CHEMICAL AND BIOLOGICAL INVESTIGATION INTO SOME SELECTED AFRICAN INDIGENOUS MEDICINAL PLANTS

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae in the Department of Chemistry, University of the Western Cape.

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Professor Ivan R. Green

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KEYWORDS

CHEMICAL AND BIOLOGICAL INVESTIGATION INTO SOME SELECTED AFRICAN INDIGENOUS MEDICINAL PLANTS

Keywords

Phytochemistry

Piliostigma reticulatum

Cissampelos capensis

Geranium incanum

Gethyllis species

Antimicrobial

Brine shrimp nauplii

Lethality

Cytotoxicity

Antioxidants

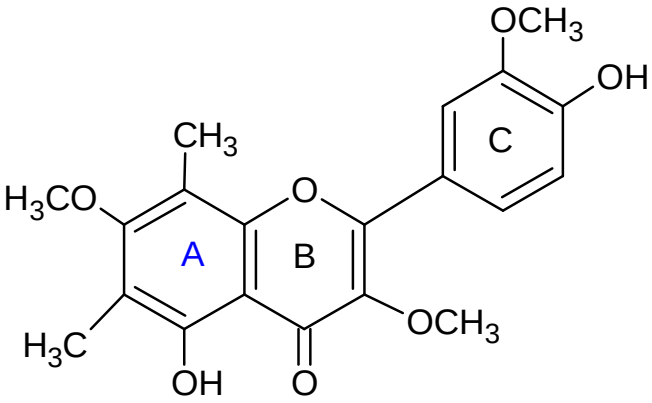
ABSTRACT

CHEMICAL AND BIOLOGICAL INVESTIGATION INTO SOME SELECTED AFRICAN INDIGENOUS MEDICINAL PLANTS

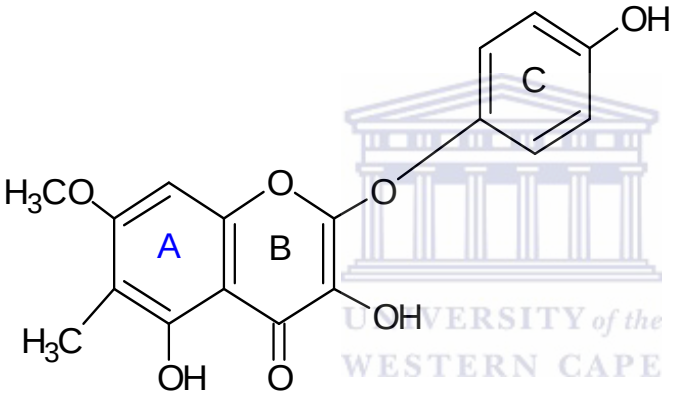
Jelili Olalekan Babajide

PhD Thesis, Department of Chemistry, University of the Western Cape,

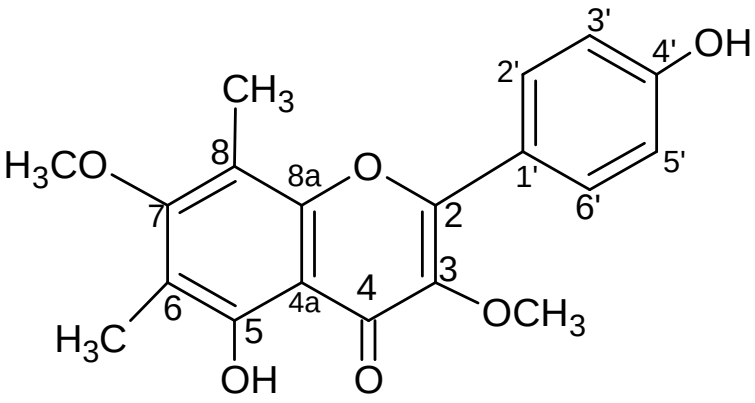
African medicinal plants are commonly used throughout Africa to treat a variety of ailments including wounds and ulcers, cough and chest complaints, gingivitis, fever and gonorrhoea, indication all related to infection and inflammation. In screening several plant species from an inventory of common medicinal plants from both South and West Africa for diverse medicinal purposes, 6 plants were selected because of their interesting and useful ethnomedicinal values. These plants are (1) *Piliostigma reticulatum* from the family *Caesalpiniaceae*, (2) *Cissampelos capensis* (family: *Menispermaceae*) (3) *Geranium incanum* (family: Geraniaceae) (4) *Gethyllis gregoriana*, (5) *Gethyllis multifolia* and (6) *Gethyllis villosa.* The three *Gethyllis* species belong to the *Amaryllidaceae* family. This study attempt to relate specific constituents present in these plants with their widespread ethnomedicinal uses. The extractions were carried out using broad spectrum of solvents (hexane, dichloromethane, ethyl acetate, methanol and water). Fractionation was carried out using standard chromatographic techniques. A total of thirty seven (37) compounds were isolated from three of the plants namely: *P. reticulatum, C. capensis and G. incanum* while only extractions were carried out on the *Gethyllis* species. Total of 18 isolates were characterised and their structures were unambiguously established by spectroscopic methods including infrared and ultraviolet spectroscopy, high resolution mass spectrometry and one- and two-dimensional nuclear magnetic resonance experiment.



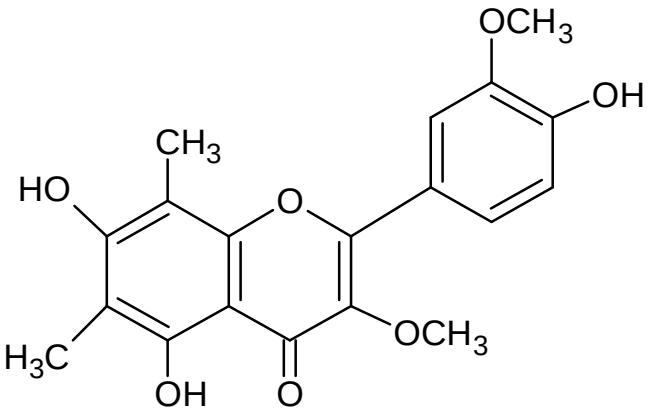
(P1) *6,8-di-C-methylquercetin- 3,7,3'-trimethyl ether* (***new compound***),



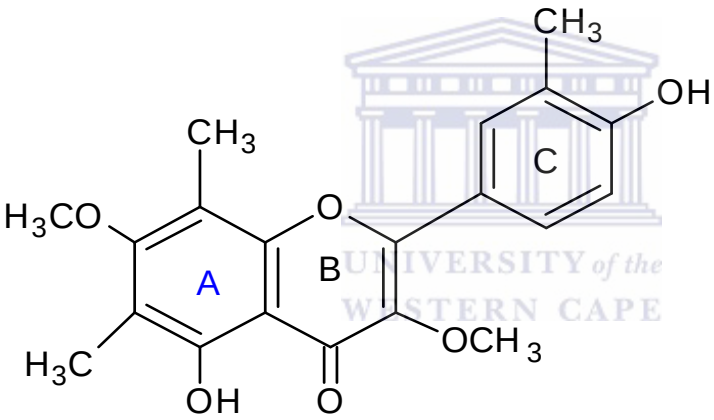
(**P2)** *Piliostigmol* (***new compound***),



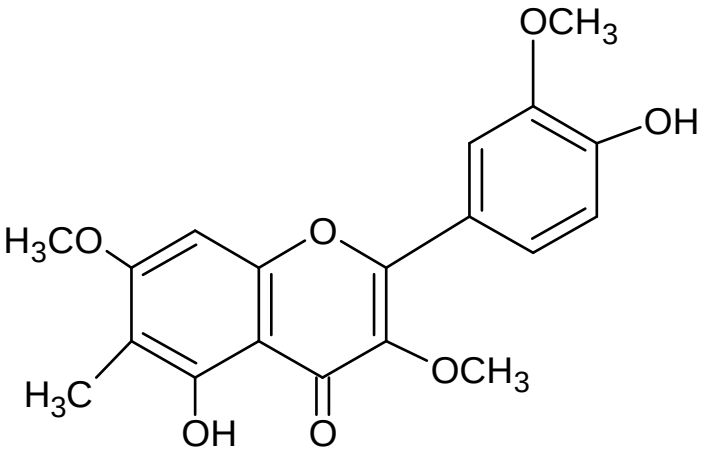
(**P3)** *6, 8-di-C-Methylkaempferol- 3,7-dimethyl ether*,



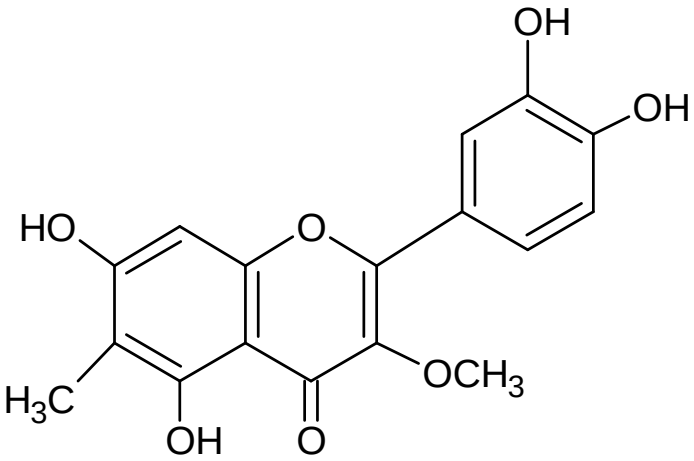
(**P4)** *6,8-di-C-methylquercetin -3,3'-dimethyl ether* (***new compound***),



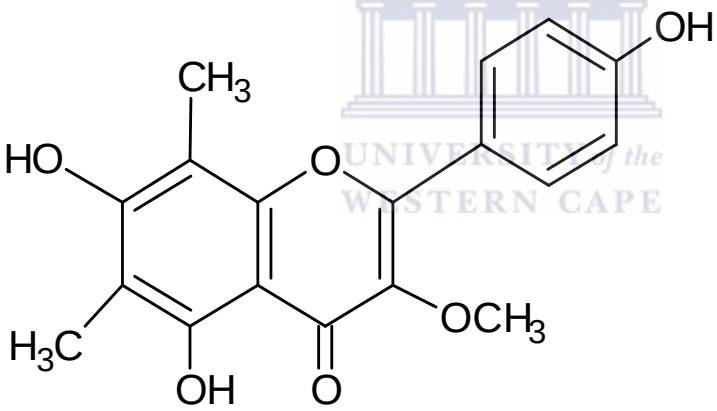
(**P5)** *6,8,3'-tri-C-methylquercetin- 3,7-dimethyl ether* (***new compound***),



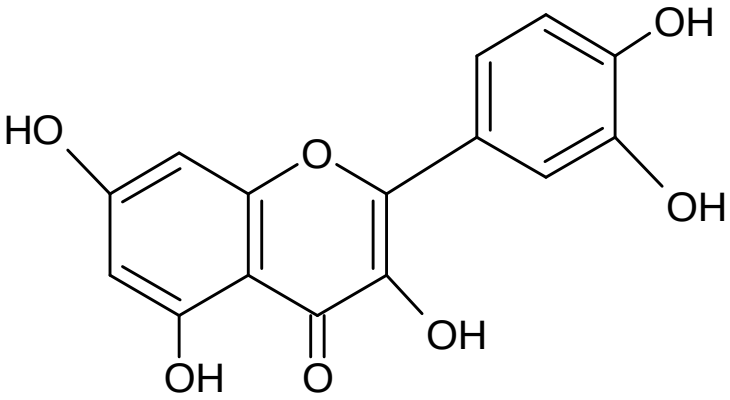
(**P7)** *6-C-methylquercetin -3,7,3'-trimethyl ether*,



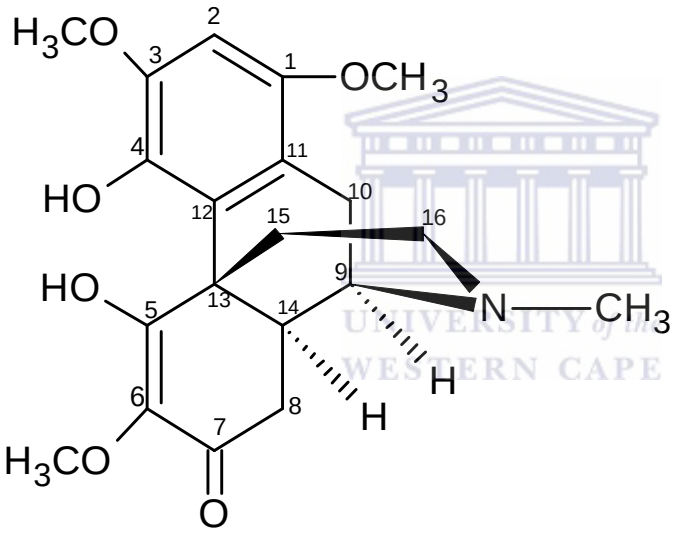
(**P8)** *6-C-methylquercetin- 3-methyl ether*,



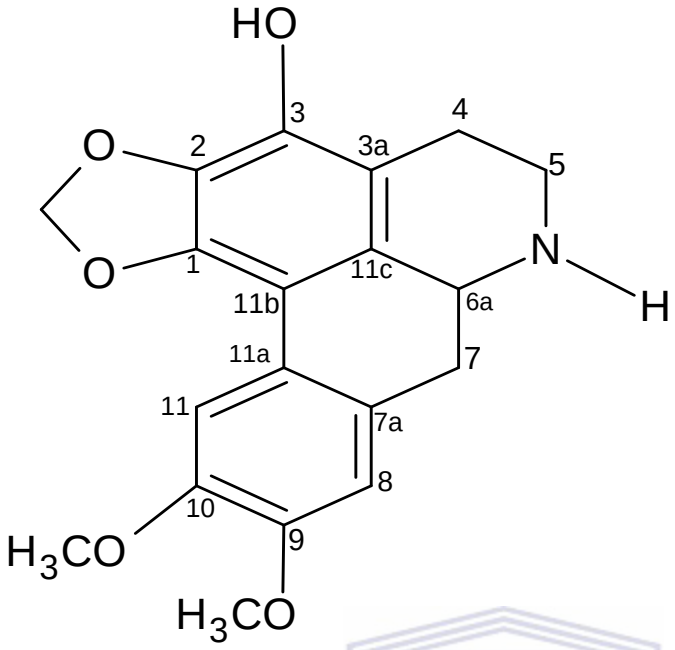
(**P9)** *6,8-di-C-methylkaempferol- 3-methyl ether* and



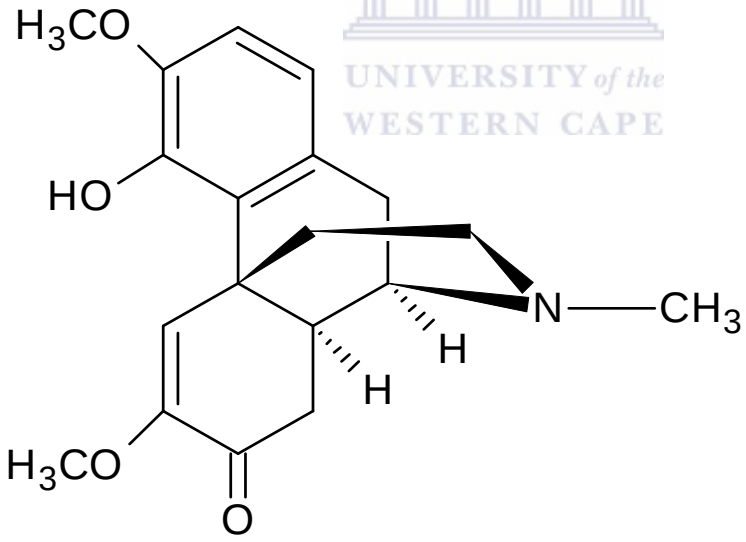
(**P13)** *quercetin* were isolated from *P. reticulatum* while



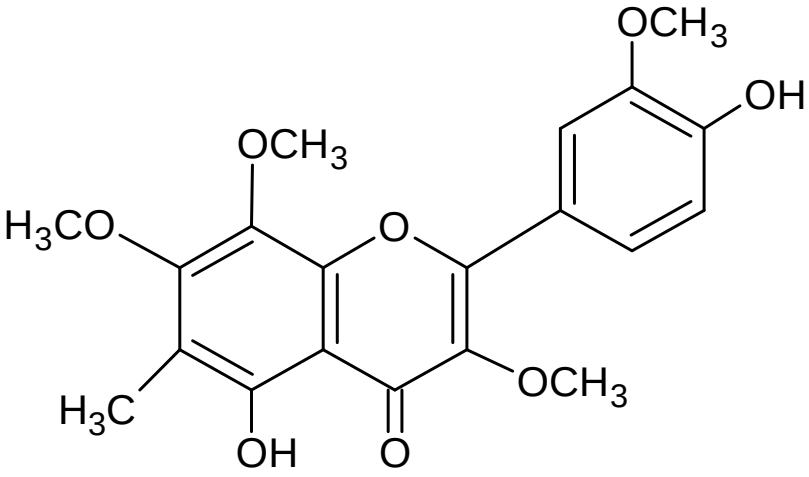
C1) *5,6-dehydro-4,5-dihydroxy-1,3,6-trimethoxy- 17-methylmorphinan-7-one* (***new compound***),



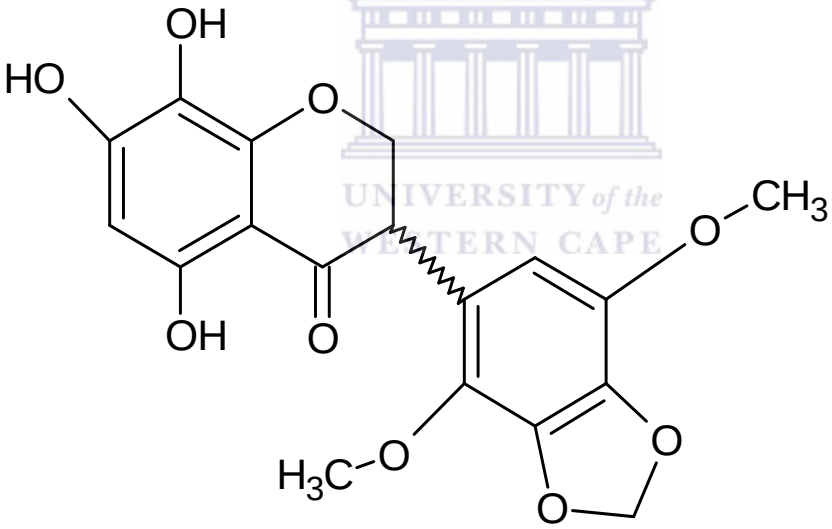
(**C2)** *1,2-methylenedioxy-3- hydroxy -9,10-dimethoxyaporphine* (***new compound***),



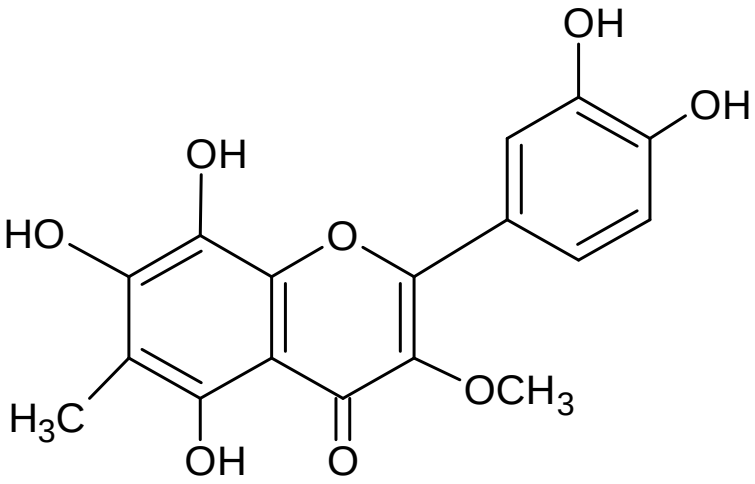
(**C3)** *5,6-didehydro-4-hydroxy-3,6-dimethoxy-17-methylmorphinan-7-one*,



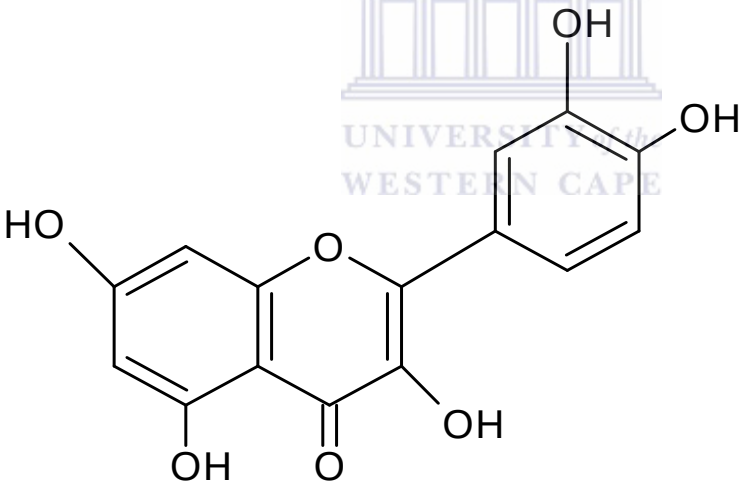
(**C5)** *3,7,8 3'-tetramethoxy- 6 - C-methyl- 5,4'-dihydroxyflavone* (*6 -C-methylquercetin 3, 3',7, 8 -tetramethyl ether*) (***new compound***),



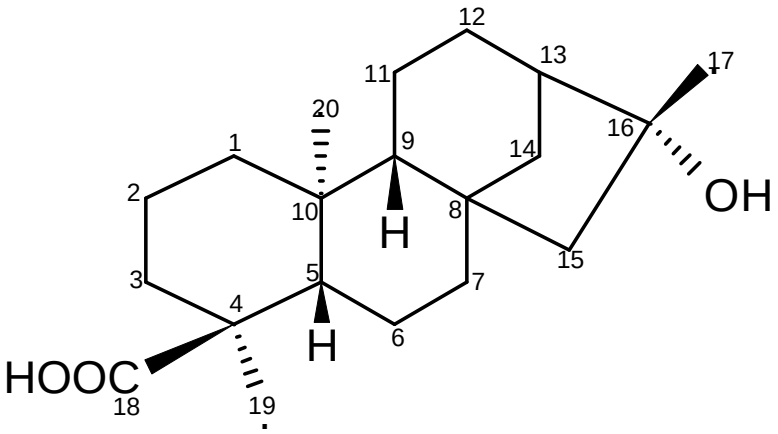
(**C6)** *5, 7, 8 -trihydroxy-2', 5'-dimethoxy-3',4'- methylenedioxyisoflavanone* (***new compound***) and



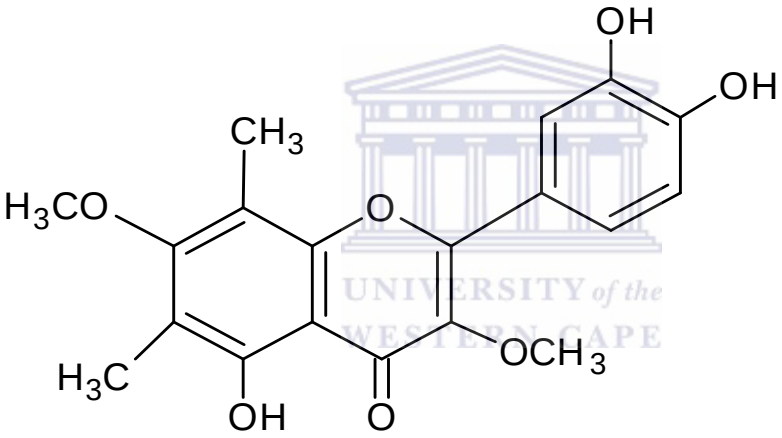
(**C11)** *3 -methoxy-6 -C-methyl-3',4',5,7,8 -pentahydroxyflavone* (*6 -C-methylquercetin -3-methyl ether*) were isolated from *C. capensis and G. Incanum* offered



(G5) *quercetin,*



(G10)*16α-hydroxy-(-) kauran-18-oic acid* (***new compound***) and



(G12) *6, 8-di-C-methylquercetin 3, 7-dimethyl ether* (***new compound***).

Ten (10) of the isolated compounds were novel, including the first ever C-methyl-2-phenoxychromonol, piliostigmol, 2-O-(p-hydroxyphenyl)-3,5-dihydroxy-6-C-methyl-7-methoxychromone, the diterpene16α-hydroxy-(-)-kauran-18-oic acid, and five C-methylflavonols, isoflavanone, aporphine and a dehydromorphinandienone. The different extracts, fractions and isolates were tested for cytotoxicity using brine shrimp lethality test and antimicrobial capability using nine microbes with three gram –ve and +ve bacteria and three fungi. The gram-negative bacteria were *Pseudomonas aeruginosa* (NCTC 10332), *Proteus vulgaris* (NCTC 4175) and *Escherichia coli sero type 1* (NCTC 09001), while the gram-positive were *Bacillus*

subtilis (NCTC 8236), *Staphylococcus aureus* (NCTC 13134) and *Bacillus licheniformis* (NCTC 01097). The fungal species used were *Candida albicans* (ATCC 90028), *Candida eropiralis* (ATCC 750) and *Aspergillus niger* (ATCC 10578). *G. gregoriana* showed the highest cytotoxicity level of LC_{50} = 0.2229 where as it was observed that all the hexane fractions were not active while most activities resides in the methanolic extracts. The total tertiary alkaloid (TTA) showed the highest activity against *B. substillis* at 45mm while same trend observed in the cytotoxicity test were also observed in the antimicrobial activity, where all the hexane fractions showed no activity against any of the nine pathogens. The *P. reticulatum* was also found to exhibit antimicrobial activity against some bacteria and fungi such as *S. aureus, E. coli, B. subtilis, P. vulgaris, A. niger* and *C. albicans*. Piliostigmol, showed the highest activity against *E. coli* (MIC = 2.57 μg/ml, 0.006 μmol), which was found out to be three times more active than the amoxicillin standard used. The total antioxidant assay also reveal a huge amount of information into the nature and properties of each extracts and isolates in which the distribution of polyphenols, flavonoids and anthocyanins present in the plant extracts were reavealed. The ORAC, FRAP and TEAC showed intensively the antioxidant capacity of the extracts and isolates in which it was evident that the methanolic extracts and isolates obtained from them are good antioxidant agents. The results obtained for this study give a useful profile for development of a good antimicrobial and antioxidant agents for the future from the results obtained. The observed antimicrobial and antioxidant activities of the isolated compounds were correlated with their structures, and the structural requirements for activity in both test systems were defined. Finally, the traditional use of the extracts in infections and inflammatory conditions was rationalized based on the content of the isolated compounds, and it has been proposed that the total crude extract, with its content of so many bioactive compounds, could be formulated for use in skin infections, microbial, viral and inflammatory conditions.

DECLARATION

I declare that "Chemical and biological investigation into some selected African indigenous medicinal plants" is my own work, that it has not been submitted before for any degree or assessment in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Jelili Olalekan Babajide November 2009

Signature _____

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I acknowledge God first and foremost for giving me the strength and wisdom to complete this work. HE has made this happen and beautiful in his own time.

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Special thanks go to my great friend, Dr Richard Akinyeye for our many friendly scientific and general discussions and who made the inception of this journey possible. You are indeed a blessing to me.

I also wish to express warmest thanks to all my colleagues and friends at the Organic Laboratory, Chemistry Dept, U.W.C for their collaboration, encouragement, and tireless help. While it is impossible to mention everyone who has been of help, I would like to single out a few who have been especially instrumental. It was pleasure to work in the same laboratory with Tanya, Sarah, Wendell, Duwelani and Lawrence

and a privilege to learn from Fani Rautenbach (CPUT) who found answers to so many of my questions and assisted me with his instrumentational skills in antioxidants.

I would like to appreciate Prof Bouic P.J.D. & Mrs Oduwole E.O. of Synax both of Department of Medical Microbiology, Faculty of Health Sciences, Stellenboch University, South Africa for providing the microbial pathogens used in this study, Mr N Coldery of the Medical biosciences department, University of the Western Cape for assisting with some of the microbial assay, Mr Gordon Summerfield of Summerfield's Indigenous Bulbs and Seeds, Cape Town, for donating the *Gethyllis* plant materials and Mr Franc Weitz for the identification and collection of some plant materials.

