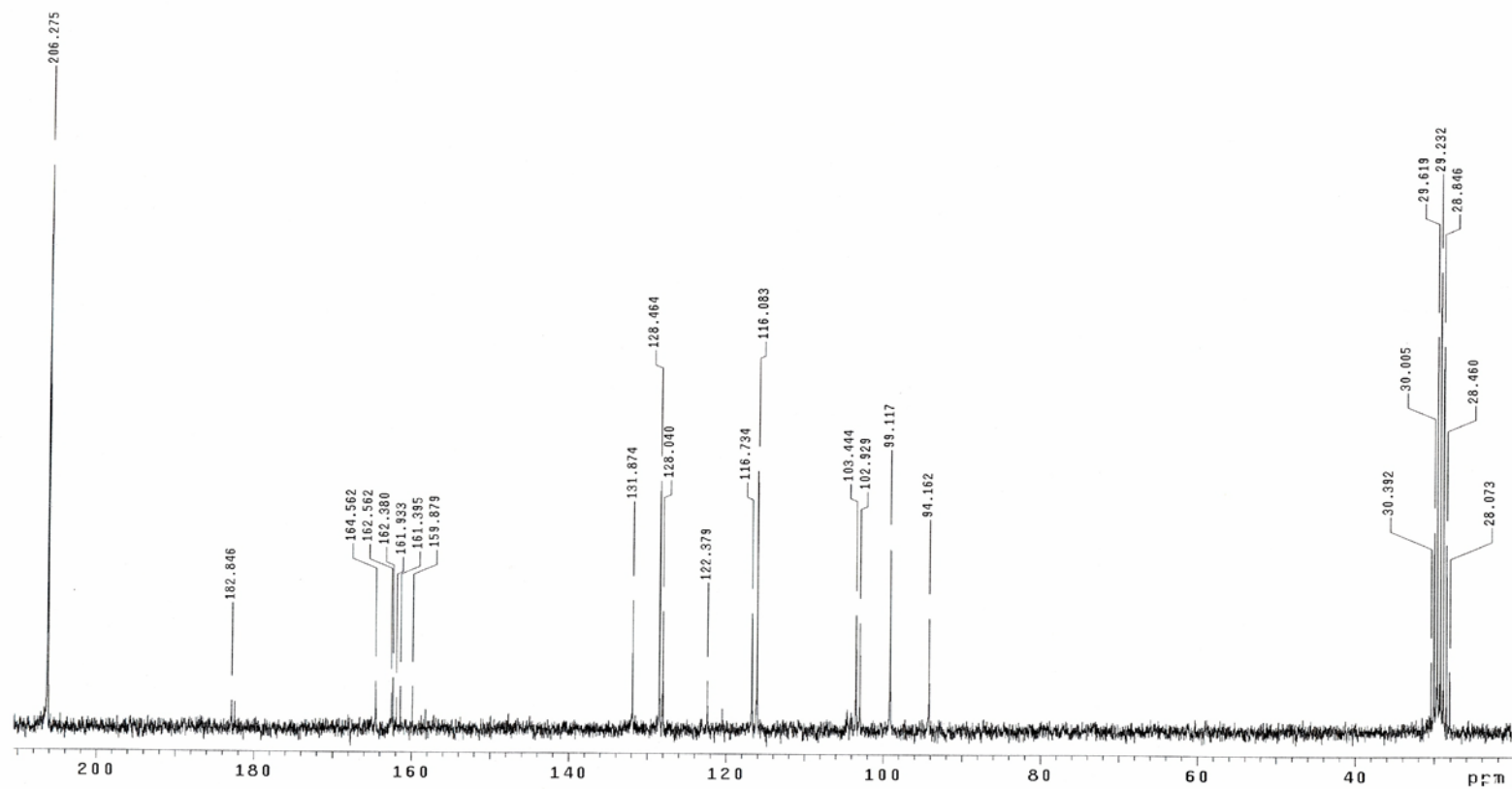**Appendix 2e:** ^{13}C NMR spectrum of compound 1 (amentoflavone)