My warmest thanks go to all my good friends, especially to Dr. Olatokunbo Abayomi, Segun Akinyemi, Dr Omotayo Arotiba, Pastors- Sola Oduwole, Lanre Fatoba, Wale Olowu and the Household of God, UWC brethren for numerous stimulating discussions, prayers and the encouragement needed through life's difficult moments.

I am deeply grateful to my parents Mr and Mrs A.A. Babajide and in–laws (Elder and Rev. Mrs. Onaneye) for their great support and encouragement.

Most of all I wish to thank my lovely wife Omotola, my daughter Oyinkansola and son Ayomikun. They have been endlessly patient and supportive, while reminding me that occasionally there are things more important than chemistry.

LIST OF PUBLICATIONS

1. Olalekan J. Babajide, Omotola O. Babajide, Abimbola O. Daramola, Wilfred T. Mabusela. (2008). Flavonols and an oxychromonol from *Piliostigma reticulatum*. *Phytochemistry*, **69**, 2245–2250

2. Olalekan J. Babajide, Wilfred T. Mabusela, Ivan R. Green, Farouk Ameer, Frans Weitz and Emmanuel I. Iwuoha. (2009). Phytochemical and biological activity studies of five South African indigenous medicinal plants. *Journal of medicinal plant research* ((JMPR-09-401) accepted for publication).

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LIST OF ABBREVIATIONS

AGC	Accelerated Gradient Chromatography
ATCC	American Typed Culture Collection
CGC	Column Gradient Chromatography
CIMS	Chemical ionization Mass Spectroscopy
^{1}H -^{13}C COSY	Heteronuclear Shift- Correlated Spectroscopy
^{1}H -^{1}H COSY	Homonuclear Shift -Correlated Spectroscopy
1D	One dimensional
2D	Two Dimensional
DEPT	Distortionless enhancement by polarization transfer
EIMS	Electron Impact Mass Spectroscopy
EtOAc	Ethyl acetate
EtOH	Ethanol
MeOH	Methanol
$CHCl_3$	Chloroform
Tolu	Toluene
HMBC	Heteronuclear multiple bond connectivity
HMQC	Heteronuclear multiple quantum correlation
HRCI(MS)	High resolution chemical ionization Mass Spectroscopy
HREI(MS)	High resolution electron impact Mass Spectroscopy
LC_{50}	Concentation that gives 50% inhibition
IR	Infrared
MBC	Minimum bacterial concentration
Me	Methyl
MIC	Minimum inhibitory concentration
MP	Melting point

MS	Mass spectroscopy
NCTC	National collection of typed cultures, central public healthlaboratory, Colindale Avenue, London NW9, England
ND	Not determined
NMR	Nuclear magnetic resonance
NOESY	Two –dimensional nuclear overhauser spectroscopy
OMe	Methoxyl
ppm	Parts per million
RP	Reversed phase
RPTLC	Reversed phase Thin-layer Cromatography
TLC	Thin- layer Cromatography
TMS	Tetramethylsilane
UV	Ultraviolet
ORAC	Oxygen radical absorption capacity by H^+ ion transfer
FRAP	Ferric reducing antioxidant power by the movement of e^- electron
TEAC	Trolox equivalent antioxidant capacity
IA	Inactive
AC	Active
VA	Very active

CHAPTER 1

INTRODUCTION

1.1 The Medicinal Use of Plants: An Overview.

Disease continues to be a great barrier to progress in the advancement of humankind. Problems of ecology, culture and habit peculiarities contribute to making people more susceptible to diseases.

Medicinal plants have been used in many forms over the years to cure, manage or control man's ailments. Any effort to further maximize the output of medicinal or natural products from the botanical florals and so to improve health-care delivery certainly deserves great attention.

Plants have been used since antiquity for medicinal purposes by diverse peoples and cultures throughout the world. Indeed, the recorded use of natural products as a source of relief from illness dates back at least four thousand years, and it can be assumed that unrecorded practices are as old as mankind (Christophersen et al., 1999). The use of plants for medicinal purposes continues to this day, usually in the form of traditional medicine, which is now recognized by the World Health Organisation (WHO) as a building block for primary health care (Akerele, 1988; WHO, 2005). The vibrant healing power of herbs had been recognized since creation and hence botanical medicine is one of the oldest practiced professions by mankind (Van Wyk and Gericke, 2000; Iwu, 1993).

It has been estimated that 25% of prescribed medicines today are substances derived from plants (Hamburger and Hostettmann, 1991). These include about 119 plant-derived chemical compounds of known structures which are currently used as drugs or as biodynamic agents that affects human health. Less than a dozen of these compounds are produced by chemical synthesis or semi-synthesis, the rest being extracted and purified directly from plants (Farnsworth, 1990). Well-known examples of drugs with plant origins includes aspirin, atropine, digoxin, ephedrine, morphine, quinine, reserpine, vincristine and vinblastine, as well as several plant steroidal

sapogenins which serve as semi-synthetic precursors to the steroidal drugs. The study of plants of medicinal importance in the first years of the nineteenth century led to the isolation in crystalline form of such complex substances as Strychnine ($C_{21}H_{22}O_2N_2$), Quinine ($C_{20}H_{24}O_2N_2$) and Morphine ($C_{17}H_{19}O_3N$) which have physiological actions in man and animals (Farnsworth and Bingel, 1977).

Herbal medicine has for too long been neglected in favour of synthetic drugs of which its misuse or abuse, and cases of side effects have become a social evil (Farnsworth, 1990). It has also been reported that 'nearly' half the prescriptions written annually in the United States of America contain a drug of natural origin, either as the sole ingredient or as one of the two main ingredients (WHO, 2002). The structure of many synthetic drugs resulted directly from an observation of some biologically active plants materials (Farnsworth, 1990). The plant Kingdom has long served as a prolific source of useful drugs, foods, additives, flavouring agents, lubricants, colouring agents and gums from time immemorial (Keay et al., 1964).

The World Health Organization (WHO) has reported that about 80% of the world's population depends mainly on traditional medicine and the traditional treatment involves mainly the use of plant extracts (WHO, 2005). This practice is commonly found in rural areas where synthetic drugs are not available or, where available, are not affordable. This estimated 80 percent still relies mainly on herbal medicine for primary health care - especially in developing nations and third world countries (WHO, 2002). The existence of traditional medicine depends on plant species diversity and related knowledge of their use as herbal medicines (Shrestha and Dhillion, 2003; Svarstad and Dhillion, 2000; Tabuti et al., 2003). People in Africa are known for using the plants growing in their backyards as part of their primary health care systems for millennia. In fact, archaeologists have discovered the remains of plants used as medicine at different archaeological dig sites all over the world which date back to 8000 B.C. (WHO, 2002). There are so many plants used for the primary health care needs in our world nowadays and many of these have never been subjected to any type of scientific research yet. In South Africa the huge diversity of tribes reflected these systems of medicine practised (Van Wyk et al., 1997). Where

traditional healers are known by the Zulu people as inyangas or herbalists and isangomas or diviners while they are known as ixwele and amaquira (Xhosa), nqaka (Sotho) and nanga, mungome or maine (Vhavenda) (Van Wyk et al., 1997).

The use of a plant in a specific way for a specific purpose for many years and in many different geographical areas is most probably an indication that it is an effective treatment. It is this rapidly growing discipline (called ethnobotany) that helps scientists target which plants to research first and what to study them for. Indigenous people originally discovered the medicinal uses of almost three-quarters of the plant-derived drugs in use today.

Since synthetic and pharmaceutical drugs continue to be available at high cost, one of the most viable alternative medicines is from plant sources. Plant-based medicines are often the most accessible and appropriate therapy for a wide diversity of health problems experienced by rural and suburban dwellers. On many occasion, people cultivate and transplant medicinal plants in and around their homes and villages, for use in the treatment of common fevers, fungal infections, respiratory problems, pain and gastrointestinal problems, sometimes as antidotes for poisonous snake bites, among many others health problems.

Medicinal plants have been found useful as antimalarial, antisickling, antihelmintic, antimicrobial, anticonvulsant, antihypertensive and antischistosomal (molluscicidal) agents etc (Coates et al., 2002; Henry, 2000). On the other hand, multiple drug resistance has become a very real issue in pharmacotherapeutics as there are an increasing number of diseases which are exhibiting various levels of drug resistance, including bacterial and fungal infections (Henry, 2000). The search for new drugs to combat this problem is nowadays receiving much attention (Coates et al., 2002). Plants used in traditional medicine have the potential to provide pharmacologically active natural products which can be used to treat various ailments. This could be achieved by taking advantage of recorded information available from traditional medicine and ethnobotanical knowledge.

Thousands of plants are known to be of medicinal importance from their screening results and local usage in traditional medicine for one ailment or the the other. In

Africa, especially in South Africa, a large part of the day –to – day medicine is still derived from plants and large volumes of plants or their extracts are actually sold in the informal and commercial sectors of the economy in all the 9 Provinces either directly as folkloric remedies or in different indigenous systems of medicine or indirectly in the pharmaceutical preparations and formulations of modern medicines in the form of creams, gels, tablets, capsules, syrups and suspensions (Mander,1998; De Wet and Van Wyk, 2008; Van Wyk, 2008; Van Wyk et al., 2008). According to National Health Experts, over 1000 different plant materials are used for medicinal preparations for both internal and external use in South Africa (Thring and Weitz, 2006; Mander,1998; Van Wyk, 2008; Van Wyk et al., 2008). The world health organization recently compiled an inventory of more than 20 000 species of medicinal plants (Galal et al., 1991; Hoffmann et al., 1993; WHO, 2005, 2002). Although some of the plant species have been tested for antimicrobial properties, the majority have not yet been adequately evaluated (Balandrin et al., 1985).

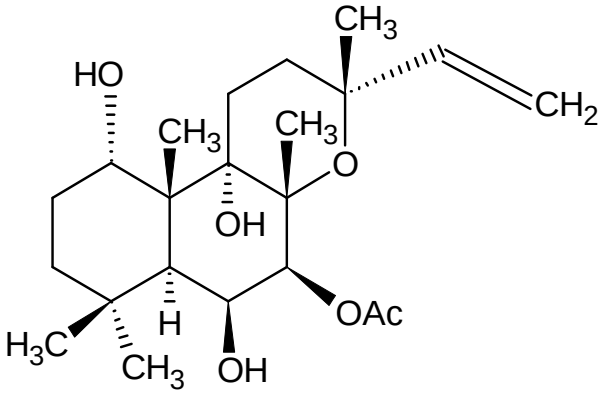
In Africa, natural medicine has never been completely abandoned and the old methods of healing are now being revived. Several outstanding examples support the value of investigating folkloric medicinal plants, e.g *Rauvolfia serpentina* has yielded a hypotensive alkaloid; *Digitalis purpurea* and *Digitalis lanata* have yielded digitoxin a powerful cardiotonic agent; Opium has given us the analgesics codeine and morphine; the smooth muscle relaxant papaverine Atropa belladona has yielded the parasympatholytics atropine, cinchona species have yielded the antimalerial quinine and the antirrhythmia quinidine; and Ammimajus has yielded the agent used to treat leukoderma (Odebiyi and Sofowora, 1979). All of these drugs are still in current use.

The extraction of bioactive agents from plants is one of the most intensive areas of natural product research today, yet the field is far from exhausted. The majority of biologically active plant principles as reported by Farnsworth and Bingel, 1977 were alkaloids, terpenes, flavonoids, steriols, coumarins, quinines, Anthraquinones, Tannins, Saponins and other simple secondary plants metabolites.

Chromatographic techniques are central to any isolation scheme, and there are several available to the natural product chemist (Joustra et al., 1967; Brooks and Keates, 1969; Harborne, 1973; Hostettmann et al., 1986; Hamburger and Hostettmann, 1991; Marston and Hostettmann, 1991), while the structure elucidation of isolated compounds is usually carried out by spectroscopic methods(Voirin, 1983 ; Sadler, 1988; Derome, 1989; Martin and Crouch, 1991; Baldwin, 1996). The advent of microbial fermentation coupled with advances in synthetic pharmaceutical chemistry in the 1950's and 1960's led to a decline in medicinal plant research. However, the developments of new chromatographic techniques for isolation, and spectroscopic methods for structure elucidation, have led to an upsurge in the last decade, resulting in the discovery and development of several new agents as important pharmacological tools or therapeutic drugs.

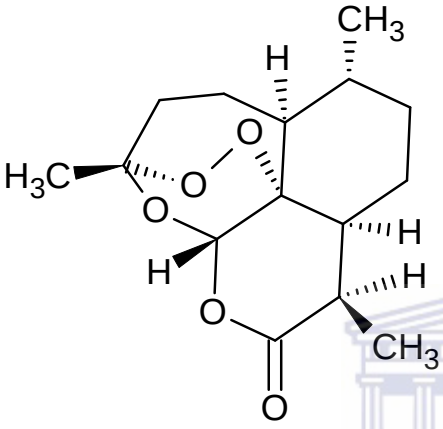
1.2 Recent Progress in Phytochemistry.

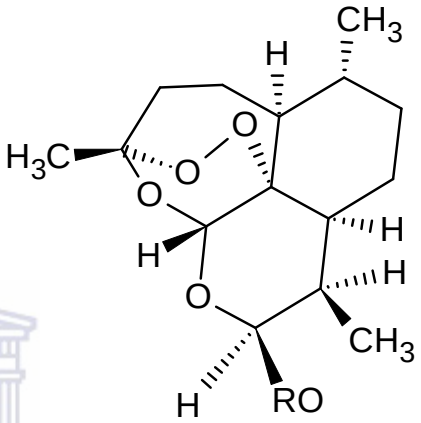
Medicinal plant research has been boosted by the clinical success of such plant-derived drugs as vincristine and vinblastine, the antitumor principles of *Catharanthus roseus* (Apocynaceae). More recently, the diterpene forskolin (**I**) isolated from *Coleus forskohlii* Brig. (Labiatae) has emerged as an important tool in investigating the physiological role of adenosine cyclic3',5'-(hydrogen) phosphate (cAMP), through its stimulation of the enzyme adenylate cyclase (Ammon and Muller, 1985).



I

A drug which has proved clinically effective in the treatment of multi-drug resistant *falciparum* malaria is the sesquiterpene endoperoxide artemisinin (**II**) also called qinghaosu, isolated from the Chinese herb qinghao (*Artemisia anmia L.*, Asteraceae). Synthetic derivatives of artemisinin such as artemether® (**III**) and Sodium artesunate (**IV**) are also available in the market (Trigg, 1989).





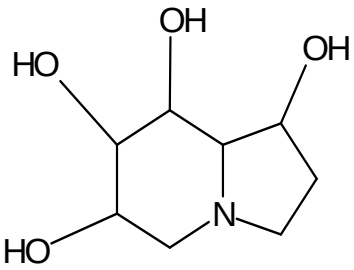
II **III** R = -CH_3

IV R = -$COCH_2CH_2COONa$

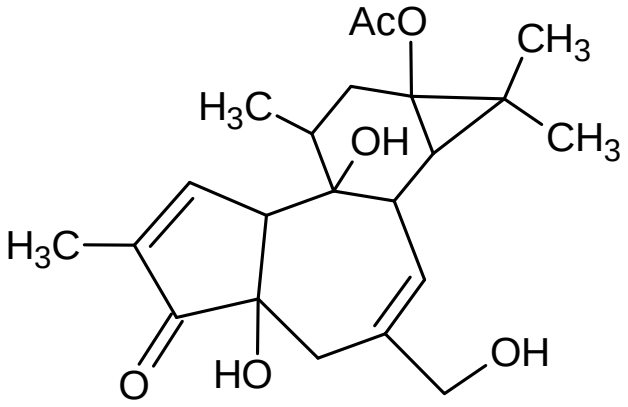
The search for phytochemicals effective against the human immunodeficiency virus (HIV), the causative agent of Acquired Immune Deficiency Syndrome (AIDS) has resulted in the isolation of several promising compounds, including the tetrahydroxyindolizidine alkaloid castanospermine (**V**) from *Castanospermum australe* (Leguminosae) and the phorbol ester prostratin (**VI**) from *Homalanthus nutans* (Euphorbiaceae). Prostratin was already known, but had not been under investigation as an antiviral agent (Hamburger and Hostettmann, 1991; Cox and Balick, 1994).

The ginkgolides, which are potent and specific inhibitors of the phospholipid mediator of inflammation and platelet activating factor (PAF), were isolated from another Chinese medicinal plant *Ginkgo biloba L.* (Ginkgoaceae). The ginkgolides, especially ginkgolide B (BN 52021, **VII** have been found to possess a wide range of

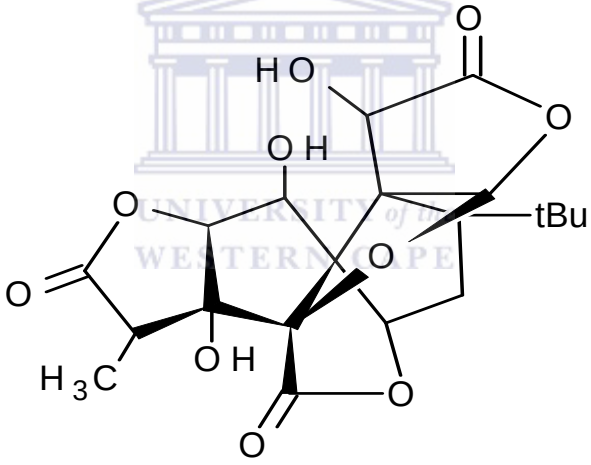
biological activities, and have also proved to be very useful pharmacological tools (Braquet, 1987; Smith et al., 1996).



V



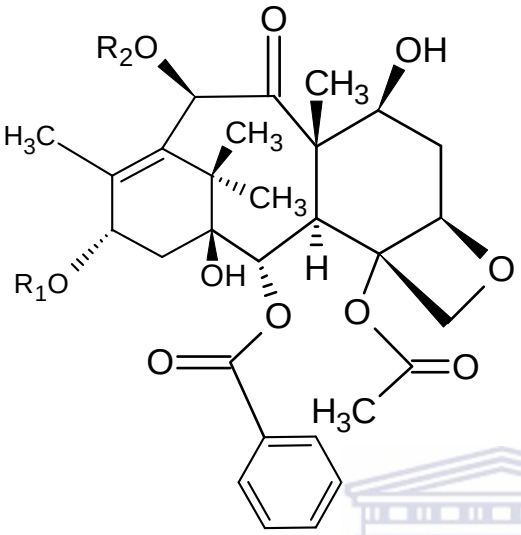
VI



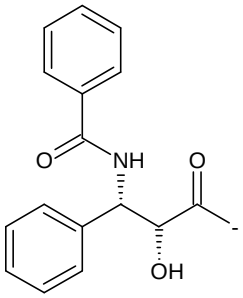
VII

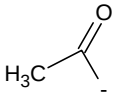
Finally, the diterpenoid paclitaxel (Taxol®, Bristol-Myers Squibb) isolated from the bark of the pacific yew tree *Taxus brevifolia* and other Taxus species is a potent antitumor agent and has been approved for the treatment of ovarian cancer as well as metastatic breast cancer unresponsive to other therapies (Cox and Balick, 1994). Taxol (**VIII**), due to its low concentration in the bark of Taxus species and the high worldwide demand which almost result in the extinction of the Pacific yew, is now obtained mostly by semi-synthesis form 10-deacetylbaccatin III (**IX**) isolated from a

more sustainable source, the needles of *Taxus baccata* and other species of *Taxus* (Appendino, 1993; Lenaz and de Furia, 1993).



VIII





	R_1	R_2
IX	H	H

African scientists have only recently begun to play a major role in research into the medicinal plants which abound on the continent, and this is yielding good results with a vast body of literature already accumulated. Thus, contributions to the phytochemistry of African indigenous medicinal plants for the period 1969 – 2008 have been reviewed by many scientist (Olaniyi, 1981; Olaniyi and Satake, 1992; VanWyk et al., 1997; Van Wyk and Gerick, 2000; De Wet and Van Wyk, 2008 ;). Recent advances in the flavonoid and alkaloid chemistry of African medicinal plants have also been reviewed (Saleh, 1994, Kauh et al., 2009) while a comprehensive bibliography of research on African medicinal plants in the 1984 – 1994 literature has also been published (Dagne, 1996). A major shortcoming of research on African medicinal plants has been the limited application of bioactivity screening and isolation procedures, resulting in fewer new drugs of proven value being isolated so far (Olaniyi and Satake, 1992).

1.3 Background information and motivation

There are several reasons why plants should be investigated for bioactive agents.

(i) The isolation of bioactive agents helps to provide chemotaxonomic evidence for the classification of genera or species, especially those whose classification on morphological grounds alone is unclear.

(ii) It enables structure-related activity studies to be carried out.

(iii) It also makes it possible to demonstrate that the reported physiological activity of the plant under screening is real.

A major constraint on the continued use of plants for medicinal purposes is the fact that the forests where a majority of these plants abound are disappearing at a very alarming rate (Cox, 1991). There is also the loss of the associated plant folklore as herbalists are not passing on the generations of accumulated knowledge which they possess. While policies aimed at ensuring a sustainable use of bioresources are now being produced on a global scale (Akerele, 1988; Bonati, 1988; Cunningham, 1988; Mander, 1998; Van Wyk, 2008), there is also an urgent need for a systematic study of our medicinal plants to isolate their active principles, before they are lost forever (Cox and Balick, 1994; Ogungbamila and Ogundaini, 1994; Van Wyk et al., 2008).

It has been estimated that only about 0.5% of the 250,000 – 500,000 species of higher plants have been adequately investigated, either phytochemically, or with respect to biological activity (Cox and Balick, 1994). Furthermore the 119 plant derived drugs in current use enumerated by Farnsworth (1990) are produced commercially from less than 90 species of plants which indicates that a large untapped resource still exists containing potential phytochemicals which could be used as new drugs, as precursors for the semi synthesis of natural products with complex chemical structures, or as potent pharmacological tools. As highlighted above, in Africa many people are still known to use medicinal plant as an alternative or supplement rather than visiting a western health care practitioner (orthodox practitioner) (van wyk et al., 1997). From literature search it has been known that over 500,000 species of medicinal plants is in existence all over the African continent which is widely spread all over this region (Van Wyk et al., 1997; Van Wyk and Gericke, 2000; Van Wyk and Wink, 2004; Irvine, 1961; Smith, 1966; Thring and Weitz, 2006; Van Wyk et al., 2008). Broad spectrum was chosen from West Africa and South Africa for review

purposes only. Among the 60 000 medicinal plants that are available in West Africa, 550 have been screened and from this total of 13 were selected based on their interesting ethnomedicinal uses, ready availability, easy accessibility and identification. Extensive literature search shows some interesting overview of the 13 plants using available data bases which can generate the required information about the plants at least up to 80%. The data bases used are (1) Science Finder Scholar (Scifinder) (2) Science Direct (3) Scopus and (4) Google Web. The results of the search are shown in Table 1.

The plants are:

(1) Phyllanthus niruri (Euphorbiaceae)

(2) Picralima nitida (Apocynaceae) or (Asclepiadaceae)

(3) Tithonia diversifolia (Compositaceae) or (Acanthaceae)

(4) Hibiscus sabdariffa (Malvaceae)

(5) Hypoestes rosea (Acanthaceae)

(6) Rauvolfia vomitoria (Apocynaceae)

(7) Alstonia bone (Apocynaceae)

(8) Trichilia emetica (Meliaceae)

(9) Uraria picta (Leguminoseae)

(10) *Hibiscus cannabinus* (Malvaceae)

(11) *Sida cordifolia* (Malvaceae)

(12) *Piliostigma reticulatum* (Caesalpiniaceae)

(13) *Combretodendron macrocarpum* (Lecythydaceae) or (Moraceae)

Table 1: Analysis of published articles on selected 13 plants using different data bases

	Medicinal plants	**Scifinder Scholar articles**	**Science Direct articles**	**Scopus articles**	**No of Patents**	**No. of reports on Chemistry**	**No. in Google Web.**
1.	*P. niruri*	(3)	(25)	(110)	(85)	(30)	(736)
2.	*P. nitida*	(63)	(8)	(23)	(5)	(8)	(170)
3.	*T. diversifolia*	(91)	(25)	(116)	(18)	(27)	(779)
4.	*H. sabdariffa*	(472)	(45)	(182)	(144)	(38)	(1377)
5.	*H. rosea*	(9)	(2)	(2)	(16)	(1)	(166)
6.	*R. vomitoria*	(27)	(5)	(9)	(3)	(3)	(157)
7.	*A. bone*	(3)	(3)	(2)	(9)	(1)	(105)
8.	*T. emetica*	(33)	(10)	(16)	(2)	(4)	(243)
9.	*U. picta*	(6)	(1)	(7)	(3)	(4)	(47)
10.	*H. cannabinus*	(1169)	(85)	(249)	(104)	(3)	(1294)
11.	*S. cordifolia*	(58)	(8)	(29)	(44)	(1)	(1253)
12.	*P. reticulatum*	(10)	(4)	(17)	(0)	(0)	(159)
13.	*C. macrocarpum*	(5)	(6)	(4)	(0)	(2)	(75)

In South Africa alone there are over 30,000 species of higher plants and over 3000 of these plants species have been found to be used in traditional medicine across the country (Van Wyk et al., 1997, 2008; Thring and Weitz 2006).

Knowledge of the biochemistry of these plants is very important, not only due to their potential for discovery of newer alternatives for the treatment of illnesses, but also to

have the blue prints of the biochemical composition of the medicinal plants. A Data bank of any plants that may be under threat due to a high demand for medicinal purposes may thus be created before the plant becomes extinct. The data bank could then be used to recreate similar biochemical composition through the diverse available Synthetic methods. (Brown, 2002; Watt and Breyer-Brandwijk, 1962; Van Wyk and Gericke, 2000; Smith, 1966; Tabuti et al., 2003; Van Wyk and Wink, 2004; Van Wyk et al., 1997; Hutchings, 1989; Cunningham, 1988; Hutchings et al., 1996). The survey of the medicinal plants of South Africa was confined to the Western Cape Province alone for reason of easy accessibility.

The literature shows that there are over 800 medicinal plants of high value in the Western Cape; however only 70 were selected based on the nature of their uses as well as percentage distribution throughout the Province (Table 2).

Table 2: The names of the 70 selected medicinal plants in Western Cape Province

SN	Name of plant	Family it belongs	% Distribution
1.	*Acacia karroo*	(Fabaceae)	80%
2.	*Acokanthera oppositifolia*	(Apocynaceae)	10%
3.*	*Agathosma betulina*	(Rutaceae)	40%
4.	*Aloe ferox*	(Asphodelaceae)	30%
5.*	*Arctopus echinatus*	(Apiaceae)	35%
6.	*Artemisia afra*	(Asteraceae)	25%
7.	*Asclepias fructicosa*	(Asclepiadaceae)	100%
8.*	*Aspalathus linearis*	(Fabaceae)	30%
9.	*Aster bakeranus*	(Asteraceae)	25%
10.	*Ballota africana*	(Lamiaceae)	80%
11.	*Berula erecta*	(Apiaceae)	15%
12.	*Boophane disticha*	(Amaryllidaceae)	40%
13.	*Carpobrotus edulis*	(Mesembryanthemaceae)	30%

14.	*Centella asiatica*	(Apiaceae)	15%
15.	*Chironia baccifera*	(Gentianaceae)	70%
16.	*Cichorium intybus*	(Asteraceae)	25%
17.	*Cissampelos capensis*	(Menispermaceae)	100%
18.	*Cnicus benedictus*	(Asteraceae)	10%
19.	*Cotyledon orbiculata*	(Crassulaceae)	100%
20.	*Curtisia dentata*	(Cornaceae)	15%
21.	*Cyclopia intermedia*	(Fabaceae)	10%
22.	*Datura stramonium*	(Solanaceae)	55%
23.	*Dicoma capensis*	(Asteraceae)	15%
24.	*Dodonaea angustifolia*	(Sapindaceae)	90%
25.	*Elytropappus rhinocerotis*	(Asteraceae)	100%
26.	*Eriocephalus africanus*	(Asteraceae)	85%
27	*Euclea undulata*	(Ebenaceae)	50%
28.	*Foeniculum vulgare*	(Apiaceae)	60%
29.	*Geranium incanum*	(Geraniaceae)	25%
30.*	*Gethyllis species*	(Amaryllidaceae)	20%
31.	*Glycyrrhiza glabra*	(Fabaceae)	10%
32.	*Gunnera perpensa*	(Gunneraceae)	40%
33.	*Helichrysum species*	(Asteraceae)	50%
34.	*Heteromorpha arborescens*	(Apiaceae)	50%
35.*	*Hypericum perforatum*	(Clusiaceae)	10%
36.	*Knowltonia vesicatoria*	(ranunculaceae)	40%
37.	*Leonotis leonurus*	(Lamiaceae)	40%
38.	*Lobostemon fructicosus*	(Boraginaceae)	50%
39.	*Melianthus comosus*	(Melianthaceae)	50%
40.	*Mentha longifolia*	(Lamiaceae)	100%
41.	*Ocotea bullata*	(Lauraceae)	30%
42.	*Olea europaea*	(Oleaceae)	60%

43.*	*Osmitopsis asteriscoides*	(Asteraceae)	15%
44.	*Pelargonium luridum*	(Geraniaceae)	20%
45.	*Pellaea calomelanos*	(Adiantaceae)	60%
46.*	*Peucedanum galbanum*	(Apiaceae)	10%
47.	*Pittosporum viridiflorum*	(Pittosporaceae)	20%
48.	*Plumbago auriculata*	(Plumbaginaceae)	10%
49.	*Polygala fruticosa*	(Polygalaceae)	40%
50.	*Protea repens*	(Proteaceae)	50%
51.	*Prunus africana*	(Rosaceae)	05%
52.	*Rapanea melanophloeos*	(Myrsinaceae)	40%
53.	*Rhoicissus tridentata*	(Vitaceae)	15%
54.	*Rhus undulata*	(Anacardiaceae)	40%
55.	*Ricinus communis*	(Euphorbiaceae)	90%
56.	*Rumex lanceolatus*	(Polygonaceae)	100%
7.	*Ruta graveolens*	(Rutaceae)	05%
58.	*Salix mucronata*	(Salicaceae)	100%
59.	*Scabiosa columbaria*	(Dipsacaceae)	95%
60.	*Scadoxus puniceus*	(Amaryllidaceae)	05%
61.	*Sutherlandia frutescens*	(Fabaceae)	70%
62.	*Tarchonanthus camphoratus*	(Asteraceae)	30%
63.	*Tulbaghia violacea*	(Alliaceae)	10%
64.	*Typha capensis*	(Typhaceae)	40%
65.	*Valeriana capensis*	(Valerianaceae)	20%
66.	*Viscum capense*	(Viscaceae)	40%
67.	*Withania somnifera*	(Solanaceae)	50%
68.	*Zantedeschia aethiopica*	(Araceae)	40%
69.	*Zanthoxylum capense*	(Rutaceae)	05%

(*) indicates that the medicinal plant is found only in Western Cape Province.

The extensive literature search for the above plants shows the overview of the results below using the same databases as that used for plants of West Africa (Table 3)

Table 3: Analysis of published articles on selected 70 plants using different data bases

	Medicinal plants	Scifinder Scholar articles	Science Direct articles	Scopus articles	No of Patents	No. of works in Chemistry	No. in Google Web.
1.	*A. karro*	(26)	(19)	(69)	(2)	(1)	(511)
2.	*A. oppositifolia*	(4)	(0)	(0)	(0)	(0)	(43)
3.	*A. betulina*	(10)	(0)	(8)	(7)	(3)	(117)
4.	*A. ferox*	(18)	(9)	(33)	(135)	(9)	(1065)
5.	*A. echinatus*	(5)	(2)	(3)	(0)	(0)	(9)
6.	*A. afra*	(10)	(15)	(29)	(2)	(12)	(308)
7.	*A. fructicosa*	(8)	(12)	(17)	(10)	(7)	(1736)
8.	*A. linearis*	(35)	(19)	(75)	(172)	(19)	(544)
9.	*A. bakeranus*	(1)	(1)	(0)	(1)	(0)	(4)
10.	*B. africana*	(5)	(1)	(2)	(2)	(1)	(146)
11.	*B. erecta*	(16)	(1)	(20)	(1)	(1)	(776)
12.	*B. disticha*	(9)	(4)	(8)	(0)	(0)	(70)
13.	*C. edulis*	(25)	(5)	(31)	(3)	(1)	(1043)
14.	*C. asiatica*	(89)	(230)	(299)	(867)	(51)	(2859)
15.	*C. baccifera*	(7)	(1)	(3)	(0)	(1)	(35)
16.	*C. intybus*	(310)	(211)	(474)	(341)	(57)	(7336)
17.	*C. capensis*	(1)	(0)	(1)	(0)	(0)	(81)
18.	*C. benedictus*	(11)	(9)	(13)	(67)	(6)	(821)
19.	*C. orbiculata*	(14)	(1)	(9)	(2)	(0)	(211)
20.	*C. dentata*	(2)	(0)	(0)	(0)	(0)	(85)
21.	*C. intermedia*	(8)	(10)	(13)	(13)	(4)	(78)
22.	*D. stramonium*	(456)	(280)	(716)	(1717)	(54)	(5955)
23.	*D. capensis*	(1)	(2)	(2)	(0)	(0)	(21)
24.	*D. angustifolia*	(6)	(3)	(0)	(0)	(0)	(12)
25.	*E. rhinocerotis*	(6)	(3)	(5)	(0)	(0)	(55)

26.	*E. africanus*	(7)	(2)	(3)	(11)	(1)	(54)
27.	*E. undulata*	(3)	(0)	(5)	(0)	(0)	(99)
28.	*F. vulgare*	(129)	(210)	(320)	(344)	(97)	(4277)
29.	*G. incanum*	(1)	(0)	(0)	(6)	(0)	(527)
30.	*G. species*	(1)	(1)	(5)	(0)	(0)	(32)
31.	*G. glabra*	(234)	(87)	(313)	(1211)	(91)	(3927)
32.	*G. perpensa*	(6)	(15)	(11)	(1)	(2)	(62)
33.	*H. species*	(56)	(110)	(107)	(174)	(12)	(2499)
34.	*H. arborescens*	(3)	(0)	(2)	(0)	(0)	(96)
35.	*H. perforatum*	(899)	(678)	(1446)	(721)	(251)	(13486)
36.	*K. vesicatoria*	(0)	(0)	(0)	(0)	(0)	(19)
37.	*L. leonurus*	(12)	(8)	(11)	(6)	(2)	(683)
38.	*L. fructicosus*	(1)	(0)	(0)	(2)	(0)	(8)
39.	*M. comosus*	(13)	(2)	(3)	(4)	(0)	(82)
40.	*M. longifolia*	(160)	(12)	(1)	(6)	(0)	(1)
41.	*O. bullata*	(10)	(8)	(14)	(0)	(3)	(240)
42.	*O. europaea*	(993)	(1002)	(1119)	(2031)	(160)	(5613)
43.	*O. asteriscoides*	(9)	(1)	(3)	(0)	(1)	(22)
44.	*P. luridum*	(3)	(0)	(0)	(0)	(0)	(32)
45.	*P. calomelanos*	(1)	(0)	(0)	(0)	(0)	(117)
46.	*P. galbanum*	(4)	(0)	(1)	(0)	(1)	(29)
47.	*P. viridiflorum*	(6)	(5)	(5)	(5)	(3)	(257)
48.	*P. auriculata*	(13)	(8)	(16)	(9)	(3)	(899)
49.	*P. fruticosa*	(2)	(0)	(2)	(4)	(1)	(1082)
50.	*P. repens*	(12)	(1)	(19)	(9)	(0)	(502)
51.	*P. Africana*	(29)	(37)	(53)	(49)	(6)	(2617)
52.	*R. melanophloeos*	(3)	(3)	(5)	(3)	(1)	(124)
53.	*R. tridentate*	(6)	(11)	(12)	(0)	(3)	(61)
54.	*R. undulata*	(3)	(1)	(3)	(8)	(1)	(446)
55.	*R. communis*	(1199)	(1087)	(2290)	(3311)	(111)	(8998)
56.	*R. lanceolatus*	(29)	(0)	(0)	(0)	(0)	(676)
57.	*R. graveolens*	(123)	(167)	(222)	(105)	(63)	(2485)
58.	*S. mucronata*	(0)	(0)	(11)	(0)	(0)	(852)
59.	*S. columbaria*	(19)	(3)	(40)	(11)	(1)	(1017)
60.	*S. puniceus*	(3)	(0)	(0)	(0)	(0)	(114)
61.	*S. frutescens*	(19)	(11)	(18)	(2)	(1)	(250)

62.	*T. camphoratus*	(29)	(2)	(8)	(10)	(2)	(101)
63.	*T. violacea*	(19)	(8)	(16)	(3)	(4)	(463)
64.	*T. capensis*	(2)	(3)	(3)	(1)	(2)	(1156)
65.	*V. capensis*	(0)	(0)	(0)	(11)	(0)	(341)
66.	*V. capense*	(3)	(1)	(1)	(3)	(0)	(44)
67.	*W. somnifera*	(301)	(144)	(331)	(162)	(72)	(1822)
68.	*Z. aethiopica*	(29)	(17)	(33)	(85)	(3)	(1325)
69.	*Z. capense*	(3)	(1)	(3)	(0)	(0)	(86)
70.	*H. asiatica*	(88)	(240)	(299)	(866)	(50)	(2850)

The final selection of plants from both West and South Africa was made based on (1) the ready availability of the plant in the sizable amount needed for the work (2) literature review (3) interest in their recorded ethnomedicinal uses (4) volume of work in chemistry that was recorded for the selected plants. These plants are (1) *Piliostigma reticulatum,* (2) *Cissampelos capensis,* (3) *Geranium incanum* and (4) *Gethyllis* species.

1.4 Justification of the study

The present study involves the screening of some selected African medicinal plants for the diverse ethnomedicinal uses acclaimed. The results obtained were used to rank the four selected plants: *Piliostigma reticulatum*, *Cissampelos capensis, Geranium incanum* and *Gethyllis* species. Each plant has specific but diverse ethnomedicinal uses which are well documented (Irvine, 1961; Gill, 1988; Hostettmann, 1997; Hutchinson and Dalziel, 1963; Bonati, 1988; Ogungbamila and Ogundaini,1994; Sofowora,1982; Van Wyk, and Gericke, 2000; Van Wyk et al., 1997; Smith, 1966; Watt and Breyer-Brandwijk, 1962; Levyns, 1934; Botha, 1980; Hilliard and Burtt, 1985; Muller-Doblies, 1986; Du Plessis and Duncan, 1989; Van Wyk and Wink, 2004; Ganguly et al., 2007; Amresh et al., 2007). There is comparatively little work which has been done on the evaluation of their chemical and biological actions, and even less on the isolation of active constituents which may explain their traditional

uses, especially the *Gethyllis* species. Very little work has been carried out on the chemistry of *C. capensis* and *Gethyllis* species to the best of our knowledge.

1.5 Aims and Objectives

The main aims of this study are:

(1) To derive useful informative data about the numerous active constituents in the four selected medicinal plants.

(2) To demostrate and identify the different spectrum of activities on diverse diseases and to provide a rationale for the widespread ethnomedicinal uses of the plants in infections and inflammatory conditions by relating their activities to the presence of specific isolated compounds.

(3) To equally contribute to the growing body of knowledge relating to the phytochemistry of some selected African medicinal plants in which these bioactive compounds can equally serve as templates for other new potent drugs (Ojo, 2001; Reinhard and Wagner, 1987; Mc Laughlin et al, 1991; Harbone et al, 1994, 1996 and 1997).

(4) To use the bioactivity guided protocol for isolating and purifying active agents contained in some of the plant extracts.

(5) To test for the antioxidant capabilities of the plants.

1.6 Scope of the present investigation

1.6.1 Experimental overview

In order to achieve the above objectives, this research will involve

(A) Collection, authentication, extractions and preliminary evaluation of the crude extracts for the four plants. Solvent extraction involves use of different organic solvents followed by bioassay evaluation of the extracts using the quick benchtop brine shrimp lethality test bioassay (Babajide, 1994; Adesanya, 1994).

(B) Isolation using various modern separation techniques such as Thin Layer Chromatography (TLC), Column Chromatography (CC), Vacuum Liquid Chromatography (VLC), Minimum Effort Liquid Chromatography (MELC) (Bækström, 1994; Babajide, 1994).

(C) Characterization and structure elucidation of the isolated constituents of the plants using all the available spectroscopic methods such as Infra red Spectroscopy (IR), Ultra Violet/Visible Spectroscopy (UV-Vis), Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectroscopy (MS) etc.

(D) Biochemical evaluation of the fractions and isolates for cytotoxicity using brine shrimp lethality test bioassay, antimicrobial and total antioxidant evaluation.

1.6.2 Brine shrimp cytotoxicity

It is very necessary that medicinal plants cytotoxicity and microbial properties are well documented to have a broad base data bank as highlighted earlier on. The cytotoxicity bioassay was carried out using the brine shrimp lethality method where the nauplii (larvae) of *Artemisia salina* were used in the analysis (Pelka et al., 2000; Chan-Bacab et al., 2003). The (LD_{50}) Lethality of substances to brine shrimp nauplii has been linked to the probable ability of such compounds to kill cancer cells (antitumor activity), possibly pesticidal and antimicrobial activity (Mc Laughlin et al., 1991; Adesanya, 1994; Babajide et al., 2008). Padmaja et al., 2002; Kapadia et al., 2002 and Moreno-Murillo et al., 2001 also showed the application of this method in screening different plant materials because of the sensitivity of the nauplii to toxic substances. This was achieved by looking at the ratio between dead larvae (no motility) and living larvae (high motility) in comparison to a control without any toxic substances which was used in estimating the toxicity of the test solutions.

1.6.3 Antioxidant evaluation

The *in vitro* chemical antioxidant evaluations were also carried out due to its importance in our day to day living because of its linkage to oxygen. **Oxygen** is essential to human life at the same time is also involved in toxic reactions and is therefore a constant threat to the well-being of all living things. This imperfection is known to induce damage to body tissues which usually accumulate with time (Langseth, 1995). This damage has been hypothesized to be a major contributor to many of the degenerative diseases of ageing, including cardiovascular disease, cancer, cataracts, the age-related decline in the immune system and degenerative diseases of the nervous system (Eric, 1993; Ames et al., 1993). In the past decade, researchers have made major strides in looking for substances that may possibly prevent, postpone or limit the severity of these diseases by enhancing the body's antioxidant defence mechanisms through improved nutrition (Packer and Glazer 1990; Langseth, 1995).

Most of the potentially harmful free radical (oxidants) generated by both endogenous and environmental factors such as exercise, Inflammation, cigarette smoke, environmental pollutants, radiation, ultraviolet light, certain drugs, pesticides, anaesthetics, industrial solvents and ozone can be prevented or eliminated by antioxidant (Machlin, 1992 ; Papas, 1992; Frei, 1994; Sies, 1991; Halliwell and Gutteridge, 1989; Yagi, 1993; Packer and Glazer, 1990; Aruoma et al., 1991; Diplock, 1991; Pfeifer, 1993; Block et al., 1992; Singh and Gaby, 1991; Ames et al., 1993).

Aruoma 2003 showed that the study of these free radicals and antioxidants in biology is producing medical revolution that promises a new age of health and disease management. Cancer, one of the diseases caused by free radical as shown by the multistep processes in figure 1 can be prevented by antioxidant as shown in figure 2.

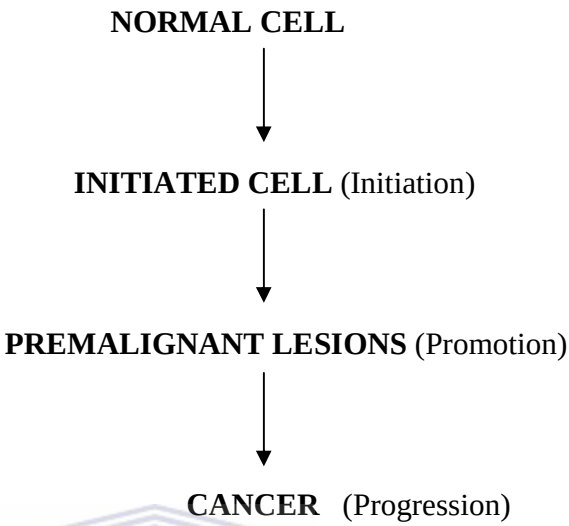


Fig 1: A multistep model for carcinogenesis

It has been known that **antioxidants** may be able to cause the regression of premalignant lesions or inhibit their development into cancer by chemoprevention.

Repair ⟶ **Regression** ⟶ **Inhibition**

Fig 2: A chemoprevention scheme

An impressive body of scientific evidence shows that people with high dietary intakes of fruits and vegetables are less likely to develop cancer than people who have low dietary intakes of these foods. Over 300 epidemiologic studies of fruits, vegetables and cancer have been published, and their results are remarkably consistent (Beecher, 2003; Langseth, 1995).

Young and Woodside, 2001 showed the effect of antioxidants on health and diseases with respect to the basic chemistry of free radical formation in the body while several literature showed how medicinal plant extracts and isolates, teas, beverages, fruits and seeds containing flavonoids and polyphenolic derivatives can be used as a

remedy and antioxidants in these free radical- scavenging capabilities (Langseth, 1995; Eric, 1993; seeram et al., 2006).

Diet is believed to play an important role in four major diseases of advanced and transitional economies: cardiovascular disease, cancer, hypertension and obesity (Silalahi 2002).

Several classes of dietary compounds have been suggested to reduce the risk of some cancers, especially those of the gut, and there is some evidence that consumption of certain foods leads to a reduction in biomarkers of oxidative damage. The active principles in extracts from a variety of plant sources, such as cocoa shells, oats, tea, olives, garlic, ginger, red onion skin, grapes, apple cuticle, wheat gliadin, licorice, nutmeg, clove, thyme and a variety of medicinal plants and seeds extracts from tropical and sub-tropical regions of the world continue to be of interest for use in complementary medicine supplements because of the fact that it contain the important antioxidants such as Vitamin E, Vitamin C, Carotene, flavonoids, alkaloids and other polyphenols (Aruoma 2003).

The major antioxidants in plants can be in form of **polyphenols, flavonols, flavanols, anthocyanins** and **flavanones** derivatives. Their measurement can be carried out using standard methods and different reference standards. The amount of total phenolics in a herb extracts can be determined with the Folin- Ciocalteu reagent according to the method of Slinkard and Singleton, 1977 using gallic acid as a standard (Waterhouse, 2005) while catechins can be use as standard for flavonoids (Delcour and Janssens, 1985; Treutter, 1989; Waterhouse, 2005; McMurrough and McDowell, 1978; Seeram et al., 2006; Cao et al., 1996; Beecher, 2003).

Anthocyanins on the other hand are pigments responsible for the attractive red to purple to blue colours of many fruits and vegetables and they are also known as good antioxidant. Interest in the anthocyanin content of foods has intensified because of their possible health benefits. They are known for their role in reduction of coronary heart disease, increased visual acuity and cancer properties (Eric, 1993; Ames et al., 1993; Francis, 1989).

Measurement of total anthocyanin pigment content is carried out by measuring absorptivity of the solution at a single wavelength. This is possible because anthocyanins have a typical absorption band in the 490 to 550 nm region of the visible spectra. (Francis, 1982 and 1989; Fuleki and Francis, 1968a and 1968b; Jackman et al., 1987; Sondheimer and Kertesz, 1948; Wrolstad et al., 1982, 1995; Somers and Evans, 1974; Swain and Hillis, 1959; Giusti et al., 1998).

The chemistry involved in the antioxidant capacity assays were highlighted by Huang et al., 2005 and Prior et al., 2005 which highlighted three major methods of evaluating the antioxidant capacity of any material. These methods include

1. ORAC (Oxygen radical absorption capacity) by H^+ ion transfer.
2. FRAP (Ferric reducing antioxidant power) by the movement of e^- electron.
3. TEAC (Trolox (a well known standard) equivalent antioxidant capacity)

ORAC assay is a fluorescence-based method which was first developed by Glazer et al. in 1989 and is based on the discovery that the fluorescence of phycoerythrin (PE) changes with respect to time upon damage caused by peroxyl or hydroxyl radical attack (Glazer and DeLange, 1989). This assay measures the ability of antioxidant compounds in test materials to inhibit the decline of R-PE fluorescence that is induced by a peroxyl radical generator, AAPH (Cao et al. 1993a and b; 1995; Cao and Prior, 1998; Prior et al., 2003; 2005; Wang and Lin, 2000). The FRAP assay uses an oxidation/reduction reaction to measure the ability of a sample to reduce Fe_{III} to Fe_{II}. It is assumed that the FRAP assay is a method for evaluating antioxidant capacity (Benzie and Strain, 1996; 1999).

The ABTS (2,2'-azino-di-3-ethylbenzthialozine sulphonate) TEAC assay is a radical cation scavenging assay which was first developed by Miller et al., 1993 for the determination of the total antioxidant status (TAS) of a material.

CHAPTER 2

LITERATURE REVIEW

2.1 *PILIOSTIGMA RETICULATUM*

2.1.1 Piliostigma reticulatum (DC.) Hochst).

Plant description

The family leguminosae has 16,000 -19,000 species in about 750 genera, and is conventionally divided into three distinct subfamilies: Mimosoidaeae, Caesalpiniodeae and Papilionoideae. More recent taxonomic restructuring has accorded full family status to each of the three subdivisions, as Mimosaceae, Caesalpiniaceae and Fabaceae in order Leguminales (Allen and Allen, 1981).

The caesalpiniaceae, to which *P. reticulatum* belongs, is a pantropical family of about 150 genera and 2000 species, and consists of about 200 species of 58 genera in tropical Africa. The Caesalpiniaceae species are usually trees or shrubs, and rarely herbs or vines. The leaves are often large and brightly coloured (Figure 3). The fruits are usually pods (Assi and Guinko, 1991).

The genus *Piliostigma* which is now recognized as distinctive from Bauhinia type which it was segregated from, consist of three species which are trees, evergreen, small to medium and generally grow up to 10 metres high as shown in Figure 4 (Index kewensis, 1953; Allen and Allen, 1981). The leaves are simple, broad, deeply bilobed at the middle and the apex, typically bauhina-like, with 9 – 15 prominent main veins radiating from the base, at the point of attachment with the long petiole (Figure 3). Members of the genus *Piliostigma* occur in tropical Africa and Indo-Malaya. *P. malabarium* (Roxb.) Benth. Var acidum de Wit, is confined to Asia, in the drier monsoon belt of India, Malaysia and the Philippiness (Djuma, 2003; Schultes and Hofmann, 1973), although it has reportedly been recently introduced into Sierra Leone (Burkill, 1995). The two African species *P. reticulatum* (DC.) Hochst. and *P. thonningii* (Schum.) Milne-Redhead, inhabit dry and moist savannahs, respectively (Allen and Allen, 1981). *P. reticulatum* has close resemblance to *P. thonningii* to the extent that they can be easily confused with each other during identification, although at very close range distinguishing morphological features become observable. *P.*

reticulatum has smaller leaves and smoothness on the lower side whereas *P. thonningii* has larger leaves and hairiness on the lower side. When they enjoys damped climate, they readily colonises abandoned land (Burkill, 1995).

Piliostigma reticulatum is a shrubs or small tree 5 – 10m high (Figure 4), with a trunk that is often twisted, and possessing a dark brown, fibrous and deeply longitudinally fissured bark. The leaves are bilobate and covered below by a reddish pubescence and are 7.5-16 cm long and 10- 18 cm broad (Figure 3). The flowers are white and fragrant (Fig. 3), while the pods are woody, flat and dark brown when ripe (Adjanohoun et al., 1991; Assi and Guinko, 1991). Other synonyms of *P. reticulatum* which were used in the past include *Bauhinia thonningii* (Schumach.), *Bauhinia reticulata* (DC.), *Bauhinia abyssinica* (A. Rich.), *Bauhinia pyrrhocarpa* (Hochst.), *Locellaria bauhinioides* (Welw.) and *Piliostigma pyrrhocarpum* (Hochst) (Watt and Breyer-Brandwijk, 1962). The traditional Nigerian names of *P. reticulatum* are Kalgo (or Kargo) in Hausa (Northern Nigeria), Abafe in Yoruba (Western Nigeria) and Okpo-atu in Igbo (Eastern Nigeria) (Dalziel, 1937; Burkill, 1995). The Yoruba name implies people choice because of its multipurpose usage while the Igbo name implies that a person who takes the plant cannot be infected with worms (Asuzu and Onu, 1994).

Fig 3: Leaves of *P. reticulatum*

Fig4: *P. reticulatum* tree in the background.

2.1.2 Ethnomedicinal uses of Piliostigma reticulatum.

P. reticulatum is used as a medicinal plant to treat various ailments throughout tropical Africa. The available data are summarized in Table 4. It can be seen that the plant has a widespread area of use, and that the indications are similar. The most common indications are cough and chest complaints (upper respiratory problems),

diarrhoea and dysentery, fever, headache, toothache, gingivitis. It is also used to treat wounds and chronic ulcers. As it may be obseved, most of these indications are related to inflammatory conditions or infections.

Ethnomedically, the bark, root, pod, young stem or leaves have been used for treating leprosy, smallpox, coughs, ulcer, heart pain, gingivitis, snake bite, dysentery, fever, wounds and a variety of closely related disease conditions. (Irvine, 1961; Asuzu and Onu, 1994; Bombardelli et al., 1994; Dalziel, 1937; Watt and Breyer-Brandwijk, 1962; Bombardelli et al., 1973; McGaw et al., 1997; Bombardelli et al., 1992; Okwute et al., 1986).

2.1.3 Reported biological activities of Piliostigma reticulatum

2.1.3.1 Actions on smooth muscles.

Most of the literature available reported work done on *Piliostigma thonningii.* A normal saline extract of the fruit of *Piliostigma reticulatum* had no effect on the isolated uterus preparation (Anokbonggo, 1972). Asuzu and Ugwuja (1989) found that the 70% ethanol extract of the bark induced persistent contractions of the isolated guinea pig ileum which were completely blocked by atropine.

2.1.3.2 Antimicrobial activity.

An aqueous decoction of the stem bark of *P. thonningii* when tested for activity against the Gram-positive organisma *Sarcina lutea*, *Staphylococcus aureus* and *Mycobacterium phlei* using the agar diffusion hole-in-plate method showed activity only against *S. lutea* (Malcolm and Sofowora, 1969). The 60% methanol extract of the root bark of same plant showed activity against the yeast *Saccharomyces cerevisiae* at a test concentration of 100µg/ml (Taniguchi et al., 1978). This extract however had low activity against *Escherichia coli*, *Bacillus subtilis* and *Penicillium crustosum* at the same test concentration. The 10% methanol extract of the leaves of *P. thonningii* had no activity against *Escherichia coli* and *Sarcina lutea* at a test concentration of 15 mg/ml (Laurens et al., 1985). The methanol extract of the dried leaf was active at a concentration of 2 mg/ml against *Pseudomonas aeruginosa* and

Streptococcus species in the agar plate method (Hussain and Deeni, 1991) in the same study, the extract had no activity against *Corynebacterium diptheriae, Neisseria species, Salmonella* species, *Staphylococcus aureus* and *Streptobacillus* species. Finally, le Grand et al., (1988) found that the 95% ethanol extract of the leaves and stem bark had activity against *Staphylococcus aureus* and *Aspergillus niger*, but no activity against *Bacillus subtilis*, all at a concentration of 50 mg/ml. From the above, extracts of *Piliostigma thonningii* seem to be active mostly against Gram-positive organisms although the literature does not show consistency as to its spectrum of activity.