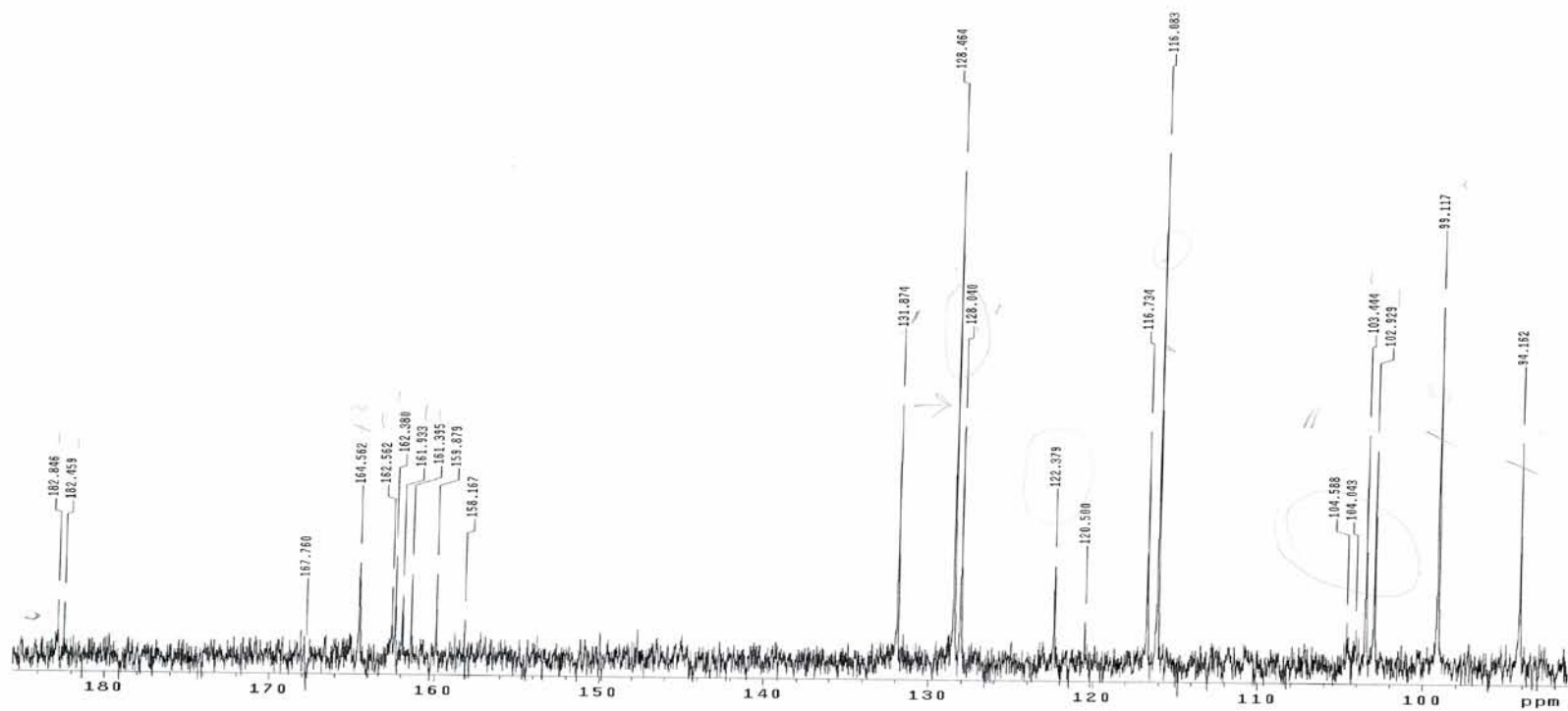Appendix 1a: ^{1}H NMR spectrum of compound 2 (4' monomethoxyamentoflavone)

Appendix 1b: ¹H NMR spectrum of compound 2 (4' monomethoxyamentoflavone)

Appendix 1c:^{1}H NMR spectrum of compound 2 (4' monomethoxyamentoflavone)

Appendix 1d: ^{13}C NMR spectrum of compound 2 (4' monomethoxyamentoflavone)

Appendix 1e: ^{13}C NMR spectrum of compound 2 (4' monomethoxyamentoflavone)

Appendix Table 2: ^{1}H NMR and ^{13}C NMR for isolated compound (**2**) 4' monomethoxyamentoflavone

Carbon position	Chemical shift			
	[1]H		[13]C	
	I	II	I	II
2	-	-	161.39	164.56
3	6.72	6.66	103.4	102.9
4	-	-	182	182.8
5	-	-	161.4	160
6	6.24	6.46	99	99.12
7	-	-	162.5	161.9
8	6.51		94.2	103.4
9	-	-	158.1	156
10	-	-	104.6	104.6
1	-	-	120.5	122.3
2	8.12	7.66	128.0	128.4
3	-	6.84	122.3	116
4	8.04	-	159.8	161.3
5	-	6.84	116.7	116.1
6	8.04	7.66	131.8	128