Akinpelu and Obuotor (2000) showed that the methanolic extract of *Piliostigma thonningii* stem bark exhibited activity against six out of eight bacterial isolates at a concentration of 20 mg/ml. In a survey of 84 medicinal plants used locally in the treatment of various diseases in Bauchi State—Nigeria, Adamua et al., 2005 showed that *Piliostigma* extracts were found to show potentially interesting activity against *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* at a concentration of 200 mg/ml

Table 4: Uses of *Piliostigma reticulatum*

Locality	Plant part	Use	Indications	References
Kano, Northern Nigeria	Leaves and young buds	Decoction	Eye wounds and infection	Hussain and Karatela, 1989
West Africa	Leaves(+ leaves of Xylopia aethiopica, Annonaceae)	Decoction taken as beverage	Fever	Assi and Guinko, 1991
West Africa	Raw shoots	Eaten raw	Dysentery	Assi and Guinko, 1991
West Africa	Leaves	Decoction	Gastric pains and as tonic	Assi and Guinko, 1991

West Africa	Crushed flower buds	Diluted in water in water	Heart pain	Assi and Guinko, 1991
West Africa	Young leaves	Chewed and applied as paste	Gingivitis	Assi and Guinko, 1991
Western Nigeria	Leaves and stem bark (in multiple plant recipe)	Decoction	Dermatomycosis and malaria	Adjanohoun et al., 1991
Nigeria	Powdered bark; leaves	External dressing; infusion used as wash	Wounds and chronic ulcers	Dalziel, 1937
Nigeria	Bark	Infusion	Toothache; astringent for diarrhoea and dysentery	Dalziel, 1937
Nigeria	Bark, root and leaves	Decoction	Leprosy; smallpox	Dalziel, 1937
East Africa	Bark	Boiled in milk or soup	Gonorrhea	Watt and Breyer-Brandwijk, 1962
East Africa	Bark	Concentration infusion	Antipyretic; as mouthwash for inflammation of the gums	Watt and Breyer-Brandwijk, 1962
East Africa	Leaves	Decoction	Cough and chest complaints	Watt and Breyer-Brandwijk,

				1962
Enda, southern Africa	Root and bark (+ root and bark of faurea saligna, proteaceae)	Decoction	Venereal disease and bilharzia	Arnold and Gulumian, 1984
Tanzania	Root (part of multiple plant recipe)	Decoction	Insanity and epilepsy	Mathias, 1982
East Africa	Root (+ root of vigna phaseolloides,papillionaceae	Infusion	Contraceptive	Brondegaard, 1973
Eastern Nigeria	Bark	Infusion	Anthelmintic	Asuzu and Onu, 1989
Senegal	Dried leaves, bark, and flowers	Hot water extract	Phadegenic ulcers, odontalgia, diarrhoea amd dysentery , cough	Le Grand, 1989
Senegal	Dried flowers	Powder is smoked	Cough	Le Grand and Wondergem, 1987
Nigeria	Roots and fruits	Decoction (2 :1 ratio of root : fruit)	Fever, skin diseases	Bhat et al.,1990
Ivory coast	Fresh leaves	Crushed and applied externally	Hemostatic	Kone-Bamba et al.,1987
Senegal	Dried leaves	Hot infusion	Conjunctivitis and headache	Tignokpa et al., 1986
Uganda	Unspecified	Decoction	Diarrhoea	Anokbonggo et al., 1990
East Africa	Root	Infusion	Miscarriage;	Kokwaro,

			prolonged menstruation	1976
Tanzania	Root	Decoction	Stomachache, rectal prolapse, menorrhagia, malignant ulcers	Chhabra et al., 1987
West Africa	Leaves, stem, roots	Infusion	Toothache; cough; wounds; ulcers	Ayensu, 1978
West Africa	Leaves	Not stated	Snakebite	Houghton and Osibogun, 1993
Angola	Bark	Internally, bark is 'cooked' while the macerated bark is used externally	Tuberculosis, cough, bronchitis, asthma; paludism and as a piscicide	Bossard, 1993
Nigeria	Stem bark, leaves	Decoction	Skin infections, smallpox, chickenpox	Burkill, 1995
Gambia, Tanganyika	Stem bark	External dressing	Wound healing	Burkill, 1995
West Africa	Stem bark, leaves	Decoction	Inflammation of the eyes, guinea worm, sore-throat, toothache and gum troubles	Burkill, 1995

2.1.4 Other activities

The leaves, pods and root of *piliostigma reticulatum* (DC.) Hochst was found to exhibit no molluscicidal activity at the test concentration of 100 ppm (Adewunmi and Sofowora. 1980). On the other hand, the 70% ethanol extract of the bark of *piliostigma reticulatum* showed significant broad spectrum of activity against the larvae of common intestinal parasites of cattle (Asuzu and Onu, 1993). The 70% ethanol extract also showed concentration-dependent anthelmintic activity in vivo in chickens, and also induced concentration-dependent contraction of *Ascaridia galli* in vitro (Asuzu and Onu, 1994). These observed activities thus supported the traditional use of the bark as an anthelmintic (Asuzu and Ugwuja, 1989). Aderogba et al., 2006 confirmed the antimicrobial and anti-inflammatory Effects of *Piliostigma reticulatum* Leaf Extract by its comparison with that of *Piliostigma thonningii.*

The 70% ethanol extract of the bark also significantly reduced pentobarbitone-induced sleeping time in mice following intraperitoneal injection, but had no significant effect on leptazole and strychnine induced convulsions (Asuzu and Ugwuja, 1989). The extract also had an oral LD_{50} of 1862 mg/kg in mice. The methanol extract of the leaves and stem bark of *Piliostigma thonningii* had an LC_{50} of 10 - 50µg/ml in an in vitro antimalarial test using the multidrug resistant k1 strain of *Plasmodium falciparum* (Weenen et al., 1990). This is significant, as Adjanohoun et al., (1991) reported the use of *Piliostigma reticulatum* as part of a multiple plant regimen used to treat malaria.

Mandibaya and Chihora, 1999 and Babajide et al., 2006 showed that *Piliostigma* pod meal has lower nutritive value but can be very useful feed supplement to communal cattle grazing poor quality forage during the tropical dry season. Akhtar et al., 2000 showed its usefulness as an antihelmintic in livestock. In a recent survey of plants used ethnomedically against cancer, *Piliostigma reticulatum* was found to be one of them as shown by the NAPRALERT database currently maintained by the Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, and University of Illinois-Chicago, USA. Which are part of the work of Jonathan

Hartwell, ‘‘Plants used against cancer: a survey’’, including previously unrepresented genera and families (Graham et al., 2000).

Extracts of 23 plant species used popularly against schistosomiasis in Zimbabwe were screened for their anthelmintic effect. Schistosomules of the trematode *Schistosoma mansoni* and cysticercoids of the cestode *Hymenolepis diminuta* were studied in vitro. The material consisted of 58 plant extracts, of which *piliostigma* is one of the 37 that killed the newly excysted cysticercoids within an hour, when incubated in a culture medium (Mølgaard et al., 2001). Ajaiyeoba et al., 2006 showed the antimalarial potentials in the plant while Rochfort et al., 2008 explain its bioactive contents for ruminant health and productivity. Because it combine reproductive efficiency, milk and meat quality improvement as well as serving as antibiotics (Mlambo et al., 2008). Asuzu (1999) showed the antihelmintic activity of D-3-*O* methylchiroinositol isolated from *Piliostigma thonningii* stem bark.

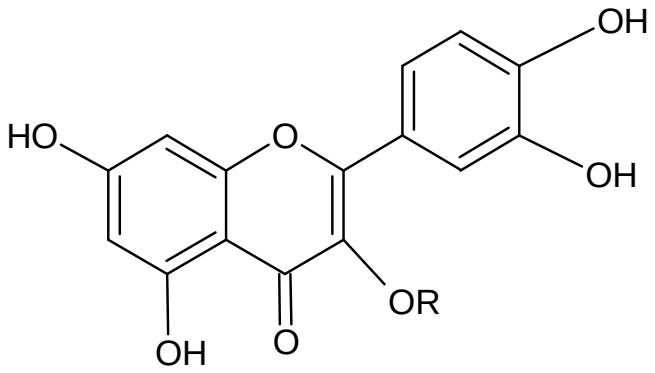
Finally, Bombardelli et al., (1992) found that the 50% acetone extract of *Piliostgma reticulatum* leaves produced a statistically significant reduction of ammonia aerosol induced cough in guinea pigs, as well as cough induced by electrical stimulation of the trachea in the anaesthetized guinea pig. This observed antitussive activity has been linked to the catechin oligomers in the polyphenolic fraction of the plant extract (Bomnardelli et al., 1994) and explains the widespread use of the plant in the treatment of cough (Table 4; Watt and Breyer-Brandwijk, 1962; le Grand, 1989; Ayensu, 1978; Bossard. 1993).

2.1.5 Chemical constituents of Piliostigma reticulatum.

Piliostigma reticulatum is a plant which is rich in tannins, and the bark is used for tanning leather. Dalziel (1937) reported that the bark of the smaller twigs contain over 20% of tannin, while the bark of larger branches and the root have up to 18% tannin, and the immature pods contain lesser amounts decreasing towards maturity.

The first systematic investigation of the chemical constituents of *Piliostigma thonningii* was by Ferreira et al., (1963) who found that the bark contained sucrose, pyrocatechic tannins, tartaric acid, citric acid, rhamnitol as well as a carotenoid and

an unidentified sterol. Subsequently, the leaves of *P. thonningii* were found to contain quercetin(**X**), quercitrin (**XI**) and quercetin-3-O-glucoside (**XII**) (Bombardelli et al., 1973).



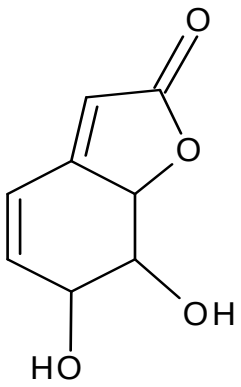
X R = H

XI R = rhamnose

XII R = glucose

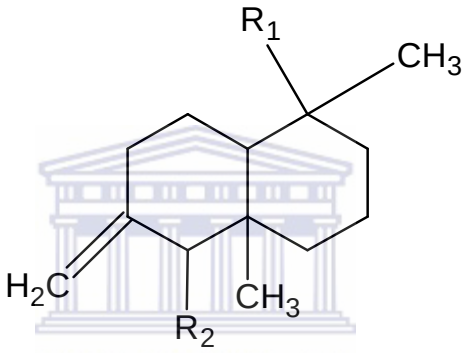
Further investigations by Bombardelli et al. (1992; 1994) showed that the plant contains (-)-epicatechin, proanthocyanidin B2 as well as catechin trimers, tetramers and higher oligomers.

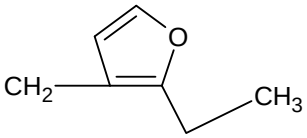
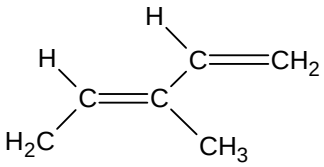
Okwute et al. (1986) isolated griffonilide (**XIII**) from the stem bark. Griffonilide, a γ-lactone, had previously been isolated from *Griffonia simplicifolia* (Baill.) (Caesalpiniaceae) by Dwuma-Badu et al, (1976).



XIII

Snatzke and Wolff (1989) isolated 5α-stigmasta -7,2,2-dien-3β-ol and four labdane derivatives from the petroleum ether extract of the root. The labdane derivatives were lambertianic acid (**XIV**), lambertianol (**XV**), lambertianol methyl stearate (**XVI**) and trans-communic acid (**XVII**).



	R1	R2
XIV	–COOH	
XV	$-CH_2OH$	,,,,
XVI	$-CH_2OCO(CH_2)_{16}CH_3$	,,,,
XVII	–COOH	

Finally, literature showed that established isolated constituents include flavonoids and proanthocyanidins (Snatzke and Wolff, 1989; Lamikanra et al., 1990; Komiya et al., 1975), labdane derivatives (Hashidoko et al., 1991; Huang et al., 1993), a kaurane diterpene (Roitman and James, 1985; Martin et al., 1997), *C*- methylflavonols (Breitmeier and Voelter, 1990; Harborne et al., 1994; Rabesa and Voirin, 1985; Voirin, 1983; Aderogba et al., 2003; Rasamoelisendra et al., 1989) and phenoxychromones (Ibewuike et al., 1996; 1997), while broad phytochemical analysis showed the presence of other classes of constituents such as saponins, tannins and polyphenols (Irvine, 1961; Bombardelli et al., 1994). Asuzu (1999) showed the isolation of D-3-*O*-methylchiroinositol from *Piliostigma thonningii* stem bark.

2.2 *CISSAMPELOS CAPENSIS*

2.2.1 Plant description 1

Cissampelos capensis is one out of 520 species in 75 genera (Watson and Dallwitz, 1992). This plant belongs to the family Menispermaceae. Well notable synonyms to *Cissampelos capensis* are *Cissempelos pareira, C. acuminate, C. cordata, C. cordifolia, C. hirsuta, C. orbiculata, Antizoma capensis* (L.f.) Diels, *pulverulenta* (Harv.) Diels, *Cissampelos fruticosa* (L.f.) Diels, and *Cissampelos humilis* (Poir). It is widely distributed in the Sandy slopes and scrub of the Northern, Western and Eastern Cape Provinces of South Africa and northwards into Namibia. It is called by different local name such as Dawidjiewortel (Afrikaans) and Mayisake (Xkhosa). Due to of its richness in bisbenzylisoquinoline alkaloids, this family is used worldwide in traditional medicine to treat a variety of ailments (Barbosa- Filho et al., 2000; DeWet et al., 2004, 2005). The literature revealed that out of the 75 genera in the Menispermaceae, twenty-one genera are used for medicinal purposes in Africa and twenty-nine genera in the rest of the world. (Neuwinger, 2000; Duke, 2007;De Wet, 2006). The southern Africa Menispermaceae comprises of seven genera and thirteen species, and among these one genus and three species are endemic to the region. In South Africa, some ethnobotanical information has been recorded (summarized in Arnold et al., 2002) but only one species, *Cissampelos capensis* (L.f.) Diels [dawidjies (wortel)], is a well-known and much used medicinal plant, particularly in the Eastern and Western Cape Provinces (Watt and Breyer-Brandwijk, 1962; Smith, 1966; Rood, 1994; Cillie, 1992; Dykman, 1891; Iwu, 1993).

Fig 5: Dawidjiewortel (Cissampelos capensis woody shrub)

2.2.2 *Plant description 2*

Dawidjiewortel is a woody dioecious perennial climbing vine with a sprawling or twining **stem** shrublet without tendrils but supports itself by twining around the stems of other plants, fence or walls (Figure 5). The vine is blackish-brown and tough. When this is freshly cut it has a waxy luster. The **leaves** are rounded, bright green, entire, alternate, almost without hairs. It posses an ovate to heart-shape and up to 2.5cm wide by 2.5cm long, on thin petioles up to 3cm long. The **flowers** which usually sprout between the month of February and May are an axillary, velvety-hairy, greenish in colour. The female flowers which are 1 to 2 are with 1 sepal and petal while the male in cymose inflorescences are produced in clusters. The **fruit** is inedible, dark, grape-sized berries or sometimes they are small fleshy orange berries. The **root** is up to 2.5cm in diameter with grey-brown bark, cream in transverse section with distinct brown medullary rays (Botha, 1980). The plant is usually confused with *Zehneria scabra* (Cucurbitaceae), which is in some parts known by the same local name (Smith, 1966). The latter resembles a cucumber and can be distinguished by the spirally coiled tendrils. Under the microscope the leaf showed the absence of calcium oxalate crystals in the lamina and the occasional unicellular clothing hairs of leaf and stem, up to 240µm long, with thin slightly roughened walls.

The cells of the leaf epidermis are with striated cuticle and thickened walls. The presence of anomocytic stomata found on both leaf surfaces with papillated cells making up the leaf margin (Botha, 1980).

2.2.*3* *Ethnomedicinal uses* of *Cissampelos capensis*

Nearly all the parts of the plant were put to use, from the whole vine, seed, bark, leaf to the root. The general ethnomedicinal uses of *Cissampelos* species all over the world is as shown in Table 5. It is traditionally used in South Africa to treat variety of ailments such as gravel and glandular swelling, gall stones. A weak brandy tincture for dysentery, mucous membrane infection, menstrual problems, prevention of miscarriage, difficult labour, expelling the placenta, appetite stimulant, blood purification, antisyphilitic use, cholera, colic, erypsipelas internally and externally, bladder problems, snakebite, stomach pain, influenza. Toothache: chew root and a sedative effect by chewing the rhizome. For treating pain, using infusions, bilious complaints, measles: mix root with half a teaspoon Epsom salt, fever: mixed root with "grandpa powder" (a headache powder, which contains aspirin, paracetamol and caffeine), vinegar and sugar, headache: smoke inhaled through nostrils to treat headache, diabetes, tuberculosis, stomach and skin cancers, purgative, good-luck charm: root is carried around, the leaves are used for: ulcers and syphilis sores: paste is used, snakebite wound: paste is used (Smith, 1895; Watt and Breyer-Brandwijk, 1962; Rood, 1994; Von Koenen, 2001; Van Wyk and Gericke, 2000; VanWyk et al., 1997; De Wet and Van Wyk, 2008;; Barbosa- Filho et al., 2000; DeWet et al., 2004, 2005).

It sometimes administered as leaf infusion or the tincture is taken orally and a poultice or paste applied externally for various ailments. Preparations of the root of this species are also extensively used, both internally and externally but it is worthy to note that high precautions must be maintained because of the presence of highly bioactive alkaloids which are known to be toxic. The vine or root is used in tropical countries to prevent a threatened miscarriage and to stop uterine hemorrhages after

childbirth. The plant extract is also believed to aid poor digestion, drowsiness after meals, and constipation.

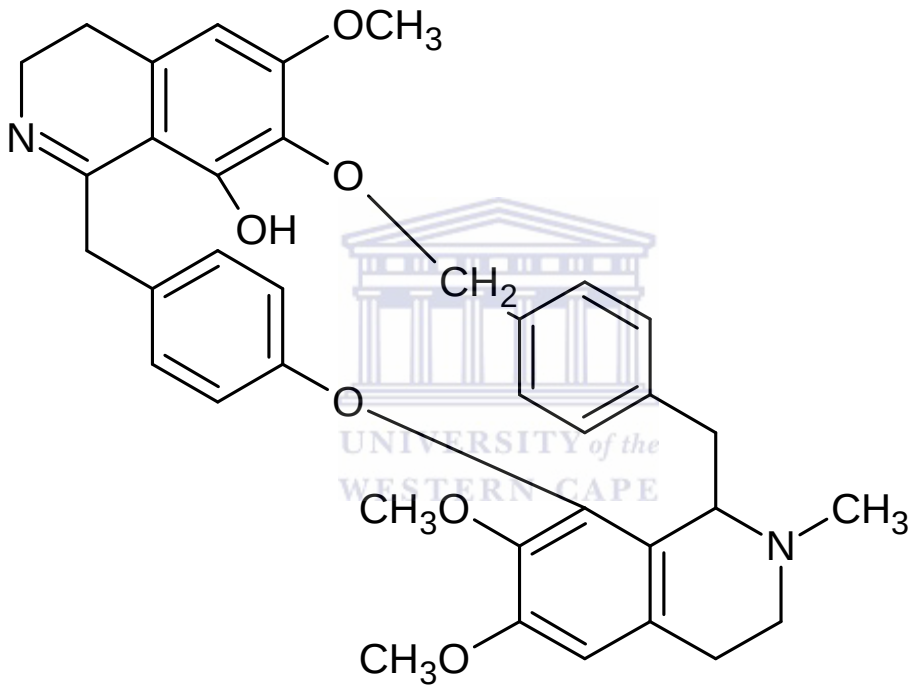
2.2.4 Reported biological activities of Cissampelos species

In 1962, it was reported that *C. pareira* demonstrated anti-inflammatory, smooth muscle relaxant, antispasmodic, and uterine relaxant actions in various laboratory animals. Subsequent studies with animals confirmed the plant's antispasmodic and anti-inflammatory actions. These documented effects are quite similar to *Cissampelos* species traditional uses for menstrual disorders (including cramping and pain) (Pillay et al., 2008; Taylor, 2005). In other animal studies, a root extract was reported to have a diuretic effect, a finding that confirms another of its traditional medicine uses (Taylor, 2005; Ssegawa and Kasenene, 2007). Other *in vivo* studies on the extracts of *Cissampelos* species indicated that the leaf has antiulcerous actions and that the root has a very mild hypoglycemic action (only at high dosages). Studies have also shown that the *Cissampelos* species root has other possible therapeutic uses: it demonstrated anticonvulsant actions in mice; and, in dogs, it was shown to significantly lower blood pressure. In addition, test-tube (*in vitro*) studies over the years of related *Cissampelos* species has reported that they have antioxidant properties; antibacterial actions against *Staphylococcus, Pseudomonas, Salmonella*, and *Klebsiella*; and antimalarial effects. One of these *in vitro* studies also reported that a root extract demonstrated a toxic effect against colon cancer cells. Alkaloids constituents have been ascribed to posess sedative, antispasmodic and antitumour properties (Dic. Nat. prod., 1996; Bruneton, J., 1995)

2.2.5 Major chemical constituents

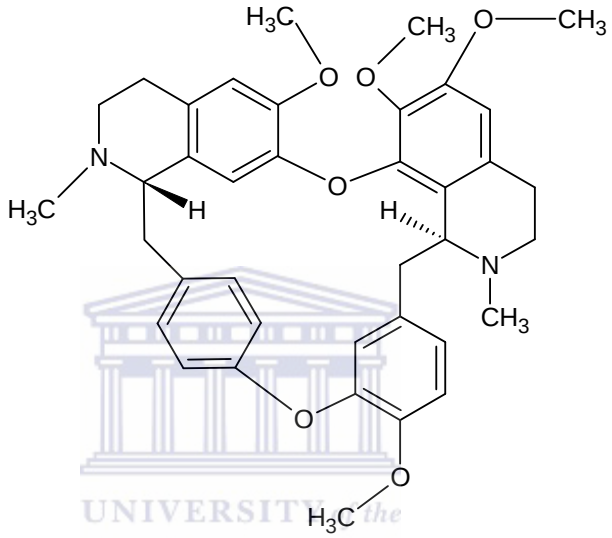
Phytochemical tests showed the presence of alkaloids, saponins and tannins but cardiac glycosides and anthraquinone were absent. The chemical profile of the family typically includes benzylisoquinoline, bisbenzylisoquinoline (e.g. tubocurarine,

cissampareine), diterpene and triterpene alkaloids, as well as saponins. A large number of biologically active alkaloids of the bisbenzylterahydroisoqunoline type have been isolated from several *Cissampelos species* and various other genera of the Menispermaceae, and among these cissampareine (**XVIII**) is a typical example (Dic. Nat. prod., 1996; Jittra et al., 2005; Maria et al., 1995)



Cissampareine (XVIII)

Cissampelos plants, including *C. pareira*, contain a group of plant chemicals called isoquinoline alkaloids. Since the late 1960s, it has been found that these chemicals have received a great deal of attention and research. One, out of thirty-eight alkaloids thus far discovered in *Cissampelos* species, called **Tetrandrine** (**XIX**) is the best documented.

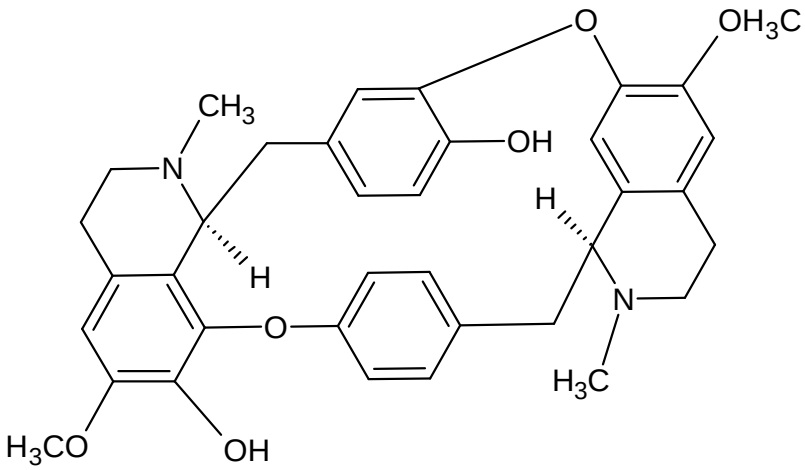


Tetrandrine (**XIX**)

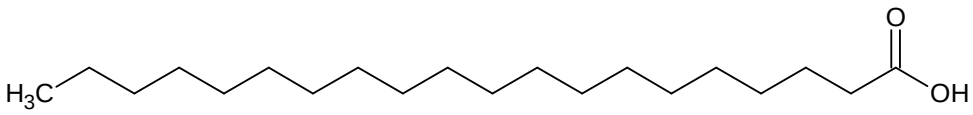
Clinical research over the years has found Tetrandrine to have pain-relieving, anti-inflammatory, and fever-reducing properties. More than one hundred recent clinical studies also describe this chemical's promising actions against leukemia and some other cancer cells. However, the therapeutic dosages of Tetrandrine used in these animal studies are much higher than one can reasonably obtain from natural *C. pareira* root or vine. Another well-known alkaloid **Berberine** (**XX**) has been documented to have hypotensive, antifungal, and antimicrobial actions. This chemical has been used for the treatment of irregular heartbeat, cancer, Candida, diarrhea, and irritable bowel syndrome. Another alkaloid called Cissampeline is sold as a skeletal muscle relaxant drug in Ecuador. The main chemicals in *Cissampelos* species, especially *C. pareira* which is well documented are alkaloids, arachidic acid **(XXI)**,

bebeerines**(XXVI)** , berberine (**XX**), bulbocapnine **(XXVII)**, cissacapine **(XXVIII)**, cissampareine**(XVIII)** , corytuberine, curine, 4-methylcurine, cyclanoline, cycleanine, dicentrine, dehydrodicentrine, dimethyltetrandrinium, essential oil, grandirubrine, hayatine, hayatinine, insularine, isochondodendrine, isomerubrine, laudanosine, linoleic acid, magnoflorine **(XXIV)**, menismine, norimeluteine, nor-ruffscine, nuciferine, pareirine, pareirubrine alkaloids, pareitropone **(XXIX)**, quercitol, stearic acid, and tetrandrine (**XIX**) (Taylor, 2005).

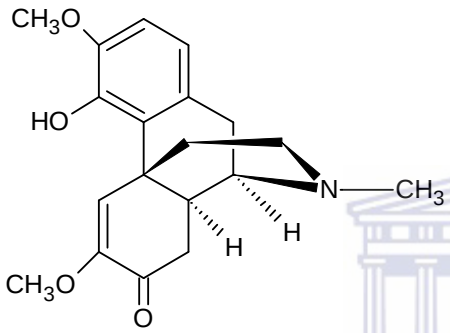
Hydrocolloids, an important thickeners and gelling stabilizers in food system were isolated from *C. Pareira* and characterized by Vardhanabhuti and Ikeda (2006). A red-brown amorphous aporphine alkaloid powder, Cissaglaberrimine **(XXIII),** Magnoflorine **(XXIV)** and Oxobuxifoline **(XXV)** were isolated from *C. glaberrima* (Barbosa-filho et al., 1997) while Milonine **(XXII)**, an 8, 14-dihydromorphinandienone alkaloid was also isolated from the dried leaves of *C. sympodialis* (Freitas et al., 1995). Similarly pareitropone **(XXIX)**, a condensed tropone-isoquinoline alkaloid with high antileukemic activity was also isolated from *C. pareira* (Morlta et al., 1995).



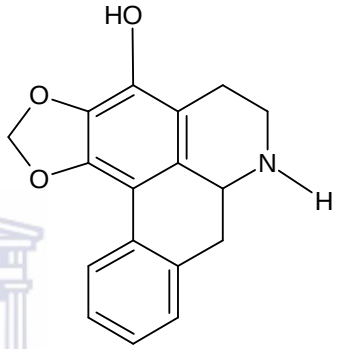
Berberine (**XX**)



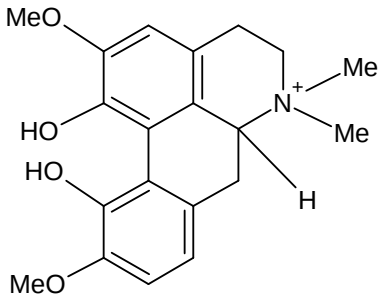
Arachidic acid (Eicosanoic acid) (XXI)



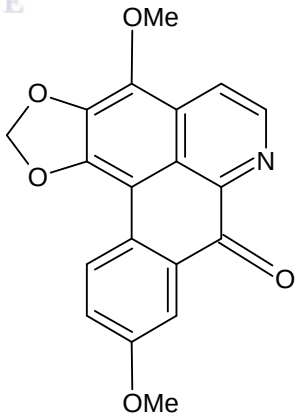
1-Milonine (XXII)



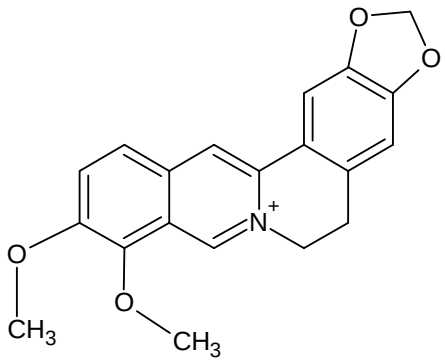
Cissaglaberrimine (XXIII)



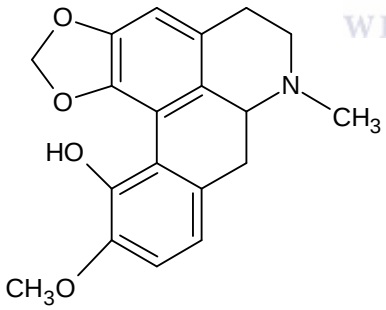
Magnoflorine (XXIV)



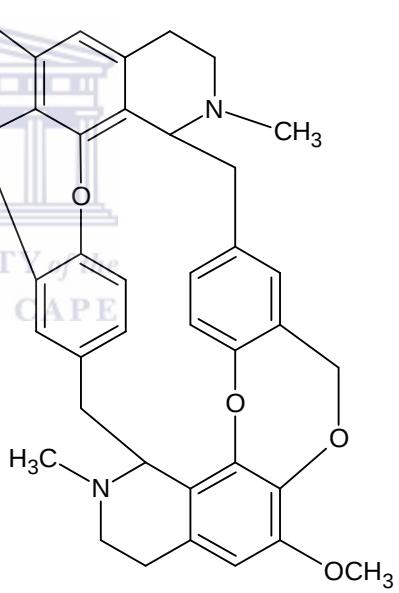
Oxobuxifoline (XXV)



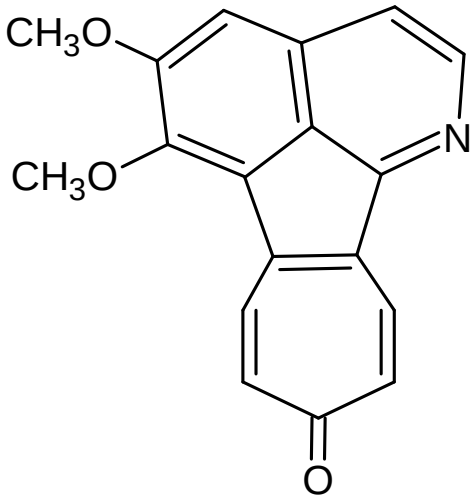
Bebeerine (XXVI)



Bulbocapnine (XXVII)



Cissacapine (XXVIII)



Pareitropone (XXIX)

2.2.6 *Current Uses*

Cissampelos is still in use in many part of the world where it can be found used for the same purposes it has been used traditionally for centuries - as a childbirth aid and for general women's ailments. South and North American natural health practitioners commonly rely on *Cissampelos* as an excellent natural remedy for menstrual difficulties, including cramping and pain, premenstrual syndrome (PMS), excessive bleeding, and fibroid tumors. Its ability to curb excessive menstrual bleeding very quickly can be quite remarkable. It is often employed in overall female balancing formulas, in kidney formulas (for its diuretic and smooth-muscle relaxant effects), and in combination with other plants, in heart tonics and hypertension remedies. Toxicity studies with animals confirm the safety of the plant; rats given 10 g of the plant per kilogram of body weight evidenced no toxic effects (Wu, 2007; Amresh et al., 2007a and b; Amresh et al., 2004; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001; Taylor, 2005; Graham et al., 2000).

Hamill et al., 2003 showed the antimicrobial properties in *Cissampelos* while Amresh et al., 2007b showed the antioxidant potential in which the roots of *C. pareira* were

found to contain a large amount of polyphenols and exhibit significant and dose-dependent reducing ability, indicative of potent antioxidant ability both in vitro and in vivo. It was also found to significantly scavenge superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide at a dose regimen of 50 to 400 μg/kg in vitro. The Antinociceptive and antiarthritic activity of *Cissampelos pareira* roots were equally highlighted by Amresh et al., 2007c while the antifertility activity and anti-inflammatory activity were showed by Ganguly et al., 2007 and Amresh et al., 2007b respectively.

The Khoi-San or Cape Dutch showed the importance of this plant in Cape herbal medicine as one of the major distinct plant used for treatment of most diseases (VanWyk, 2008) while in the evaluation of some South African medicinal plants for antimalarial properties it was discovered that *C. capensis* posses antiplasmodial activity (Pillay et al., 2008). The medicinal values of *Cissampelos* species in Sango bay area in Southern Uganda was also recorded by Ssegawa and Kasenene (2007).

TABLE 5: Uses of *Cissampelos capensis*

Locality	Plant part	Indications	References
Amazonia	Leaves and young buds whole vine, seed, bark, leaf, root.	Childbirth, colic, fever, muscle spasms and pain, nervous children, pinta, snakebite.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.
Argentina	Whole vine, seed, bark, leaf, root.	Diarrhoea, menstrual disorders, respiratory tract infections, urinary tract infections.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Amresh et al., 2004; Caceres et al., 1987; Gessler et al., 1995 Ramirez et al., 2003; Sanchez Medina et al., 2001.
Brazil	Whole vine, seed, bark, leaf, root.	Abortions, anemia, asthma, bladder problems, colic, congestion, constipation, contusions, cramps, cystitis, digestive problems, detoxification (by inducing sweating), dysentery, dyspepsia, drowsiness, edema, excessive phlegm and mucous, fever, gallbladder problems (to stimulate bile), hepatitis, inflammation, kidney	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Amresh et al., 2004; Caceres et al., 1987; Gessler et al., 1995; Morita et al., 1993; Morita et al., 1993b; Chapuis et al., 1988; Ramirez et al., 2003; Sanchez Medina et al., 2001.

		stones, menstrual disorders, muscle aches, pains and spasms, testicular inflammation, threatened miscarriage, pre-and postnatal pain, rheumatism, snakebite, stomach problems, urinary tract disorders, uterine hemorrhages, water retention.	
Guatemala	Leaves whole vine, seed, bark, leaf, root.	Cramps, erysipelas, fever, menstrual disorders, rheumatism, snakebite, water retention, and to increase perspiration.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.

Mexico	Crushed flower buds, whole vine, seed, bark, leaf, root.	Bladder problems, dermatitis, diarrhea, dysentery, edema, excessive phlegm and mucous, fever, insect bites, jaundice, menstrual disorders, muscle inflammation, nephritis, pain, pimples, rheumatism, snakebite, urogenital problems, vaginal discharge, water retention, and as a female balancing aid.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Amresh et al., 2004; Caceres et al., 1987; Gessler et al., 1995; Morita et al., 1993; Morita et al., 1993b; Chapuis et al., 1988; Ramirez et al., 2003; Sanchez Medina et al., 2001.
Nicaragua	Young leaves, whole vine, seed, bark, leaf, root.	Bites, fever, skin rash, sores, stings, venereal disease.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.
South Africa	Leaves and stem bark (in multiple plant recipe) whole vine, seed, bark, leaf, root.	Gravel and glandular swelling, Gall stones, dysentery, infections, Menstrual problems, Prevention of miscarriage, Appetite stimulant, Blood purification, Cholera, Colic, Bladder problems,	Barbosa- Filho et al., 2000; DeWet et al., 2004, 2005; VanWyk et al., 1997; De Wet and Van Wyk, 2008; Van Wyk and Gerick, 2000. Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Amresh et al., 2004; Caceres et al., 1987; Gessler

		Snakebite, pains, Toothache, Measles, Fever, Diabete, Tuberculosis Stomach and skin cancers,Purgative, Good-luck charm, Ulcers and syphilis sores.	et al., 1995; Morita et al., 1993;Chapuis et al., 1988; Ramirez et al., 2003; Sanchez Medina et al., 2001.
United States	Powdered bark, leaves, whole vine, seed, bark, leaf, root.	hemorrhages and excessive bleeding, constipation, kidney stones, menstrual disorders, muscle spasms, premenstrual syndrome (PMS), testicular inflammation, urinary tract irritation, water retention.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.
Venzuela	Bark, whole vine, seed, bark, leaf, root.	Bladder problems, kidney stones, snakebite, also used as a diuretic.	Taylor, L., 2005; Feng et al., 1962; Amresh et al., 2004; Caceres et al., 1987; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.

Other places	Bark, root and leaves, whole vine, seed, bark, leaf, root.	Abortions, anemia, arrow poisoning, asthma, boil, childbirth, constipation, cough, cystitis, diabetes, diarrhea, dyspepsia, excessive phlegm and mucous, edema, eye problems, fetal growth problems, fever, hemorrhages, hypertension, indigestion, itch, kidney stones, malaria, menstrual disorders, pain, post-menstrual hemorrhages, rheumatism, snakebite, sores, sterility, threatened miscarriage, urogenital inflammation, uterine hemorrhage, venereal disease, water retention, wounds and as a female balancing aid.	Taylor, L., 2005; Feng et al., 1962; Wu, 2007; Amresh et al., 2006; Adesina, 1982; Amresh et al., 2004; Caceres et al., 1987; Gessler et al., 1995; Ramirez et al., 2003; Sanchez Medina et al., 2001.

2.3 *GERANIUM INCANUM*

2.3.1 Plant description 1

Geranium incanum is a shrublet plant that belongs to the family Geraniaceae. It is refered to as Vrouebossie – amarabossie (Afrikaans) and Ngope-sethsoha, Tlako (Sotho). This plant is commonly found along the southern coastal areas of the Western and Eastern Cape Provinces of South Africa (Hilliard and Burtt, 1985).

Fig 6: *Geranium incanum* plant

2.3.2 Plant description 2

This is an attractive plant and is a sprawling perennial shrublets with finely divided silvery leaves (Figure 6). The white, pale pink, violet or magenta flowers are borne on long, slender stalks, followed by a characteristically elongated fruit resembling a stork's bill (Figure 6). The hairs on the exterior of the flowers and flower stalks lie flat and are not spreading as in related species (Hilliard and Burtt, 1985).

Species of *Geranium* are all very similar and have been much confused in the past (Hilliard and Burtt, 1985). It is therefore possible that some medicinal records apply to species other than true *G. incanum* and *G. canescens*. *G. incanum* has been divided into two varieties: *var. incanum*, with small, white or pale pink flowers, and *var.*

multifidum, with larger, light violet to magenta pink flowers (Hilliard and Burtt, 1985).

Akpulat and Celik 2005, Clark et al., 2009 and Alireza et al., 2009 showed how the climatic environment, soil structure, the water and mineral intake that is available in the soil can affect both the floristic diversity and characteristic as well as the phytochemical composition in a plant. This phytochemical variation is found to be due to soil pH, the slope of the terrain which can affect the amount of nutrient it can retain due to wash off of nutrients and altitudinal belts.

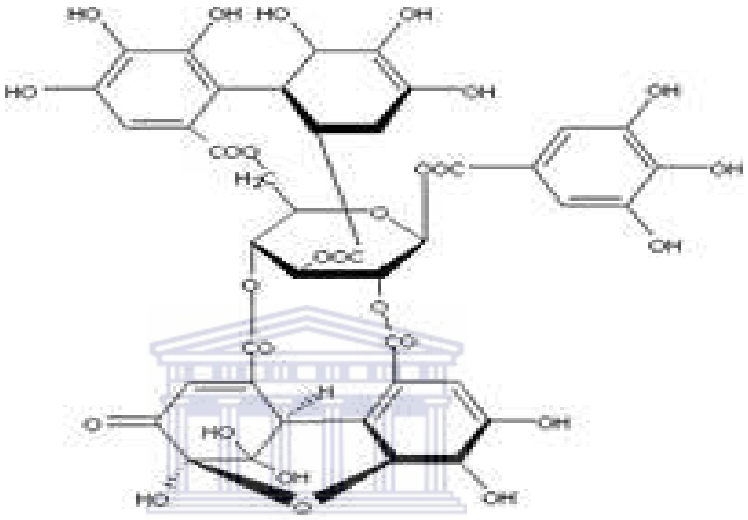
2.3.3 *Ethnomedicinal uses of Geranium incanum*

The leaves have been used as a tea substitute (Rood, B., 1994; Watt and Breyer-Brandwijk 1962) for treating bladder infections, venereal diseases and menstruation-related ailment, hence the common name vrouebossie ("vroue" = women; "bossie" = small bush) (Smith, C.A., 1966). It is used also for colic, diarrhoea, fever and bronchitis (Watt and Breyer-Brandwijk 1962). It is very interesting to note that other *Geranium species* such as *G. robertianum* (Robert Herb) are traditionally used in Europe and America to treat diarrhoea (Bruneton, J., 1995; Grieve, M., 1967). The indication common to all tannin-containing drugs is the symptomatic treatment of diarrhoea (Bruneton, J., 1995; Amabeoku, G.J., 2009). Steenkamp, 2003 highlighted the fact that traditional remedies are part of the cultural and religious life of the African people and that *G. incanum* is used extensively for gynaecological complaints in South Africa which confirm the earlier observation of VanWyk for use of the plant extract for menstruation associated problem (Van Wyk and Gericke (2000), Van Wyk et al. (1997).

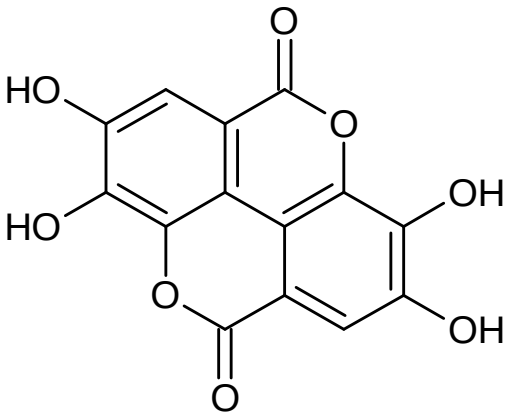
2.3.4 Major chemical constituents

Phytochemical tests reveal the presence of saponins, flavonoids and tannins such as Ellagic acid **(XXXI)** and Gallic acid derivatives **(XXXII)** but alkaloid, cardiac glycosides and anthraquinone were absent. The chemical profile of the leaves of

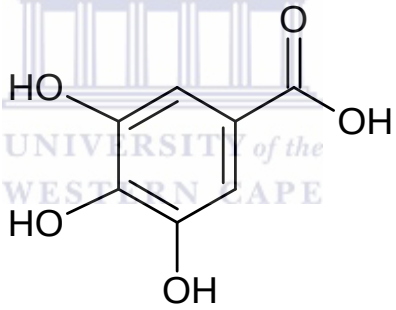
Geranium species is known to contain tannins, in which geraniin **(XXX)** is likely to be one of the compound in the plant (Dic. Nat. prod., 1996; Bruneton, J., 1995; Amabeoku, G.J., 2009). Several flavonoids have also been presumed to be present in *Geranium* species. Amabeoku, (2009) also showed that the aqueous leaf extract of *Geranium incanum* possess antidiarrhoeal activity.



GERANIIN (XXX)



ELLAGIC ACID (XXXI)



GALLIC ACID (XXXII)

2.4 *GETHYLLIS SPECIES*

2.4.1 Plant description

Gethyllis species are from the family Amaryllidaceae and the species are *gregoriana, multifolia and villosa.* They are generally referred to as Koekemakranka (Khoi, Afrikaans) or Kukumakranka (English). It is noteworthy that *Gethyllis* is considered a rare and endangered genus in the Amaryllidaceae family that are found mostly in the winter rainfall region of southern Africa and they are most numerous in the Western and Northern Cape Provinces (Muller – Doblies, 1986; Du Plessis and Duncan, 1989; Elgorashi and Van staden, 2004; Elgorashi et al., 2007).

Fig 7 *Gethyllis* plant showing the spiral appearance of the leaves

Fig 8 *Gethyllis* plant showing the flowering time without the leaves

It has an unusual foliage, flowers and fruits that are very fascinating because of the fact that they produces their leaves, flowers and fruit all at different times of the year which never co-occur (Van Wyk and Gericke, 2000). Koekemakranka is an underground bulb of which the scales form a distinctive neck at ground level (Figure 7). The long, thin leaves are usually spirally twisted, tattooed or coiled (Figure 7). The attractive flowers appear in summer when the leaves have already died. They have a narrow tube which extends below the ground, where the fruit eventually develop. *Gethyllis* flowers (Figure 8) are produced in summer and have a subterranean ovary which remains cool and protected underground. The attractive white or pink flowers (Figure 8) are followed by fragrant club-shaped berries that emerge in mid-winter, long after the flowers and leaves have wilted (Van Wyk and Gericke, 2000). There are about 38 *Gethyllis* species, in which *G. afra* (also called "bramakranka") and *G. spiralis* are perhaps the most fragrant and commonly available. (Muller – Doblies, 1986; Du Plessis and Duncan, 1989) but more and more

new *gethyllis* are being discovered and the list is increasing by the day (Smith, 1966). *G. spiralis* is perhaps the most fragrant and commonly used one.

2.4.2 Ethnomedicinal uses of Gethyllis species

Koekemakranka brandy is one of the early Cape remedies used in traditional medicine to treat colic, flatulence and indigestion (Smith, C.A., 1966; Cillie, A.M., 1992; Rood, B., 1994; Watt and Breyer-Brandwijk 1962; Du Plessis and Duncan, 1989; Forbes, V.S., 1986). The ripe edible fruit was highly valued to perfume rooms and linen because of their powerful sweet and fruity odour (Watt and Breyer-Brandwijk 1962; Forbes, V.S., 1986). For children, the gathering of the inconspicuous fruit (which they refer to as "koekemakrankies") is a special occasion, and success depends to a large extent on a good sense of smell. (Smith, C.A., 1966). The fruits of many species of *Gethyllis* are administered as an alcohol infusion or as a brandy to treat stomach disorders. The fruits are much sought after for their fragrance and reported medicinal properties. In the past, the odourous dried fruits were often used to scent handkerchiefs and cupboards (Van Wyk and Gericke, 2000). Today the edible fruits of *Gethyllis* are still used to make "kukumakranka" brandy, a popular drink (Van Wyk and Gericke, 2000).

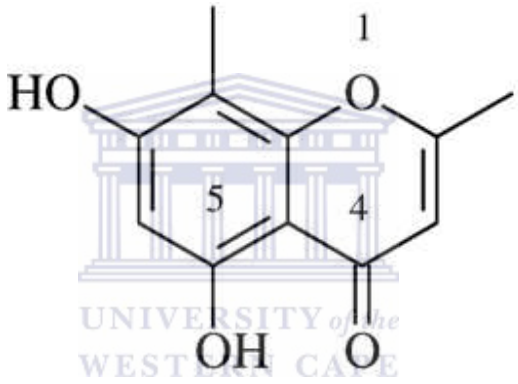
Traditionally, an alcoholic infusion or tincture is made from a few ripe fruits (Dic. Nat. prod., 1996) which are presumed to contains some oils and esters of low molecular weight alcohols (Watt and Breyer-Brandwijk 1962),

2.4.3 Major chemical constituents

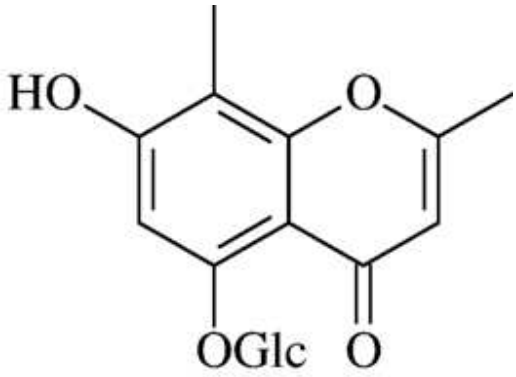
Kamatou et al 2008 reported the presence of 29 volatile organic compounds in the fruit of *G. ciliaris* which represented 96.5% of the total composition. The major compounds detected include pentacosane (19.2%); ethyl octanoate (18.0%); ethyl isovalerate (11.7%); ethyl hexanoate (9.1%) and ethyl benzoate (7.4%). It was concluded that these compounds may be the major contributors to the fruity-sweet odour of *G. ciliaris*. 43 compounds were identified in the fruit of *G. afra* which represented 87.9% of the total composition with α-pinene (11.2%), n-butyl n-butyrate

(8.5%), isoamyl acetate (8.1%), β-pinene (6.4%) and 2-methylbutyl butyrate (5.8%) as its main constituents. These major constituents were equally associated with the banana/piney/fruity odours found in *G. afra*.

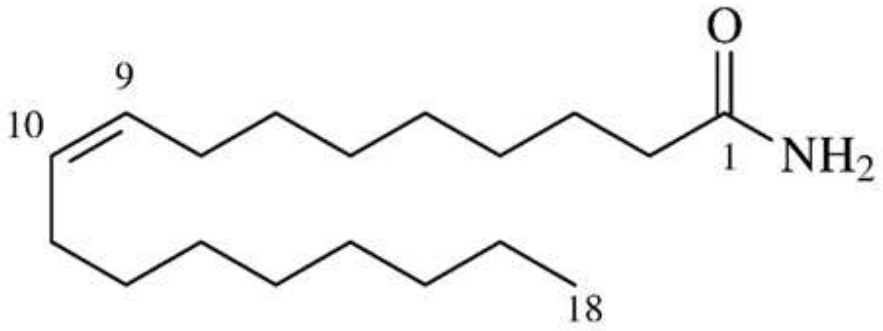
Elgorashi et al., 2007 showed how bioassay guided fractionation of the dichloromethane extract of the underground parts of *Gethyllis ciliaris*, using a cyclooxygenase-1 enzyme assay, has resulted in the isolation of the dihydroxydimethylbenzopyran-4-one (isoeugenitol) **(XXXIII)** and how 90% methanolic extract of same plant yielded 9Z-octadec-9-enamide **(XXXV)** and isoeugenitol glycoside **(XXXIV)** respectively.



Isoeugenitol (XXXIII)



Isoeugenitol glycoside (XXXIV)



9Z-octadec-9-enamide (XXXV)

It is noteworthy that alkaloids were not detected in either the dichloromethane or 90% methanolic extracts using Dragendorff's reagent as a conventional method of detection. The majority of compounds found in the Amaryllidaceae family are alkaloids (Fennell and Van Staden, 2001). Specific alkaloids, which are unique to the family, are associated with its members. Amaryllidaceae alkaloids repeatedly indicated antitumour potential and amongst other characteristics, showed in vivo activity against various human viruses (Duri et al., 1994; Hutchings et al., 1996). The bulbs also contain flavonols, organic acids, carbohydrates and soluble nitrogen compounds. Plants belonging to the family Amaryllidaceae, including some *Gethyllis* species, are known to be the exclusive source of Amaryllidaceae alkaloids (Viladomat et al., 1997). However, the fact that alkaloids were not detected in *G. ciliaris,* does not necessarily mean that this species does not contain Amaryllidaceae alkaloids. More emphasis, in future research, needs to be paid to the ontogenic and environmental factors that affect the production of alkaloids just as it was mentioned in the case of *Geranium incanum* (Viladomat et al., 1997).

2.4.4 Reported biological activities of Gethyllis species

The use of *Gethyllis* species as a potential medicinal plant was broadly highlighted in the literature (VanWyk, 2008, Nielsen et al., 2004, Thring and Weitz, 2006, Helme and Trinder-Smith, 2006). The anti-inflammatory and antibacterial activities in *Gethyllis* species were confirmed by Elgorashi and Van Staden, 2004 and Louw, et al., 2002. Dichloromethane extracts showed high anti-inflammatory activity and a moderate antibacterial activity. The detected anti-inflammatory and antibacterial activities are in line with the uses of some of these plants as reported in their investigation and uses in traditional medicine (Elgorashi et al., 2003). Several members of the Amaryllidaceae are toxic and can cause symptoms such as headaches, excessive salivation, nausea, dizziness, heartbeat irregularities, visual disturbances and dermatitis. Some toxic principles include lycorine and haematin, and the inappropriate use of a number of species can be fatal (Hutchings et al., 1996). Nevertheless, other species are administered orally as medicine to children or eaten in porridge by local people (Crouch et al., 1999).

CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS AND INSTRUMENTATION

Plant Materials:- *Piliostigma reticulatum* leaves

Cissampelos capensis areial shoot and root

Geranium incanum (whole plant)(winter and summer collections)

Gethyllis gregoriana (whole bulb)

Gethyllis multifolia (whole bulb)

Gethyllis villosa (whole bulb)

All laboratory grade solvents were distilled prior to use. Spectroscopic grade solvents were used as such. Adsorption column chromatography was performed using the following methods: Column Gradient Chromatography (CGC), Vacuum Liquid Chromatography (VLC) and Accelerated Gradient Chromatography (AGC). AGC is a modification of conventional medium pressure liquid chromatography (Pongprayoon et al., 1991a; BaeckstrÖm, 1993; Dunstan, 1995). The ascending mode was employed, using Merck Silica gel 60 H (0.040 -0.063 mm particle size, Merck). The equipment for the AGC workstation was from BaeckstrÖm Separo AB, LidingÖ, Sweden. Unless otherwise specified, a column with internal diameter of 25 mm was used and volumes of 10 ml were collected in Pyrex test tubes. Size-exclusion column chromatography and Gel filteration were performed using Sephadex LH-20 (Sigma) pre-swollen in the specified solvent before loading onto the column. Unless otherwise specified, a glass column (2.5 * 50 cm) was used and 5 ml volumes were collected.

Preparative TLC was performed using Merck Silica gel 60 PF_{254} on glass plates (20 cm x 20 cm) and with a thickness of 0.5 mm. Analytical TLC was conducted on normal-phase Merck Silica gel 60 PF_{254} on precoated aluminium plates. Separated compounds on TLC and PTLC plates were visualized under UV light at (254 and 366 nm), and spraying of the plates where required was carried out using 2% vanillin in H_2SO_4 followed by heating at 110^oC for 2– 4 mins. Quercetin, Rutin and Catechin were used as reference standards for flavonoids, Garlic acid for tannins and Oleanolic

acid for essential oils. In the case of the alkaloids, dragendorff was used as the spraying reagent while Quinine and Berberine were used as standards.

Reversed phase TLC (RPTLC) was performed using pre-coated plates (Merck, RP-18. F254 2, 0.25 mm thickness) and spots were detected as described above. R_f values were determined at room temperature using different solvent systems as applicable to each of the plants. Melting points were determined on a Baird and Tatlock laboratory melting point apparatus as well as a Fisher – John's melting point apparatus (Fisher-Scientific). All melting points are uncorrected.

For *P. reticulatum*, IR spectra were recorded on a Mat. Galaxy 5000 series FT-IR spectrometer. MS were obtained using JEOL JMS-AX505HA double-focusing probe at 70 eV, while NMR spectra (both 1D and 2D) were obtained on a Bruker AMX-400 (at 400 MHz for 1H NMR and 100 MHz for 13C NMR) spectrometer in DMSO-d6. Ultraviolet spectra of other isolated compounds were obtained with a Unicam UV 4 - 100 UV/Vis recording spectrophotometer. The spectra were recorded over the range of 200 – 550 nm, at a concentration of 0.02 mg/ml in spectroscopic grade methanol. All infra-red (IR) spectra were recorded on a Perkin Elmer universal ATR (Precisely) Spectrum 100 series FT-IR spectrometer while Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM-400 or AMX-600 spectrometer, using a triple resonance probe head with self-shielded gradient coils and a Bruker Z-gradient accessory delivery squared gradients. The chemical shifts are expressed in ppm relative to TMS.

3.2 PLANT MATERIALS COLLECTION AND AUTHENTICATION

3.2.1 Plant materials

Fresh leaves of *P. reticulatum* (Schum.) were collected in March 2005 at Ajebambo Village, close to Ondo Town, Ondo State (lat. 7^0 05' 08.55"N long. 4^0 47' 49.61"E) in the South Western part of Nigeria and authenticated by a botanist, Dr. A. Ayoola of the Department of Biology, Obafemi Awolowo University, (ACE Campus, Ondo).

A voucher specimen (specimen no.: ACEBH 6266) has been prepared and deposited at the College Herbarium.

Whole plants of *G. multifolia* and *G. gregoriana* were obtained in September 2007 from Mr Gordon Summerfield of Summerfield's Indigenous Bulbs and Seeds, in Summerset West, Cape Town, South Africa. The plants had previously been collected from Rawsonville (lat. 33^0 41' 16.75"S long. 19^0 18' 41.87"E) and Vanrynsdorp (lat. 31^0 36' 50.27"S long. 18^0 44' 36.24"E) respectively, both locations found in Western Province.

Whole plants of *G. villosa* was obtain from a horticulturist in the Cape Peninsula University of Technology in Cape Town, South Africa (lat. 33^0 55' 57.96"S long. 18^0 25' 56.50"E).

Whole plants of *G. incanum* were collected from an open field close to Erica drive in Belhar area of Bellville in Cape Town, South Africa. One collection was made during early March 2007 and another collection was made during late August 2007(lat. 33^0 56' 22.90"S long. 18^0 38' 00.41"E)

Aerial shoot and root of *Cissampelos capensis* were collected in May 2007 from the University of the Western Cape (UWC) Cape Nature Conservation Reserve, Bellville, Cape Town, South Africa (lat. 33^0 56' 09.94"S long. 18^0 37' 33.01"E).

All the plants were authenticated by a taxonomist, Mr Frans Weitz of the Department of Biodiversity and Conservation Biology, University of the Western Cape. Voucher specimens of all the plants were prepared and deposited at the University Herbarium with voucher numbers: - Weitz 1013(UWC) for *G. incanum*; Weitz 1056(UWC) for *C. capensis*; Summerfield in UWC 6964(UWC) for *G. gregoriana*; Summerfield in UWC 6965(UWC) for *G. multifolia*; Summerfield in UWC 6966(UWC) for *G. villosa*.

3.3 PLANT PREPARATION

All the plants collected were separately washed with distilled water and dried at room temperature in a ventilated room for two to three weeks based on the nature of the plant materials viz., bulbs and the roots take a longer time to dry. They were then milled separately to a fine powder. All the powdered plant materials were stored in well labeled air-tight containers prior to analysis.

P. reticulatum Leaves = 2.5Kg

C. capensis Areial shoot = 3.5Kg and Root = 2.6Kg

G. incanum (whole plant)(winter collections = 3.9Kg and summer collections = 4.0Kg)

Gethyllis gregoriana (whole plant) = 342g

Gethyllis multifolia (whole plant) = 229g

Gethyllis villosa (whole plant) = 111g

3.4 EXTRACTION AND ISOLATION

General sequential extraction

The plant materials were sequentially extracted with hexane (Hex) follow by dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH) and finally with water (H_2O). Each extraction was repeated three times for 24 hours respectively by continuous stirring with a mechanical stirrer except *Piliostigma reticulatum* which was sequentially extracted with hexane, ethyl acetate and methanol in a soxhlet apparatus.

Each of the extract was separately evaporated under reduced pressure at 40°C while the (water) aqueous extracts were concentrated by freeze-drying. The Gethyllis species were extracted with methanol, 70% ethanol and water only.

3.5. PHYTOCHEMICAL SCREENING

For the purpose of phytochemical screening, portions of the milled samples were soaked separately in four solvents, namely: hexane, dichloromethane, methanol and water at room temperature (25°C) for 24 hours, and the extracts so derived were evaporated on a rotary evaporator at ≤ 45°C. Phytochemical screening for the detection of natural products such as tannins, phenolics, glycosides, saponins, flavonoids, alkaloids anthraquinones, steroids, essential oils and terpenes was performed according to the method of Wagner (Wagner and Bladt, 2001).

3.6. *PILIOSTIGMA RETICULATUM*

Plant material (2 kg) was sequentially extracted in 4 batches of 500g with hexane, ethyl acetate and methanol in a Soxhlet apparatus. The solid residues remaining in the Soxhlet extractor were bulked together and extracted by maceration in 6L of water for 7days by continuous mechanical stiring at intervals of 8hours. Similar extracts were bulked together and concentrated while the water extract was freeze dried. These afforded hexane extract (78.45g, 3.92%), ethyl acetate extract (109.27, 5.46%), methanol extract (246.28g, 12.31%) and water extract (124.21g, 6.21%).

3.6.1 *Fractionation of the hexane extract*

The hexane extract (28.13g) was adsorbed on silica gel 60 (Merck, 50g, 0.040- 0.063 mm particle size) and fractionated by AGC using the following solvent mixtures X: petroleum ether (PE) 200ml and then 100ml each of PE – EtOAc (95 : 5), (90 : 10), (80 : 20), (60 : 40) and (20 : 80). This was followed by EtOAc 100ml and 100ml each of EtOAc – MeOH (96: 4), (92: 8), (84: 16), (68: 32), (36: 64) and finally washed with MeOH.

Fractions collected were analyzed by TLC using toluene – EtOAc (19: 1) as the mobile phase. Fractions showing the same TLC characteristics were bulked together and concentrated in vacuo. 4 fractions coded A – D were obtained and the results were summarized in Table 6.

TABLE 6: Fractions A – D

Tubes	Yield (g)	Fractions
1 – 12	1.91	A
13 – 26	0.62	B
27 – 45	1.68	C
46 – 89	9.67	D

Fraction D (8.5g) was rechromatographed using the same solvent mixtures X. 78 tubes were collected, analysed by TLC and those showing similar characteristics were bulked together, giving rise to five fractions coded DI – DV. The results are presented in Table 7.

TABLE 7: Fractions DI – DV

Tubes	Yield (g)	Fraction
1 - 7	0.32	DI
8 – 13	1.40	DII
14 – 29	2.13	DIII
30 – 48	0.44	DIV
49 - 78	2.31	DV

Fraction DII (1.20 g) was adsorbed on silica gel 60 (20.0 g) and the constituents eluted by AGC, using the following solvent mixtures Y: 100ml of Hex and then 60ml each of Hex – Tolu (87.5 : 12.5), (75 : 25) and (50 : 50). This was followed by Tolu 100ml and 60ml each of Tolu – EtOAc (99.69 : 0.31), (99.38 : 0.62), (98.75 : 1.25), (97.5 : 2.5), (95 : 5), (90 : 10), (80 :20), (60 : 40), (20 : 80) and finally with 100ml of EtOAc.

38 tubes were collected and analysed by TLC. These were bulked into three fractions coded DIIa to DIIc as follows DIIa (tubes 1 – 10, 0.23 g); DIIb (tubes 11 – 20, 0.41 g) and DIIc (tubes 21 – 38, 0.32 g). Column chromatography of fraction DIIb (0.38 g) on a sephadex LH-20 column, using toluene – EtOH (7: 3) as the eluent gave 36 tubes which were analysed by TLC. Tubes 13 – 23 were found to show one major spot on TLC, and were bulked together. Concentration to dryness *in vacuo* gave a residue (0.19 g) which was further purified by preparative TLC using Tolu – EtOAc (19 : 1).

The compound, a yellow powder (0.08 g) was recrystallized from EtOH (96%), to give **P1** as yellow waxy powder (0.0272 g). Similar fractionation of fraction DIII (2.0 g) using Tolu – EtOH (1 : 1) gave three fractions coded DIIIa (tubes 1 – 6, 0.71 g), DIIIb (tubes 7 – 9, 0.92 g) and DIIIc (tubes 10 – 30, 0.19 g). Further chromatography on sephadex LH-20 of fraction DIIIb using Tolu – EtOH (7 : 3) gave an off-white creamy powder (0.136 g) which contained compound **P2** as the major component, as shown by TLC analysis in $CHCl_3$ – EtOAc (19 : 1). AGC of this powder using a gradient of hexane (100 ml), toluene in hexane (12.5, 25 and 50%, 50ml each), toluene (100ml), EtOAc in toluene (0.625 – 80% in doubling concentrations, 50 ml each) and EtOAc (100 ml) gave **P2** as a creamy powder (0.088 g) which was purified by recrystallization from EtOH (96%) to give compound **P2** as a creamy needle like crystal (0.0133 g). Fraction DIV (0.29g) was subjected to column chromatography on silica gel 60 (20.0 g) using MeOH as the eluent. This gave compound **P3** as a yellow powder which was further purified by preparative TLC in Tolu – EtOAc (19: 1). The compound was recrystallized from 96% EtOH (bright yellow needles, 0.027 g). Fraction DV (2.0 g) was chromatographed on sephadex LH-20 using Tolu – EtOH (7 : 3), the fractions were analysed by TLC in toluene – EtOAc – CH_2Cl_2 (4 : 5 : 1) and bulked into 2 fractions coded DVa and DVb as follows: DVa (tubes 1 – 6, 0.05g) and DVb (tubes 7 – 22, 0.41 g). Fraction DVb (0.41 g) was subjected to AGC using the following eluents: Tolu (100 ml) and then 50ml each of Tolu – $CHCl_3$ (95: 5), (90: 10) (80 : 20), (60 : 40), and (80 : 20). This was followed by $CHCl_3$ 50ml and 50ml

each of $CHCl_3$ - EtOAc (90: 10), (80 : 20), (60 : 40), (20 : 80) and finally with 100ml of EtOAc. 51 tubes were collected, analysed by TLC in Tolu -EtOAc (5 : 4) and the tubes containing compound **P4** were bulked together and concentrated to dryness *in vacuo* (tubes 29 – 46, 0.056 g). Recrystallization of this product from toluene – EtOAc afforded **P4** as a yellow fluffy powder (0.0105 g).

3.6.2. *Fractionation of the ethyl acetate extract*

The ethyl acetate extract (25.21g) was adsorbed on silica gel 60 and chromatographed by AGC using solvent mixtures X. The fractions collected were also analyzed by TLC. Fractions showing same TLC characteristics were bulked together and concentrated in vacuo. 6 Fractions coded E – J were obtained and the results were summarized in Table 8.

TABLE 8: Fractions E - J

Tubes	Yield (g)	Fractions
1 – 19	1.30	E
20 – 48	0.96	F
49 – 82	8.79	G
81 – 90	1.96	H
91 – 103	0.98	I
104 – 123	0.65	J

Fraction G (8.0 g) was adsorbed on silica gel 60 (40 g) and subjected to AGC, using 100ml in each step of solvent mixtures Y. Fractions collected were analysed by TLC and those showing the same TLC characteristics were bulked together, giving four fractions coded GI – GIV as shown in Table 9.

TABLE 9: Fractions GI – GIV

Tubes	Yield (g)	Fractions
1 – 13	1.40	GI
14 – 29	2.20	GII
30 – 38	1.10	GIII
39 - 66	2.12	GIV

Fraction GI (1.13 g) was adsorbed on silica gel 60 (27.0 g) and the constituents eluted by AGC, using solvent mixtures Y. 40 tubes were collected, analysed by TLC and bulked into three fractions coded GIa to GIc as follows:- GIa (tubes 1 – 9, 0.14 g); GIb (tubes 10 – 22, 0.43 g) and GIc (tubes 23 – 40, 0.34 g).

Fraction GIb (0.38 g) was chromatographed on a sephadex LH-20 using toluene – EtOAc (5: 1), the fractions were analysed by TLC. Fractions 13 – 23 were found to show one major spot on TLC. This residue (0.19 g) was further purified by preparative TLC using PE: EtOAc (7: 3) and recrystallized from EtOH (96%). This gave compound **P5** as a bright yellow amorphous powder (0.0127 g)

Fraction GII (2.0 g) was eluted on silica gel 60 (30.0 g) using PE : toluene : MeOH (2:5: 1). 25 tubes were collected, analysed by TLC and bulked into three fractions coded GIIa (tubes 1 – 7, 0.31 g), GIIb (tubes 8 – 14, 0.83 g) and GIIc (tubes 15 – 25, 0.19 g). Successive column chromatography on sephadex LH-20 of fraction GIIb using PE: toluene: MeOH (2 : 5 : 1) gave an off-white powder (0.128 g) which was further purified by preparative TLC using Toluene – EtOAc (19: 1). Compound **P6**, a yellow waxy powder (0.088 g) was recrystallized from EtOH (96%) to give compound **P6** as a yellow waxy powder (0.0054 g).

Fraction GIIc (0.19g) was chromatographed on sephadex LH-20 using PE: toluene: MeOH (2: 5: 1) as the eluent. This gave compound **P7** as a yellow powder which was recrystallized from 96% EtOH. **P7** (bright yellow needle like crystal, 0.075 g).

Fraction GIII (0.85 g) was chromatographed on sephadex LH-20 using toluene – EtOAc (7: 3). 30 tubes were collected, analysed by TLC and bulked into three Fractions coded GIIIa to GIIIc as follows: GIIIa (tubes 1 – 6, 0.05g); GIIIb (tubes 7 – 16, 0.11 g) and GIIIc (tubes 17 – 30, 0.26 g). Fraction GIIIc (0.18 g) was further subjected to AGC using the following solvent mixtures Z: 100ml of toluene and then 50ml each of toluene – EtOAc (99.84 : 0.16), (99.69 : 0.31), (99.38 : 0.62), (98.75 : 1.25), (97.5 : 2.5), (95 : 5), (90 : 10), (80 : 20), (60 : 40) and (20 : 80). This was followed by 100ml of EtOAc and 100ml of EtOAc – MeOH (95: 5) and finally with 200ml of MeOH.

50 tubes were collected, analysed by TLC in toluene – EtOAc (5: 4). Fractions 30 – 46 (0.051 g) was further purified by preparative TLC using PE: EtOAc (7: 3) and recrystallized from toluene – EtOAc which afforded compound **P8** as a yellow powder (0.0084 g).

AGC of fraction GIV (1.80 g) was performed using solvent mixtures Z. 46 tubes collected were analysed by TLC in toluene -EtOAc (5: 4) and bulked into three fraction coded G1Va (tubes 1 – 18, 0.022 g), GIVb (tubes 19 – 26, 0.31 g) and GIVc (tubes 27 – 46, 0.208g). Fraction G1Vb (0.20 g) was chromatographed on sephadex LH-20 using 5% MeOH in toluene and finally 100% MeOH. Fractions collected were analysed by TLC in PE: EtOAc (7:3) and bulked into five fractions coded GIVb1 to GIVb5 as follows: GIVb1 (tubes 1- 10, 0.02 g); GIVb2 (tubes 11 – 17, 0.05 g); GIVb3 (tubes 18 – 23, 0.04 g); GIVb4 (tubes 24 – 31,0.025 g) and GIVb5 (tubes 32 – 40, 0.01 g). Fraction GIVb2 give a single spot which was further purified by preparative TLC using PE: EtOAc (7 : 3) and recrystallized from 96% EtOH to give **P9**, a bright yellow waxy amorphous solid, (0.0076 g).

3.6.3 *Fractionation of the methanol extract*

The methanol extract (32.5g) was adsorbed on silica gel 60 (Merck, 70g) and subjected to AGC using the following solvent mixtures R: 100ml of toluene and then 100ml each of toluene – EtOAc (95 : 5), (90 : 10), (80 : 20), (60 : 40) and (20 : 80). This was followed by 100ml of EtOAc and 100ml of EtOAc – MeOH (96 : 4), (92 : 8), (84 : 16), (68 : 32), (36 : 64) and finally with 200ml of MeOH. Fractions collected were analyzed by TLC using toluene: EtOAc: MeOH (10: 7: 3). Fractions showing the same TLC characteristics were bulked together and concentrated in vacuo. 4 fractions coded K – N were obtained and the results were summarized in Table 10.

TABLE10: Fractions K - N.

Tubes	Yield (g)	Fractions
1 – 14	2.0	K
15 – 41	2.14	L
42 – 82	10.3	M
83 – 95	3.0	N

Fraction M (9.50 g) was adsorbed on silica gel 60 (45 g) and subjected to AGC using solvent mixtures R. 60 tubes were collected, analysed by TLC in toluene: EtOAc: MeOH (10 : 7 : 3). Fractions showing same TLC characteristics were bulked into five fractions coded MI – MV as shown in Table 11.

TABLE11: Fractions MI – MV

Tubes	Yield (g)	Fractions
1 - 10	0.62	MI
11 – 13	1.30	MII
14 – 29	2.20	MIII
30 – 38	1.12	MIV
39 – 60	2.16	MV

AGC of fraction MI (0.42 g) was performed using solvent mixtures Z. 38 tubes collected were analysed by TLC and bulked into three fractions coded MIa (tubes 1 – 18, 0.022 g); MIb (tubes 19 – 30, 0.23 g) and MIc (tubes 31 – 38, 0.051g). Fraction MIb (0.20 g) was further chromatographed on sephadex LH-20 using toluene-MeOH (8: 2). Fractions collected were analysed by TLC and bulked into five fractions coded MIb1 to MIb5 as follows: MIb1 (tubes 1- 10, 0.012 g); MIb2 (tubes 11 – 16, 0.05 g); MIb3 (tubes 17 – 22, 0.03 g); MIb4 (tubes 23 – 31, 0.071 g) and MIb5 (tubes 32 – 42, 0.02 g). Fraction MIb4 was found to show one major spot on TLC. This fraction was further purified by preparative TLC using toluene: EtOAc: MeOH (10: 7: 3). The yellow amorphous powder (0.03 g) was recrystallized from EtOH (96%). This gave compound **P10** as bright yellow amorphous powder (0.0100 g).

Fraction MII (1.0 g) was eluted on a RP- 18 pre – packed column (lobar Lichroprep RP- 18 column) (Merck, size B, 40 – 63 µm) using MeOH-H_20 (4: 1). 68 tubes were collected, analysed by TLC in toluene : EtOAc : MeOH (10 : 7 : 3). **P11** and **P12** were also purified on a Prep pre-coated RP-18 TLC plates (Merck) using 80% aqueous methanol. Compound **P11** is a yellow needle (0.0189 g) while **P12** is a

yellow waxy amorphous solid which was recrystallized from EtOH – H_20 (1: 1) (0.0065 g).

AGC of fraction MIII (2.0 g) was performed using solvent mixtures R. 48 tubes were collected, analysed by TLC and fractions exhibiting similar TLC profiles were bulked together. Fractions 15 – 23 on TLC showed essentially one spot and were concentrated to dryness *in vacuo*. This fraction (0.049 g) was purified by RP -18 prep TLC using MeOH-H_20 (8: 2), compound **P13** as a bright yellow powder (0.0073 g) was observed.

AGC of fraction MV (1.60 g) was also performed using solvent mixture R. 91 tubes collected reveals dull yellow crystals in fractions 48 – 59. The crystals (0.53g) were futher purified by sephadex LH-20 using $CHCl_3$ – MeOH (9 : 1). Fractions analysed by TLC. 41 tubes were collected and fractions 25 – 37 showed a single spot. The resultant powder (0.18 g) was recrystallized from EtOAc – MeOH, giving compound **P14** as a yellow powder (0.075 g).

The final R_f values were similarly determined for all the isolates at room temperature using the underlisted solvent systems, and are uncorrected.

Solvent A: toluene – ethyl acetate (19: 1);

Solvent B: toluene – ethyl acetate – methanol (10: 7 : 3);

Solvent C: methanol – water (8: 2, RP – 18).

3.7 *CISSAMPELOS CAPENSIS*

The major targets for *Cissampelos capensis* were alkaloids and flavonoids.

A total tertiary alkaloid (TTA) fraction was generated by extracting 500g of the aerial shoot with 80% EtOH for 7 days by maceration. The dried ethanolic extract (100 g), was redissolved in 3% HCI and extracted several times with $CHCI_3$. The aqueous fraction was basified with NH_4OH to pH 9 and again extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_20, dried ($MgSO_4$) and the solvent evaporated to afford the TTA (1.85g, 0.37%). The residual aqueous fraction was neutralized to pH 7 with 3% HCl and concentrated to dryness to afford BTA (48.7g, 9.74%).

3.7.1. *Fractionation of the TTA*

The TTA (1.6g) was adsorbed on silica gel 60 (Merck, 30g) and the constituents separated by CGC using the following solvent mixtures E: 200ml of Hex and then 100ml each of Hex – $CHCl_3$ (90 : 10), (80 : 20), (70 : 30), (60 : 40) and (20 : 80). This was followed by 200ml of $CHCl_3$ and 100ml each of $CHCl_3$ – MeOH (90: 10), (80 : 20), (70 : 30), (50 : 50), (20 : 80) and finally washed with MeOH. The fractions collected were analyzed by TLC using Hex – $CHCl_3$ – MeOH (1 : 5 : 1). Tubes showing similar TLC characteristics were bulked together and concentrated *in vacuo*. 7 fractions coded A – G were obtained and the results were summarized in Table 12 with their brine shrimp cytotoxicity activities.

TABLE 12: Fractions A – G and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
1 – 12	0.021	A	989.29	IA
13 – 48	0.320	B	36.913	AC
49 – 97	0.062	C	1784.3	IA
98 – 111	0.027	D	2.4050	AC
112 – 130	0.300	E	3.6648	VA
131 – 149	0.019	F	1233.2	VA
150 – 170	0.023	G		IA

Fraction E (0.25 g) was adsorbed on silica gel 60 (20 g) and subjected to CGC, using solvent mixtures E. 62 tubes were collected , analysed by TLC in Hex – $CHCl_3$ – MeOH (1 : 5 : 1) and fractions showing the same TLC characteristics were bulked together, giving five fractions coded TI – TV. The results are presented in Table 13.

TABLE 13: Fractions TI – TV and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
		E	2.4050	VA
1 - 10	0.004	TI	1478.5	IA
11 – 15	0.0101	TII	899.79	IA
16 – 28	0.122	TIII	3.0226	VA
29 – 37	0.047	TIV	0.8655	VA
38 - 62	0.031	TV	65.335	AC

Successive repeated prep TLC of TIII in Hex – $CHCl_3$ – MeOH (1: 5: 1) was carried out until a clean one spot sample was obtained at R_f of 0.72. This gave compound **C1** as a brownish yellow powder (0.058 g). The repeated prep TLC of TIV in Hex – $CHCl_3$ – MeOH (1 : 5 : 1) afforded compound **C2** as a brown amorphous solid (0.021g).

3.7.2 *Sequential extraction of the aerial shoot*

The aerial shoot (1 kg) was sequentially extracted with hexane (3L x 2); dichloromethane (3L x 2); ethyl acetate (3L x 2) and methanol (3L x 2) by maceration for 24hours, 2 times each and finally with 4L of water for 7days as did for *P. reticulatum*. All extracts were individually bulked together and concentrated. These afforded hexane (17.35g, 1.74%), dichloromethane (26.33, 2.63%), ethyl acetate (32.84, 3.28%), methanol (56.24g, 5.62%) and water extracts (25.71g, 2.57%).

3.7.3. *Fractionation of the methanol extract of the aerial shoot*

The methanol extract (25.6g) was adsorbed on silica gel 60 (Merck, 60g) and the constituents separated by CGC using solvent mixtures E. Fractions collected were analysed by TLC using Hex – $CHCl_3$ – MeOH (1 : 5 : 1). Fractions showing the same TLC characteristics were bulked together and concentrated in vacuo. 10 fractions coded A – J were obtained and the results were summarized in Table 14.

TABLE 14: Fractions A – J and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
1 – 12	1.91	A	989.29	IA
13 – 26	0.62	B	1369.13	IA
27 – 35	0.68	C	17.843	AC
36 – 39	0.67	D	2405.7	IA
40 – 45	1.30	E	36.646	AC
46 – 48	1.96	F	1.2332	VA
49 – 55	3.29	G	84.346	AC
56 – 64	1.96	H	1148.23	IA
65 – 70	0.98	I	997.25	IA
71 – 81	0.65	J	73.563	AC

Fraction F (1.5 g) was adsorbed on silica gel 60 (35 g) and subjected to CGC, using solvent mixtures E. 62 tubes were collected, analysed by TLC and those showing the same TLC characteristics were bulked together into five fractions coded FI – FV as shown in Table 15.

TABLE 15: Fractions FI – FV and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
		F	1.2332	VA
1 - 10	0.032	FI	1124.6	IA
11 – 15	0.520	FII	66.734	AC
16 – 28	0.211	FIII	3.4420	VA
29 – 37	0.115	FIV	17.563	AC
38 - 62	0.106	FV	45.455	AC

Fraction FII (0.5 g) was adsorbed on silica gel 60 (9.0 g) and the constituents eluted by CGC using same solvent mixture E. Fractions collected were analysed by TLC and bulked into three fractions coded FIIa to FIIc as follows: FIIa (tubes 1 – 10, 0.08 g); FIIb (tubes 11 – 20, 0.21 g) and FIIc (tubes 21 – 38, 0.14 g).

Fraction FIIb (0.20 g) was chromatographed on a sephadex LH-20 using $CHCl_3$ – MeOH (7 : 3). 35 tubes were analysed by TLC. Fractions 13 – 23 were found to show one major spot on TLC and were bulked together. This product (0.19g) was further purified by repeated prep TLC in Hex – $CHCl_3$ – MeOH (1 : 5 : 1). This gave a golden brown powder (0.04g) as compound **C3**.

Fraction FIII (0.20 g) was eluted on silica gel using toluene – $CHCl_3$ – MeOH (7: 5: 3). 24 tubes were collected, analysed by TLC and bulked into three fractions coded FIIIa (tubes 1 – 7, 0.013 g), FIIIb (tubes 8 – 15, 0.091 g) and FIIIc (tubes 16 – 24, 0.024 g). Successive repeated prep TLC of fraction FIIIb using Hex – $CHCl_3$ – MeOH (1: 5: 1) gave a brownish yellow powder (0.052 g) which was recrystallized from 96% EtOH to give compound **C4** (a brownish yellow powder, 0.028 g).

Fraction FIV (0.10 g) was chromatographed using solvent mixtures E. 50 tubes collected were analysed by TLC in $CHCl_3$ – MeOH (5 : 3) and the tubes containing compound **C5** were bulked together and concentrated to dryness in vacuo (tubes 33 – 38, 0.038 g). Recrystallization from 96% EtOH afforded **C5** as a yellow amorphous Powder (0.026 g).

Fraction FV (0.10 g) was chromatographed, using toluene – $CHCl_3$ – MeOH (3: 5: 1). 33 tubes were collected, analysed by TLC and bulked into 4 fractions coded FVa to FVd as follows: FVa (tubes 1-12, 0.0120 g); FVb (tubes 13-17, 0.0430 g); FVc (tubes 18-23, 0.022 g) and FVd (tubes 24-33, 0.0133 g). Fraction FVb (0.0430g) was further purified by prep TLC using $CHCl_3$ – MeOH (9 : 2). This gave compound **C6** as a yellow powder (0.024g).

3.7.3.1 *Sequential extraction of the root of Cissampelos capensis*

The same extraction procedure was repeated for the root (1 kg). This afforded hexane (7.38g, 0.74%), dichloromethane (12.65, 1.27%), ethyl acetate (27.19, 2.72%), methanol (53.05g, 5.31%) and water extract (26.44g, 2.64%).

3.7.3.2 *Fractionation of the methanol extract of the root*

The methanol extract (30.0g) was adsorbed on silica gel and the constituents separated by using solvent mixtures E. Fractions collected were analyzed by TLC and fractions showing same TLC characteristics were bulked together. 5 fractions coded K – O were obtained and the results are summarized in Table 16.

TABLE 16: Fractions K - O and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
1 – 12	1.81	K	997.27	IA
13 – 26	2.62	L	79.458	AC
27 – 45	5.68	M	5.2375	VA
46 – 102	3.67	N	19.296	AC
103 - 146	1.82	O	33.668	AC

Fraction M (5.0 g) was adsorbed on silica gel and subjected to CGC, using solvent mixtures E. 58 tubes collected were analysed and fractions were bulked together based on their TLC characteristics. Three fractions coded MI – MIII were obtained as shown in Table 17.

TABLE 17: Fractions K - O and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
		M	5.2375	VA
1 - 12	0.62	MI	15.236	AC
13 – 32	2.10	MII	55.178	AC
33 – 58	1.20	MIII	16.776	AC

Fraction MII (1.7 g) was chromatographed using solvent mixture E. 40 tubes collected were analysed by TLC and bulked into three fractions coded MIIa to MIIc as follows: MIIa (tubes 1 – 10, 0.14 g); MIIb (tubes 11 – 22, 0.41 g) and MIIc (tubes 23 – 40, 0.64 g).

Fraction MIIb (0.38 g) was chromatographed using toluene – $CHCl_3$ – MeOH (3: 5: 1). 36 tubes were analysed by TLC and fractions 13 – 23 were found to show one major spot on TLC. These were bulked and concentrated (0.19 g). The product was further purified by repeated prep TLC in Hex – $CHCl_3$ – MeOH (1: 5: 1). This gave a brownish yellow powder which was recrystallized from EtOH (96%) to give compound **C7** (0.078 g).

Fraction MIIc (0.50 g) was chromatographed using toluene – MeOH (1: 1). 21 tubes were collected, analysed by TLC and bulked into three fractions coded MIIc1 (tubes 1 – 6, 0.071 g), MIIc2 (tubes 7 – 9, 0.24 g) and MIIc3 (tubes 10 – 21, 0.0411 g). The repeated prep TLC of MIIc2 afforded compound **C8** as a brownish yellow powder (0.065 g).

Fraction MIII (1.0g) was chromatographed using toluene – MeOH (1: 3). After repeated prep TLC in $CHCl_3$ – MeOH (1: 5), a yellow powder was obtained which when recrystallized from 96% EtOH afforded compound **C9** (bright yellow powder (0.088 g).

Fraction N (3.2 g) was chromatographed using solvent mixtures E. 80 tubes were collected, analysed by TLC and fractions showing same TLC characteristics were bulked together. Four fractions coded NI – NIV were obtained as shown in Table 18.

TABLE 18: Fractions NI – NIII and their LC_{50} values

Tubes	Yield (g)	Fractions	LC_{50} value	Class activity
		N	19.296	AC
1 - 10	1.22	NI	20.634	AC
11 – 15	0.11	NII	1688.6	IA
16 – 53	0.73	NIII	76.112	AC
54 – 80	0.28	NIV	21.225	AC

Fraction NI (1.2 g) was chromatographed using solvent mixtures E. 56 tubes collected were analysed by TLC and bulked into three fractions coded NIa to NIc as follows: NIa (tubes 1 – 15, 0.14 g); NIb (tubes 16 – 28, 0.43 g) and NIc (tubes 29 – 56, 0.04 g).

Fraction NIb (0.40 g) was rechromatographed using toluene – MeOH (7 : 4). 30 tubes were analysed by TLC and bulked into four fractions coded NIb1 to NIb4 as follows: NIb1 (tubes 1 – 5, 0.0250 g); NIb2 (tubes 6 – 10, 0.043 g); NIb3 (tubes 11 – 19, 0.215 g) and NIb4 (tubes 20 – 30, 0.014 g).

Fraction NIb3 (0.20 g) was further purified by prep TLC using toluene – MeOH (7: 3). Recrystallization of the product from 96% EtOH afforded compound **C10** as a yellow amorphous Powder (0.068 g).

CGC of Fraction NIII (0.70 g) was performed using solvent mixtures E. 45 tubes collected were analysed by TLC and bulked into three fractions coded NIIIa (tubes 1 – 18, 0.022 g), NIIIb (tubes 19 – 26, 0.21 g) and NIIIc (tubes 27 – 45, 0.058g). Fraction NIIIb (0.20 g) was chromatographed on sephadex LH-20 using toluene – MeOH (7: 3). Fractions collected were bulked into four fractions based on their TLC

characteristic as follows: NIIIb1 (tubes 1 – 9, 0.0121 g); NIIIb2 (tubes 11 – 18, 0.051 g); NIIIb3 (tubes 19 – 26, 0.021 g) and NIIIb4 (tubes 27 – 40, 0.0112 g). Repeated prep TLC of NIIIb2 using Hex – $CHCl_3$ – MeOH (1: 5 : 1) afforded a creamy yellow solid which was labeled compound **C11**.

3.8. *GERANIUM INCANUM*

3.8.1 *Sequential extraction of the summer collections*

The same procedural steps were followed for the extraction of the summer collection (2kg) which afforded hexane (29.45g, 1.47%), dichloromethane (37.69g, 1.89%), ethyl acetate (125.28g, 6.26%), methanol (183.25g, 9.16%) and water extract (86.11g, 4.31%).

3.8.2 *Fractionation of the ethyl acetate extract of the summer collections*

The ethyl acetate extract (25.0g) was chromatographed using the following solvent mixtures F: 200ml of toluene and then 100ml each of toluene – EtOAc (95 : 5), (90 : 10), (80 : 20), (60 : 40) and (20 : 80). This was followed by 100ml of EtOAc and 100ml of EtOAc – MeOH (96 : 4), (92 : 8), (84 : 16), (68 : 32), (36 : 64) and finally with 150ml of MeOH. Fractions collected were analyzed by TLC using toluene : EtOAc : MeOH (7 : 5 : 3). Fractions showing the same TLC characteristics were bulked together and concentrated *in vacuo*. 6 fractions coded A – F were obtained and the results were summarized in Table 19.

TABLE 19: Fractions A – F

Tubes	Yield (g)	Fractions
1 – 11	1.31	A
12 – 18	1.74	B
19 – 28	6.29	C
29 – 40	1.14	D
41 – 59	2.21	E
60 – 115	3.16	F

Fraction C (5.7 g) was chromatographed using solvent mixtures F. 59 tubes were collected, analysed by TLC in toluene: EtOAc: MeOH (7: 5: 3) and fractions showing the same TLC characteristics were bulked together. Five fractions coded CI – CV was obtained and the results are summarized in Table 20.

TABLE 20: Fractions CI – CV

Tubes	Yield (g)	Fractions
1 – 10	0.120	CI
11 – 13	0.111	CII
14 – 29	2.100	CIII
30 – 38	0.610	CIV
39 - 59	0.452	CV

Fraction CIII (1.7g) was chromatographed using solvent mixtures Y. 40 tubes collected were analysed by TLC in toluene : EtOAc : MeOH (7 : 5 : 3) and bulked into three fractions coded CIIIa to CIIIc as follows : CIIIa (tubes 1 – 12, 0.04 g); CIIIb (tubes 13 – 25, 0.64 g) and CIIIc (tubes 26 – 40, 0.31 g).

Fraction CIIIb (0.60 g) was chromatographed using toluene: EtOAc: MeOH (7: 5: 3). 38 tubes obtained were analysed by TLC. Tubes 12 – 26 were found to show one major spot and were bulked together. The residue (0.19 g) was further purified by prep TLC. This gave compound **G1** as bright yellow powder (0.049 g).

Fraction CIV (0.60 g) was chromatographed using Toluene: EtOAc: MeOH (7: 5: 3). 23 tubes collected were analysed and bulked into three fractions coded CIVa (tubes 1 – 6, 0.022 g), CIVb (tubes 7 – 9, 0.082 g) and CIVc (tubes 10 – 23, 0.245 g). Successive column chromatography of fraction CIVb (0.24 g) using Toluene: EtOAc: MeOH (7: 5: 3) gave an off-white powder (0.116 g) which was purified by prep TLC in the same solvent system. This gave compound **G2** as an off white powder (0.088 g) after recrystallization from EtOH (96%).

Fraction CV (0.45g) was also chromatographed using toluene: EtOAc: MeOH (7: 5: 3). This gave compound **G3** as a creamy powder after repeated prep TLC in same eluent. This was recrystallized from 96% EtOH to give **G3** a creamy off white powder (0.044 g).

3.8.3 *Fractionation of the methanol extract of the summer collections*

The methanol extract (20.0g) was chromatographed using the solvent mixtures F. Fractions collected were analyzed by TLC using toluene: EtOAc: MeOH (7: 5: 3). Fractions showing the same TLC characteristics were bulked together and concentrated *in vacuo*. 4 fractions coded G – J were obtained as summarized in Table 21.

TABLE 21: Fractions G - J

Tubes	Yield (g)	Fractions
1 – 15	0.31	G
16 – 34	6.33	H
35 – 78	3.19	I
79 – 116	1.14	J

Fraction H (6.0 g) was chromatographed using solvent mixture F. 59 tubes were collected, analysed by TLC and fractions showing the same TLC characteristics were bulked together. Three fractions coded HI – HIII were obtained as shown in Table 22.

TABLE 22: Fractions HI – HIII

Tubes	Yield (g)
1 - 14	0.132
15 – 47	2.610
48 – 59	0.622

Fraction HII (2.30 g) was chromatographed using toluene : EtOAc : MeOH (7 : 5 : 3). Fractions obtained were bulked into three fractions coded HIIa to HIIc as follows: HIIa (tubes 1 – 8, 0.14 g); HIIb (tubes 8 – 19, 0.87 g) and HIIc (tubes 20 – 35, 0.35 g).

Fraction HIIb (0.7 g) was further rechromatographed and the 50 tubes collected were analysed by TLC using toluene: EtOAc: MeOH (5: 4: 3). Fractions 31 – 39 (0.05 g) was recrystallized from methanol which afforded compound **G4** as a white powder (0.048 g).

CGC of fraction HIII (0.50 g) was performed using the solvent mixtures Z. 56 tubes collected were analysed by TLC and bulked into three fractions coded HIIIa (tubes 1 – 18, 0.022 g), HIIIb (tubes 19 – 26, 0.21 g) and HIIIc (tubes 27 – 56, 0.058g).

Fraction HIIIb (0.20 g) was further chromatographed. It was observed that tubes 18 – 29 showed a single spot which were bulked together and concentrated to dryness *in vacuo*. The recrystallization from methanol of the products formed afforded compound **G5** as a light yellow amorphous Powder (0.038 g).

3.8.4 *Sequential extraction of the winter collections*

The procedure was also repeated for the extraction of the winter collections (2kg) which gave hexane (48.35g, 2.42%), dichloromethane (34.43g, 1.72%), ethyl acetate (107.26g, 5.36%), methanol (211.77g, 10.59%) and water extract (79.32g, 3.97%).

3.8.5 *Fractionation of the ethyl acetate extract of the winter collections*

The ethyl acetate extract (25.0g) was adsorbed on silica gel 60 and the constituents separated by CGC using the solvent mixtures R. Fractions collected were analyzed by TLC using toluene: EtOAc: MeOH (5: 4: 3). Fractions showing the same TLC characteristics were bulked together and concentrated in vacuo. 5 fractions coded K – O was obtained and the results were summarized in Table 23.

TABLE 23: Fractions K - O.

Tubes	Yield (g)	Fractions
1 – 12	0.91	K
13 – 26	6.42	L
27 – 35	4.18	M
36 – 39	2.07	N
40 – 98	1.11	O

Fraction L (6.0g) was chromatographed using the solvent mixtures F. 75 tubes were collected, analysed by TLC and bulked into four fractions based on their TLC profile. The four fractions were coded LI – LIV as shown in Table 24.

TABLE 24: Fractions LI – LIV

Tubes	Yield (g)	Fractions
1 - 9	0.22	LI
10 – 26	1.21	LII
27 – 49	2.11	LIII
50 – 75	0.13	LIV

Fraction LII (1.0 g) was chromatographed using solvent mixtures Y. 77 tubes were collected and analysed by TLC. The fractions were bulked into three fractions coded LIIa to LIIc as follows: LIIa (tubes 1 – 14, 0.023g); LIIb (tubes 14 – 41, 0.46g) and IIc (tubes 42 – 77, 0.041g).

When fraction LIIb (0.43 g) was rechromatographed using toluene – MeOH (7: 3), the 50 tubes analysed by TLC showed that fractions 12 – 23 essentially showed one major spot. These were bulked, concentrated and further purified by repeated prep TLC ran in toluene: EtOAc: MeOH (5: 4: 3).

The compound **G6** obtained was recrystallized from EtOH (96%). This gave **G6** as a brownish yellow powder (0.038 g).

Fraction LIII (2.0 g) was chromatographed using solvent mixtures F. 74 tubes were collected, analysed by TLC and bulked into three fractions coded LIIIa (tubes 1 – 8, 0.05 g), LIIIb (tubes 9 – 32, 0.11 g) and LIIIc (tubes 33 – 74, 0.98 g). Successive chromatography followed by prep TLC of fraction LIIIc using toluene – MeOH (7: 3) gave an off-white powder which was labeled **G7** (0.055 g) after recrystallization from EtOH (96%).

Fraction M (4.0 g) was chromatographed using solvent mixtures R. 70 tubes collected were analysed by TLC and fractions showing same TLC characteristics generated three fractions coded MI – MIII as shown in Table 25.

TABLE 25: Fractions MI – MIII.

Tubes	Yield (g)	Fractions
1 - 20	0.11	MI
21 – 37	0.25	MII
38 – 70	1.71	MIII

Fraction MIII (1.5 g) was chromatographed using solvent mixtures Y. 55 tubes collected were bulked into three fractions as follows: - MIIIa (tubes 1 – 13, 0.033 g); MIIIb (tubes 14 – 29, 0.54 g) and MIIIc (tubes 30 – 55, 0.12 g).

Fraction MIIIb (0.50 g) was rechromatographed using solvent mixture R. 58 tubes were collected, analysed by TLC and the tubes containing compound **G8** were bulked together and concentrated to dryness *in vacuo* (tubes 30 – 38, 0.19 g) which showed single spot.

This yellow powder was recrystallized from EtOH (96%) to give compound **G8** (0.077 g) as bright yellow amorphous powder.

3.8.6 *Fractionation of the methanol extract of the winter collections*

The methanol extract (25.0g) was chromatographed using solvent mixtures F. Fractions collected were analyzed by TLC and those showing same characteristics were bulked together. 6 fractions coded P – U was obtained and the results were summarized in Table 26.

TABLE 26: Fractions P - U

Tubes	Yield (g)	Fractions
1 – 15	0.62	P
16 – 33	2.12	Q
34 – 55	5.28	R
56 – 68	3.64	S
69 – 97	1.35	T
98 – 146	0.44	U

Fraction Q (2.0g) was chromatographed using the solvent mixtures F. 80 tubes were collected, analysed by TLC and those showing the same TLC characteristics were bulked into three fractions coded QI – QIII as shown in Table 27.

TABLE 27: Fractions QI – QIII

Tubes	Yield (g)	Fractions
1 - 24	0.122	QI
25 – 50	0.801	QII
51 – 80	0.072	QIII

CGC of fraction QII (0.6 g) was performed using solvent mixtures Z. 56 tubes were collected, analysed by TLC and those showing same TLC characteristics were bulked together. Three fractions coded QIIa – QIIc as follows:- QIIa (tubes 1- 12, 0.025 g); QIIb (tubes 13 – 34, 0.051 g) and QIIc (tubes 35 – 56, 0.241 g) were obtained. Further purification of QIIc by repeated prep TLC gave a bright yellow powder (0.0911 g) which was recrystallized from EtOH (96%). This gave compound **G9** (0.067g) as a bright yellow powder.

Fraction R (5.0g) was chromatographed using solvent mixtures F. 79 tubes collected were analysed by TLC and those showing the same TLC characteristics were bulked together. Four fractions coded RI – RIV were obtained as shown in Table 28.

TABLE 28: Fractions RI – RIV

Tubes	Yield (g)
1 - 12	0.022
13 – 36	1.420
37 – 49	1.052
50 – 79	0.116

Fraction RII (1.40 g) was chromatographed using toluene: EtOAc: MeOH (7: 5: 3). 74 tubes were collected, analysed by TLC and bulked into three fractions coded RIIa to RIIc as follows: RIIa (tubes 1 – 15, 0.04 g); RIIb (tubes 16 – 39, 0.46 g) and RIIc (tubes 40 – 74, 0.15 g). Fraction RIIb (0.45 g) was further rechromatographed, 52 tubes were collected, analysed by TLC in Toluene: EtOAc: MeOH (5: 4: 3) and the tubes containing compound **G10** were bulked and concentrated (tubes 30 – 41, 0.0891g). The product was recrystallized from methanol which afforded **G10** as an off white powder (0.0532 g).

CGC of fraction RIII (1.0 g) was performed using solvent mixtures Z. 70 tubes collected were analysed by TLC and bulked into three fractions coded RIIIa (tubes 1 – 17, 0.023 g), RIIIb (tubes 18 – 37, 0.0152 g) and RIIIc (tubes 38 – 70, 0.48g). On further chromatography of fraction RIIIc (0.40 g) it was observed that tubes 21 – 29 showed a single spot which were bulked together and concentrated to dryness *in vacuo*. The recrystallization from methanol of the products formed afforded **G11** as a light yellow amorphous powder (0.098 g).

Fraction S (3.6g) was chromatographed using solvent mixtures F. 98 tubes collected were analysed by TLC and those showing the same TLC characteristics were bulked together. Five fractions coded SI – SV were obtained as shown in Table 29.

TABLE 29: Fractions SI – SV

Tubes	Yield (g)	Fractions
1 - 14	0.155	SI
15 – 41	0.211	SII
42 – 59	0.422	SIII
60 – 72	1.022	SIV
73 – 98	0.0213	SV

Fraction SIV (1.0 g) was chromatographed using toluene: EtOAc: MeOH (7: 5: 3). 55 tubes were collected, analysed by TLC and bulked into three fractions coded SIVa

to SIVc as follows: SIVa (tubes 1 – 11, 0.03 g); SIVb (tubes 12 – 32, 0.441 g) and SIVc (tubes 33 – 55, 0.102 g).

Fraction SIVb (0.40 g) was further rechromatographed. The 52 tubes collected were analysed by TLC and the fractions were further bulked into three fraction coded SIVb1 (tubes 1 – 14, 0.022 g), SIVb2 (tubes 15 – 27, 0.05 g) and SIVb3 (tubes 28 – 52, 0.125g). Further purification of SIVb3 by repeated prep TLC ran in toluene: EtOAc: MeOH (5: 4: 3) gave a creamy yellow powder which was recrystallized from methanol. This afforded **G12** (0.0658g) as a creamy yellow powder.

3.9. *GETHYLLIS SPECIES*

3.9.1 *Extraction of the constituent in Gethyllis gregoriana*

The whole plant (320g) was extracted with methanol (3L x 2) by maceration for 24hours, 2 times each and the residue with 3L of water for 4days. The two extracts were individually bulked together and concentrated to afford methanol (26.79g, 8.37%) and water extracts (18.92g, 5.91%).

3.9.2. *Extraction of the constituent in Gethyllis multifolia*

The above procedure was repeated on 215g of ***G. multifolia***. This afforded methanol (17.98g, 8.36%) and water extract (21.11g, 9.82%).

3.9.3. *Extraction of the constituent in Gethyllis villosa*

The same procedure as described above was also repeated for ***G. villosa*** (105g) in which methanol (10.65g, 10.14%) and water extract (7.43g, 7.08%) were also obtained.

3.10 BIOLOGICAL EVALUATION

3.10.1 *Brine shrimp lethality biossay*

The brine shrimp lethality biossay was carried out as previously described (Babajide, 1994 and Babajide et al., 2008) where about 2g of the brine shrimp eggs (*Artemia*

salina Leach) were hatched in 2L of sea water using a large plastic case as an artificially partitioned dam.

Incubation of the shrimp eggs was conducted at room temperature for 48 hours with the help of a light source and an aquarium aerator pump (for agitation and aeration of the eggs). The larvae (nauplii) were observed to be attracted to one side of the vessel toward the light source where they were easily collected for the assay after about 24 hours.

All the crude extracts, fractions and isolates of the 6 plants were dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration not exceeding 0.05%, and then diluted with sea water for testing at final concentrations of 10, 100 and 1000 μg/ml. Each test was conducted in triplicate where ten nauplii were used for each test. Ten nauplii were counted into the vials with the aid of a pasture pipette under a magnifying glass after 24 hours of incubation and still maintaining the vials under illumination. The control experiment was prepared in the same way except that the test samples were omitted.

The number of dead nauplii were counted and recorded (lethality data) after vials 24 hours. The numbers of dead nauplii were used for calculating the LC_{50} at 95% confidence limit by the Finney Probit analysis program. LC_{50} values greater than 1000 ppm or in that range were considered inactive. (Babajide et al., 2008; Mc Laughlin et al., 1991).

3.10.2 *Determination of antimicrobial activity*

Generally two methods were used for the antimicrobial assay

1. The diffusion method.
2. The serial dilution method

The organisms used in the screening tests were obtained from both National Collection of Type Cultures (NCTC) and American Type Culture Collection (ATCC).

The Gram-negative bacteria used were

Pseudomonas aeruginosa (NCTC 10332)

Proteus vulgaris (NCTC 4175)

Escherichia coli Sero type 1 (NCTC 09001),

Escherchia coli NCTC 10418

The Gram-positive bacteria used were

Bacillus subtilis (NCTC 8236)

Staphylococcus aureus (NCTC 13134)

Bacillus licheniformis (NCTC 01097)

Staphylococcus aureus NCTC 6571

The Fungal species used were:

Candida albicans (ATCC 90028)

Candida eropiralis (ATCC 750)

Candida albicans ATCC 10231

Aspergillus Niger ATCC 10578

3.10.3. *The diffusion method*

The organisms used in the screening tests are as follows:- The Gram-negative bacteria were *Pseudomonas aeruginosa* (NCTC 10332), *Proteus vulgaris* (NCTC 4175) and *Escherichia coli* Sero type 1 (NCTC 09001), while the Gram-positive were *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (NCTC 13134) and *Bacillus licheniformis* (NCTC 01097). These species are considered the most important pathogens (NCCLS, 1990). Fungal species used were *Candida albicans* (ATCC 90028), *Candida eropiralis* (ATCC 750) and *Aspergillus Niger* ATCC 10578. Cultures were grown in sabouraud dextrose (SD) broth at 37 ◦C and maintained on SD agar at 4 ˚C.

A colony of each bacterial strain was suspended in 1ml of Mueller–Hinton broth and incubated for 18 hrs at 37 ˚C. A subculture was again made after 6 hrs. The subculture was diluted 1/50 in the same broth before use. A disc diffusion assay was used to determine the inhibition of bacterial growth by the plant extracts, fractions, sub fractions and isolates (Rasoanaivo and Ratsimamanga-Urverg, 1993). Plant extracts, fractions and isolates were dissolved in appropriate solvents at a concentration of 100 mg/ml for extracts and 10 mg/ml for isolates. 20 µl were dispensed on a 9mm sterile paper disc (Munktell/Lasec, Numb. FLAS3526009).

Amoxicillin was used as a positive control (40µg/ml) for bacteria while Fluconazole (120µg/ml) for fungi. The diluted cultures were spread on sterile Muller–Hinton agar plates. The plates were then incubated at 37°C for 18–24 hrs for bacterial pathogens and 3 days for fungal pathogens. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone. The experiment was carried out in triplicate and the mean of the diameter of the inhibition zones were calculated. Antimicrobial inhibition activities measured were compared to that of Amoxicillin and Fluconazole standards (Vlietinck, 1997).

3.10.4 The serial microdilution method

Antimicrobial analysis was equally carried out using the microdilution titre techniques (Drummond and Waigh, 2000; Rahman and Gray, 2005) with little modification in which 96 well plates were used for the testing against the Gram-positive bacteria and Gram-negative bacteria as well as the fungi. All the experiments were performed on a laminar flow bench to avoid contamination.

1 microtitre plate of 96 wells was used per bacteria / fungal species while 4 plant extracts were tested per microtitre plate. The positive and negative controls were indicated periodically to check the growth of the bacteria and fungi. All the working cultures of the bacteria are not allowed to be older than 14 days. This was attained by reculturing them at the beginning of every experiment. Recultured organisms were always used. Bacteria were diluted in the ratio of 1:100 (1 ml bacteria and 99 ml fresh Mueller Hinton (MH) broth at room temperature.

Bacterial cells were inoculated into fresh Müller–Hinton (MH) broth (Fluka, Switzerland) and incubated at 37 ◦C for 14 h prior to the screening procedures. Densities of bacterial cultures after incubation overnight were calculated. Fungal species cultures were grown in sabouraud dextrose (SD) broth at 37 ◦C and maintained on SD agar at 4 ◦C. Densities of the fungal cultures used in the assays were also calculated.

The determination of minimum inhibitory concentration (MIC) values was performed using the listed microorganisms above, (INT) *p*-iodonitrotetrazolium violet, (MH) Müller–Hinton and (SD) Sabouraud dextrose. All the microplates were clearly

labeled. 100µl of distilled H_2O was placed in each of the wells and 100µl of the extracts (at 10 mg/ml) were added to the first row (A) of the microplate. 100 µl of the positive and negative (normally the solvent) controls were put on the microplate, similarly to the extracts. Using a multi-channel pipette, a series of two-fold dilutions of the extract and controls were made, by mixing the first row (A) 3x, followed by removing 100µl from row A to row B. This step was repeated until row H. 100µl was then removed from the last row (H) after mixing.

Thereafter 100µl of the relevant bacterial culture was placed into each well which was mixed by squirting bacteria into the wells, but care was taken by not allowing it to splash, as well as allowing the pipette tips to touch the solution inside the micro-well. The microtitre plate was covered, placed inside a plastic bag and incubated overnight at 37^{o}C. The plates were then sprayed with 2mg/mL of *p*-iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 12 h (Begue and Kline, 1972). The emergence of purple-red colour resulting from the reduction of INT into its respective formazan was a positive indicator of cell viability (Begue and Kline, 1972). Clear zones were indicative of antifungal or antibacterial activity of compounds. The microplates were examined for colour after 30, 60 120 and 180 minutes. The MIC values were determined using the serial microplate method in which the MIC was taken as the lowest concentration of the test compounds inhibiting the growth of microorganisms. The total activity (mL) of the extracts was calculated as the total mass (mg) of the extract divided by the MIC value (mg/mL). Total activity value indicates the volume to which the extract can be diluted and still inhibit the growth of microbial cells (Eloff, 1998, 2004; Masoko et al. 2005). The lowest concentration where growth was inhibited (MIC = minimal inhibitory concentration) was calculated from the original concentration of the extract e.g. (10 mg/ml); i.e. Starting with 10 mg/ml and the growth is inhibited in the first to the fifth wells, the MIC would be [10/5/2.5/1.25/0.625] = 0.625 mg/ml.

3.11. CHEMICAL EVALUATION

3.11.1 *Antioxidant assay*

Total antioxidant evaluation was carried out on all the 6 plant extracts, fractions and isolates by first evaluating the antioxidant content of the following:-

1. Polyphenols (gallic acid)
2. Flavonols (quercetin)
3. Flavanols (catechin)
4. Anthocyanins
5. Flavanones (naringenin)

B. Antioxidant capacity

1. ORAC (Oxygen radical absorption capacity) by H^+ ion transfer.
2. FRAP (Ferric reducing antioxidant Power) by the movement of e^- electron.
3. TEAC (Trolox (a well known standard) equivalent antioxidant capacity)

All readings were taken after 40 minutes unless otherwise stated and the Microsoft excel workbook program was used in all the analysis and calculations.

3.11.2. Evaluation of polyphenolic content

3.11.2.1 Methodology

The analysis makes use of the Folin Ciocalteu reagent with gallic acid as the standard to measure the total polyphenols in the samples. Since a very slight amount of turbidity can interfere with the determination, all the samples used was clarified by centrifugation and then filtered. All reagents and standards used are prepared freshly on the day of analysis unless otherwise stated for use the next day.

3.11.2.2. Equipment: - Multiskan plate reader

3.11.2.3. Sample preparation

Approximately 30 mg (0.030 g) (noting the exact weight) of the sample was weighed into a 50 mL screw-cap tube. IMPORTANT: The sample weight of 30 mg is not fixed and this was changed for some samples depending on the nature of the sample. This weight was reduced if it was suspected that the sample contains high amounts of

antioxidants or increased if it was suspected to contain a low concentration of antioxidants. Water or ethanol, 50 mL, was added with the aid of a Gilson pipette depending on which of the two dissolved the sample the best. This was then mixed thoroughly, sonicated and then centrifuged at 4000 rpm for 5 minutes. The supernatants were used directly after a suitable dilution for the entire assay.

3.11.2.4. Sample analysis

Preparation of the standard series: - 6 eppendorf tubes marked A-F were used for the standard series and prepared as shown in Table 30 below.

Table 30: Standard series of gallic acid standard wells

Tube	gallic acid stock solution (µl)	10% EtOH (µl)	Concentration (mg/L)	Well number
A	0	1000	0	A1-A3
B	25	975	20	A4-A6
C	62.5	937.5	50	A7-A9
D	125	875	100	A10-A12
E	312	688	250	B1-3
F	625	375	500	B4-6

Gallic acid standard wells – 25 µl of standard was added (tubes A-E) per well in the designated wells in a clear well plate.

Control wells – 25 µl of the control was added to the wells (B7-B12).

Sample wells – 25 µl of sample was added IN TRIPLICATE to the wells (C1-H12).

This was followed by adding 125 µl Folin reagent to each well using the 12 point multichannel pipette. After 5 minutes, 100µl Na_2CO_3 was further added to each well. The plate was left for 2 hours at room temperature before taking a reading on the multiskan spectrum plate reader.

3.11.2.5 Data analysis and calculations

All the data were expressed as mg Gallic acid equivalents per gram of sample (mg Gallic acid/g of sample). Note: - All the sample with high values of polyphenol were diluted either to 5-fold or to 10-fold as the case may be. For 10-fold dilution- the samples was diluted by pipetting 100 µl of the sample supernatant into a new eppendorf and 900 µl of 10% ethanol was added to effect the 10-fold dilution. The Phenolics assay was then repeated with the diluted sample.

3.11.3 Evaluation of flavonol Content

3.11.3.1 Methodology

This analysis makes use of quercetin as the standard for evaluating the amount of flavonols in the sample at 360nm.

3.11.3.2. Equipment and sample preparation is as in Polyphenols.

3.11.3.3. Sample analysis

The multiskan spectrum plate reader was set to read at a wavelength of 360nm and the temperature was equally set at 25°C.

Preparation of standards – The standard series was prepared as shown in Table 31 below.

Table 31: Quercetin standard wells

Tube	Quercetin stock (µl)	95% EtOH (µl)	Quercetin (mg/L)	Wells
A	0	1000	0	A1-A3
B	75	925	5	A4-A6
C	125	875	10	A7-A9
D	250	750	20	A10-A12
E	500	500	40	B1-B3
F	1000	0	80	B4-B6

Quercetin standard wells – 12.5 µl of standard (tubes A-E) was added per well in the designated wells in a clear well plate. The same amount (12.5 µl) of sample and

control were added to the sample wells and the control wells respectively. 12.5 µl 0.1% HCl in 95% EtOH was added to each well followed by 225 µl 2% HCl. This was left for 30 minutes at room temperature before taking the reading.

3.11.3.4. Data analysis and calculations

The path was set to the flavonol mode on the multiskan spectrum plate reader programme on the computer. The data were expressed as mg of Quercetin equivalents per gram of sample (mg Quercetin/g weight of sample).

3.11.4. ***Evaluation of flavanol Content***

3.11.4.1 Methodology

This analysis makes use of 4-dimethylaminocinnamaldehyde (DMACA) which reacts with flavanols to form a characteristic light blue colour that can only be measured at 640nm.

3.11.4.2 Equipment and sample preparation is as in Polyphenols.

3.11.4.3. Sample analysis:

The path was set to the flavanol mode on the multiskan programme on the computer

Preparation of standards – The standard series was prepared as shown in Table 32 below.

Table 32: Catechin standard wells

Tube	Catechin stock (µl)	Methanol (µl)	Catechin (µM)	Catechin (mg/L)	Wells
A	0	1000	0	0	A1-A3
B	5	995	5	1.36	A4-A6
C	10	990	10	2.72	A7-A9
D	25	975	25	6.8	A10-A12
E	50	950	50	13.6	B1-B3
F	100	900	100	27.2	B4-B6

Catechin standard wells – 50 µl of standard (tubes A-F) was added per well in the designated wells in a clear well plate. Same 50 µl of sample was added to the designated sample wells and control solution to Control wells. The reaction was initiated by adding 250 µl of DMACA to all the wells using a multichannel pipette. This was left for 30 minutes at room temperature before taking a reading at 640nm.

3.11.4.4. Data analysis and calculations

The data was analysed the same way as in polyphenol and was expressed as µmole Catechin equivalents per gram of sample (µmole Catechin/g of sample).

3.11.5. Evaluation of anthocyanins

3.11.5.1 Methodology

In this assay the following major precautions were taken:

(1) All the reagents and standards were prepared fresh on the day of analysis

(2) The pH of buffers were checked and adjusted prior to use.

(3) All spectral measurements were made between 15 min and 1 hr after the dilutions were prepared. This is because the observed readings tend to increase with time.

3.11.5.2 Sample preparation:

The preparation is same as in polyphenols but a 10-fold dilution was made with 10% EtOH because of the sensitivity of this assay.

3.11.5.3. Sample analysis

The measurement was made using TOTAL MONOMERIC ANTHOCYANIN MEASUREMENT BY THE pH-DIFFERENTIAL METHOD. This was carried out by first determining the appropriate dilution factor for the sample by diluting with potassium chloride buffer, pH 1.0, until the absorbance of the sample at the $\lambda_{vis\text{-}max}$ was within the linear range of the spectrophotometer (i.e. 1 – 1.2). The spectrophotometer was then standardized with distilled water at the used wavelengths ($\lambda_{vis\text{-}max}$ and 700 nm). Two dilutions of the samples were prepared, one with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5, diluting each by the previously determined dilution factor. These dilutions were left for 15 min to equilibrate. The absorbance of each dilution at the $\lambda_{vis\text{-}max}$ and at 700 nm was measured against a blank cell filled with distilled water.

3.11.5.5. Data analysis and calculations

After inputing all the appropriate dilution values for each sample, the absorbance (A) values were calculated as follows: $A = (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 4.5}$

The monomeric anthocyanin pigment concentration in the original sample was calculated using the following formula: Monomeric anthocyanin pigment (mg/liter) = (A x MW x DF x 1000)/(ε x1), where MW is the molecular weight, DF is the dilution factor (for example, if a 0.2 ml sample is diluted to 3 ml, DF = 15), and ε is the molar absorptivity.

3.11.6. Evaluation of flavanones

3.11.6.1. Methodology

This analysis makes use of Naringenin as the standard for measuring total flavanones in the sample using 2, 4-dinitrophenylhydrazine (DNPH)

3.11.6.2.1 Sample analysis:

The standard series was prepared as shown in Table 33 below.

Table 33: Naringenin standard

Tube	Naringenin stock (µl)	Methanol (µl)	Naringenin (µM)	Naringenin (mg/L)	Wells
A	0	1000	0	0	A1-A3
B	50	950	5	1.36	A4-A6
C	100	900	10	2.72	A7-A9
D	200	800	25	6.8	A10-A12
E	500	500	50	13.6	B1-B3
F	1000	0	100	27.2	B4-B6

Naringenin standard: – 50 µl of standard (tubes A-F) were added per well in the designated wells in a clear well plate. 50 µl of samples and control were equally used for the sample well and the control well respectively. The reaction was initiated by adding 250 µl of DNPH to all the wells. The plate was left for 30 minutes at room temperature before taking the reading at 495nm.

3.11.6.3 Data analysis and calculations

The data were analysed the same way as in polyphenol and are expressed as µmole Naringenin equivalents per gram of sample (µmole Naringenin/g of sample).

The measurements were carried out using a PU 8625 UV-Visible spectrophotometer (Diode-array, Philips, Netherlands).

Statistical analysis: - The results were expressed as mean SD obtained upon three independent analyses.

Statistical significance of the differences was evaluated using Student's t-test for all the samples (Kosalec et al. 2004).

3.11.7. Oxgen radical absorbance capacity assay (ORAC)

3.11.7.1. Methodology

The ORAC method was performed using a fluorescence spectrophotometer.

The chemicals used are:Phosphate buffer: 75mM, pH 7.4 (1.035 g of $NaH_2PO_4.H_2O$ in 100 mL H_2O as 1st solution; 1.335 g $Na_2HPO_4.2H_2O$ in 100 mL H_2O as 2nd solution. 18 mL of 1st solution was mixed with 82 mL of 2nd solution).

Fluorescein sodium salt (0.0225 g $C_{20}H_{10}Na_2O_5$ in 50 mL Phosphate buffer).

Peroxyl radical: AAPH (2,2'-Azobis (2-methylpropionamidine) dihydrochloride

PCA (70% Perchloric acid

Trolox (standard): 500 µM Stock solution (0.00625 g 6-Hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid was added to 50 mL phosphate buffer).

Trolox (control): 250 µM Stock solution (0.00312 g 6-Hydroxy-2, 5, 7, 8-tetra-methylchroman-2-carboxylic acid was added to 50 mL phosphate buffer).

3.11.7.2. Sample analysis

For the analysis, "ORAC" is selected and the excitation wavelength is set at 485nm while the emission wavelength set at 530nm. Trolox standard series were prepared as shown in Table 34 below.

Table 34: Trolox standard series

Tube	Standard concentration µM	Trolox stock solution µL	Phosphate Buffer µL	Well number
A	0	0	750	A1-A3
B	83	125	625	A4-A6
C	167	250	500	A7-A9
D	250	375	375	A10-A12
E	333	500	250	B1-3
F	417	625	125	B4-6

Trolox standard wells – 12 µl of standard (tubes A-E) was added per well in the designated wells in a black 96-microwell plate. Same amount (12 µl) of control were added into the control wells (B7-B12) and samples into sample wells (C1-H12) IN TRIPLICATE. 10 µl of the fluorescein stock solution was added to 2 mL phosphate buffer (in eppendorf tube) and 240 µl of this solution in 15 mL Phosphate buffer. 138 µl of this solution with a multichannel pipette was added into each well of the black microwell plate. 6 mL of the phosphate buffer was now added to the AAPH weighed earlier. 50 µl of this solution was transferred into each well. The final volume of the assay in each well is 200 µl.

The multiwell plate was inserted into the fluorometer and the reading was taken.

3.11.7.3 Data analysis and calculations

The ORAC values were calculated using a regression equation ($Y = a + bX + cX^2$) between Trolox concentration (Y) (µM) and the net area under the fluorescence decay curve (X). Data are expressed as micromoles of Trolox equivalents (TE) per milligram of sample (µmole Trolox/g of sample). It should be noted that Trolox was used as a control sample.

3.11.8. Ferric reducing antioxidant power assay (FRAP)

3.11.8.1. Methodology

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant present in stoichiometric excess.

3.11.8.2. Chemicals required

Acetate buffer: 300 mM, pH 3.6 (1.627 g Sodium acetate was added to 16 mL Glacial acetic acid and make up with distilled water to 1 L).

TPTZ (2,4,6-tri[2-pyridyl]-s-triazine): 10 mM (0.0093g TPTZ was added to 3 mL of 40 mM HCl).

Iron (III) chloride hexahydrate: 20 mM (0.054 g $FeCl_3.6H_2O$ was added to 10 mL distilled water).

Standard L-Ascorbic acid 1.0 mM solution.

3.11.8.3 Sample analysis

FRAP was selected from the computer programme of the multiskan which was set to read at 593nm and the FRAP reagent was prepared by adding together: 30 mL Acetate buffer + 3 mL TPTZ solution + 3 mL $FeCl_3$ solution + 6.6 mL distilled water. The expected straw colored solution was obtained.

The FRAP standard series were prepared as shown in Table 35 below.

Table 35: Ascorbic acid standard wells

Tube	Ascorbic acid stock solution µL	Distilled water µL	Standard concentration µM	Well number
A	0	1000	0	A1-A3
B	50	950	50	A4-A6
C	100	900	100	A7-A9
D	200	800	200	A10-A12
E	500	500	500	B1-3
F	1000	0	1000	B4-6

Ascorbic acid standard wells – 10 µl of standard (tubes A-E) were added per well in the designated wells in a clear well plate. 10 µl of control solution were equally added to the control wells (B7-B12) and samples to the sample wells (C1-H12) IN TRIPLICATE. 300µl of the FRAP reagent was added to each well with a multichannel pipette. The final volume of the assay in each well was 310 µl. The plate was incubated for 30 minutes in the incubating oven set at 37°C after which the plate was ran on the machine.

3.11.8.4 Data analysis and calculations

The data were expressed as micromoles of Vitamin C equivalents per gram of sample (µmole Vitamin C/g of sample).

3.11.9. Abts Trolox equivalent antioxidant capacity (TEAC) assay

3.11.9.1. Methodology: - Same as ORAC

*3.11.9.2. Equipmen*t: - Same as that in Polyphenols

Chemicals: - ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium salt: 7mM (0.0192 g of ABTS to 5 mL distilled water). This was prepared over night (24 hours) before starting the assay: 88µl of the potassium-peroxodisulphate solution was added to 5 mL of the ABTS solution. This was left in the dark at room temperature for 24 hours before use.

3.11.9.3 Sample analysis

3.11.9.4 Data analysis and calculations

The TEAC Readings were taken at 734nm on the multiskan spectrum plate readerand the TEAC standard series were prepared as shown in Table 36 below. Data was expressed as µmole Trolox equivalents per gram of sample (µmole Trolox /g of sample).

Table 36: TEAC standard series

Tube	Trolox standard µl	Ethanol µl	Trolox conc. µM	Well number
A	0	1000	0	A1-A3
B	50	950	50	A4-A6
C	100	900	100	A7-A9
D	150	850	150	A10-A12
E	250	750	250	B1-3
F	500	500	500	B4-6

Trolox standard wells – 25 µl of standard (tubes A-E) were added per well in the designated wells in a clear well plate. 25 µl of control solution and samples were added to the Control wells (B7-B12) and Sample wells (C1-H12) respectively IN TRIPLICATE. 300µl of this ABTS solution was added to each well using a multichannel pipette after a 2X dilution was made to the ABTS by mixing 1ml ABTS solution with 20mL EtOH. The plate was run after it was left for 30 minutes at room temperature.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *PILIOSTIGMA RETICULATUM*

The dried powdered leave material (2 kg) was sequentially extracted with hexane, ethyl acetate, methanol and finally with water. The hexane (78.45g), ethyl acetate (109.27g), methanol (246.28g) and water (124.21g) extracts were **Phytochemically screened** for detection of secondary plant metabolites such as tannins, phenolics, glycosides, saponins, flavonoids, alkaloids anthraquinones and essential oils using Wagner TLC method (Wagner and Bladt, 2001). Results of the screening as shown in Table 37 clearly demonstrate the presence of saponins, tannins, flavonoids, phenolics as well as cardiac glycosides; while alkaloids, anthraquinones, essential oils, phylobatannins and triterpenes were absent.

Table 37:- Phytochemical screening result of the *P. reticulatum* extracts

Test materials	**Tann**	**Phen**	**Glyc**	**Sapo**	**Flav**	**Alka**	**Anth**	**Esse**
PRH	P+ve	P+ve	P+ve	N-ve	P+ve	N-ve	N-ve	N-ve
PRE	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	N-ve	N-ve
PRM	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	N-ve	N-ve
PRW	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	N-ve	N-ve

PRH = *P. reticulatum* Hexane extract; **PRE** = *P. reticulatum* Ethyl acetate extract; **PRM** = *P. reticulatum* Methanol extract; **PRW** = *P. reticulatum* Water extract. Tannins (**Tann**), Phenolics (**Phen**), Glycosides (**Glyc**), Saponins (**Sapo**), Flavonoids (**Flav**), Alkaloids (**Alka**), Anthraquinones (**Anth**) and Essential oils (**Esse**)

It should be noted that all the plant parts used in this study were air-dried. This avoids the decomposition of thermo labile constituents which could occur at high temperatures, and is in keeping with the practice whereby handling and processing of collected plant materials are usually designed to closely parallel those employed by the herbalists (Samuelsson et al., 1985). The sequential extraction method utilized was found to be adequate because of the partial preliminary separation that was observed in the profile for all the plants from the hexane to water extracts. The hexane and ethyl acetate solvents sufficiently retaining the lypophilic compounds while methanol and water takes care of the hydrophilic parts (water-soluble compounds).

4.1.1 Isolation of constituent of the hexane extract fraction

Accelerated gradient chromatography (AGC) of the hexane extract (28.13g) afforded four fractions A – D (Table 6) in which A, B and C fractions were presumed to be pigments from their TLC profile. Fraction D (8.50g) was rechromatographed because it is the only fraction that showed a high presence of flavonoids and is in high yield. This afforded compounds **P1** (0.0272g) **P2** (0.0133g), **P3** (0.027g) and **P4** (0.0105 g) as shown in section 3.6.1.0 – 3.6.1.6.

4.1.2. Isolation of constituent of the ethyl acetate extract

AGC of the ethyl acetate extract (25.21g) gave 6 fractions coded E – J (Table 8). Fraction G (8.0g) was rechromatographed because of the size of the yield and evidence of the presence of flavonoids from the TLC analysis. This yielded compounds **P5** (0.0827g), **P6** (0.0054g), **P7** (0.075g), **P8** (0.0084g) and **P9** (0.0076 g) as shown in section 3.6.2.0 – 3.6.2.7.

4.1.3 Isolation of constituent of the methanol extract

On running the AGC of the methanolic extract (32.5g), four fractions were obtained which were coded K – N (Table 10). Fraction M was rechromatographed due to its high percentage yield. This afforded compounds **P10** (0.0198g), **P11** (0.0189g), **P12** (0.0065g), **P13** (0.0073g) and **P14** (0.075g) as shown in section 3.6.3.0 – 3.6.3.5. After critical examination and comparison of compounds **P1** – **P14**, it was evident that **P1** and **P6** were identical, likewise **P3** and **P11**, **P5** and **P10**, **P8** and **P14**, **P9** and **P12.**

This resulted in a total of 9 compounds **P1, P2**, **P3 P4**, **P5, P7**, **P8**, **P9** and **P13**

Solvent A: toluene – ethyl acetate (19: 1);

Solvent B: toluene – ethyl acetate – methanol (10: 7: 3);

Solvent C: methanol – water (8: 2, RP – 18).

4.2. PHYSICO-CHEMICAL DATA OF ISOLATED COMPOUNDS

4.2.1 P1 (6, 8-di-C-methylquercetin- 3, 7, 3'-trimethyl ether)

Yellow waxy powder with m.p.191-193 °C.

R_f (Solvent): 0.32 (A), 0.65 (B), 0.14(C)

UV λ_{max} nm (log ε): 257 (4.64), 355 (4.62); +NaOAc: 270, 365; +NaOH: 276, 342, 410. +$AlCl_3$: 279, 440.

IR (ν cm^{-1}) pronounced peaks: 3401, 2932, 1653, 1612 and 1556.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 372.1103. (calculated frag. gave $C_{20}H_{20}O_7$).

^{1}H NMR (400 MHz in DMSO-d_6): δ 2.12 (3H, s, Me-6), 2.36 (3H, s, Me-8) 3.78 (3H, s, OMe-3), 3.97 (3H, s, OMe-3'), 4.03 (3H, s, OMe-7); 6.91 (1H, d, J= 8.5 Hz, H-5'), 7.60 (1H, *dd*, J = 2.3, 8.4 Hz, H-6'), 7.70 (1H, d, J= 2.3 Hz, H-2'), 10.20 (1H, s, 4'-OH), 12.92 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) : see Table 38.

4.2.2 P2 (Piliostigmol)

Cream needle-like crystals with m.p. 220-224 °C.

R_f (Solvent): 0.16 (A), 0.69 (B), 0.28(C)

UV λ_{max} nm (log ε): 230 (4.89), 286 (4.59); +NaOAc: 232, 287; +NaOH: 244, 288; +A1C1$_3$: 234, 252 (sh), 308, 351.

IR (ν cm^{-1}) pronounced peaks: 3435, 3212, 1658, 1606 and 1566.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 330.0681. (calculated frag. gave $C_{17}H_{14}O_7$)

^{1}H NMR (400 MHz in DMSO-d$_6$) : δ 2.01 (3H, s, Me-6), 3.90 (3H, s, OMe-7), 6.72 (1H, s, H-8), 6.85 (2H, d, J= 8.8Hz, H-3', 5'), 7.19 (2H, d, J= 8.8Hz, H-2', 6'), 9.78 (1H, s, 4'-OH), 11.65 (1H, s, 3-OH), 12.95 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d$_6$) see Table 38.

4.2.3 P3 (6, 8-di-C-methylkaempferol- 3,7-dimethyl ether)

Yellow needles, mp. 285 – 287 ^{o}C,

R_f (Solvent): 0.19 (A), 0.73 (B), 0.18 (C).

UV λ_{max} nm (log ε): 275.5 (4.79), 332.0 (4.78); +NaOAc: 274.5, 331.5; +AlCl3: 277.0, 346.0; +NaOH: 274.0, 398.5.

IR (ν cm^{-1}) pronounced peaks: 3182, 1642, 1602 and 1545.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 343.0035. (calculated frag. gave $C_{19}H_{18}O_6$).

^{1}H NMR (250 MHz, DMSO-d6): δ 2.13 (3H, s, Me-6), 2.30 (3H, s, Me-8), 3.73 (3H, s, OMe-7), 3.80 (3H, s, OMe-3), 6.97 (2H, d, J = 8.9 Hz, H-3',5'), 8.00 (2H, d, J = 8.9 Hz, H-2',6'); 12.80 (IH, s, 5-)H). ^{13}C NMR (100 MHz in DMSO-d$_6$): see Table 38.

4.2.4 P4 (6, 8-di-C-methylquercetin -3,3'-dimethyl ether)

Yellow fluffy powder with m.p. 265-267 °C.

R_f (Solvent): 0.35 (A), 0.47 (B), 0.24(C)

UV λ_{max} nm (log ε): 262 (4.62), 354 (4.60); +NaOAc: 260, 355; +NaOH: 270, 405; +A1C1$_3$: 277, 435.

IR (ν cm^{-1}) pronounced peaks: 3429, 1652, 1613, 1568 and 1556.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 360.0954. (calculated frag. gave $C_{19}H_{18}O_7$).

^{1}H NMR (400 MHz in DMSO-d_6) : δ 2.11 (3H, s, Me-6), 2.41 (3H, s, Me-8); 3.89 (3H, s, OMe-3), 3.98 (3H, s, OMe-3'), 7.03 (1H, d, J=8.6Hz, H-5'), 7.62 (1H, dd, J= 2.3, 8.6 Hz, H-6'), 7.75 (1H, d, J= 2.3Hz, H-2'), 10.23 (1H, s, 4'-OH), 12.95 (1H, s, 7-OH).13.05 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) : see Table 38.

4.2.5 P5 (6,8,3'-tri-C-methylquercetin- 3,7-dimethyl ether)

Yellow amorphous powder with m.p 243-246 °C.

R_f (Solvent): 0.56 (A), 0.51 (B), 0.35(C).

UV λ_{max} nm (log ε): 263 (4.65), 363 (4.62); +NaOAc: 262, 367; +NaOH: 267, 412; +A1C1$_3$: 281, 447.

IR (ν cm^{-1}) pronounced peaks: 3356, 1642, 1610, 1578, and 1546

EIMS: the molecular ion base peak $(M)^+$ *m/z* 356.3821. (calculated frag. gave $C_{20}H_{20}O_6$).

^{1}H NMR (400 MHz in DMSO-d_6) : δ 2.16 (3H, s, Me-6), 2.29 (3H, s, Me-3'), 2.42 (3H, s, Me-8), 3.84 (3H, s, OMe-3), 3.92 (3H, s, OMe-7), 7.02 (1H, d, J= 8.6Hz, H-5'), 7.62 (1H, dd, J= 2.3, 8.6Hz, H-6'), 7.74 (1H, d, J= 2.3Hz, H-2'), 10.13 (1H, s, 4'-OH), 13.02 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) see Table 38.

4.2.6 P7 (6-C-methylquercetin -3,7,3'-trimethyl ether)

Yellow needle-like crystals with m.p. 184-187 °C

R_f (Solvent): 0.23 (A), 0.58 (B), 0.20 (C).

UV λ_{max} nm (log ε): 256.5 (4.78), 272.0sh, 346.0 (4.86); +NaOAc: 258.0, 346.0; +AlCl3: 268.0, 370.0; +NaOH: 267.5, 410.5.

IR (ν cm^{-1}) pronounced peaks: 3400, 2953, 1651, 1610, and 1559.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 358.4133 (calculated frag. gave $C_{19}H_{18}O_7$).

^{1}H NMR (400 MHz in DMSO-d_6) : δ 2.08 (3H, s, Me-6); 3.91 **(**3H**,** s**,** OMe-3); 3.98 (3H, s, OMe-3'); 4.01 (3H, s, OMe-7); 6.90 (1H, s, H-8); 7.07 (1H, d, J=8.6Hz, H-5'); 7.72 (1H, dd, J = 2.3, 8.6 Hz, H-6'); 7.77 (1H, d, J = 2.3 Hz, H-2'). 10.10 (1H, s, 4'-OH), 12.95 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) : see Table 38.

There was spectral comparison with that of Ref. (Rabesa and Voirin, 1985).

4.2.7 P8 (6-C-methylquercetin- 3-methyl ether)

Yellow powder with m.p. 261-263 °C.

R_f (Solvent): 0.61 (A), 0.41 (B), 0.44(C)

UV λ_{max} nm (log ε): 260.5 (4.41), 359.5 (4.45); +NaOAc: 263.5, 377.0; +AlCl3: 266.5, 376.0; +NaOH: 273.5, 409.5.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 330.6242 (calculated frag. gave $C_{17}H_{14}O_7$).

^{1}H MMR (400 MHz in DMSO-d_6) : δ 2.17 (3H, s, M-6), 3.92 (3H, s, OMe-3), 6.66 (1H, s, H-8), 7.10 (1H, d, J= 8.6Hz, H-5'), 7.64 (1H, *dd*, J = 2.3, 8.6 Hz, H-6'), 7.76 (1H, d, J= 2.3 Hz, H-2'), 10.23 (1H, s, 4'-OH), 13.10 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) : see Table 38.

There was spectral comparison with that of Ref. (Harborne et al., 1994).

4.2.8 P9 (6,8-di-C-methylkaempferol- 3-methyl ether)

Yellow waxy amorphous solid with m.p. 250-253 °C.

R_f (Solvent): 0.12 (A), 0.60 (B), 0.32(C)

UV λ_{max} nm (log ε): 275.5 (4.76), 334.5 (4.69); +NaOAc: 277.5; +AlCl3: 282.0, 359.0;+NaOH: 281.0, 332.0, 407.0.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 329.2110 (calculated frag. gave $C_{18}H_{16}O_6$).

^{1}H NMR (400 MHz in DMSO-d_6) : δ 2.15 (3H, s, Me-6); 2.35 (3H, s, Me-8); 3.88 (3H, s, OMe-3); 7.10 (2H, d, J = 9 Hz, H-3',5'); 8.05 (2H, d, J = 9 Hz, H-2', 6'); 13.00 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) : see Table 38.

There was spectral comparison with that of Ref. (Voirin, 1983).

4.2.9 P13 (Quercetin)

Bright yellow powder, m.p. 310 - 311˚C, (Lit. 314˚C, Windholz, 1983),

Rf(Solvent): 0.76 (A), 0.37 (B), 0.60(C).

IR (ν cm^{-1}) pronounced peaks: 3400, 3292, 1672, 1614, 1513.

UV λ_{max} nm (log ε): 256.0 (4.78), 374.0 (4.82); +NaOAc: 260.5, 387.5; +AlCl3: 270.0, 436.0; +NaOH: 281.0, 425.0.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 302.2112 (calculated frag. gave $C_{15}H_{10}O_7$).

^{1}H NMR (400 MHz in DMSO-d_6) : δ 6.74 (1H, d, J = 1.98 Hz, H-8), 6.78 (1H, d, J= 1.98 Hz, H-6), 7.40 (1H, d, J = 8.25 Hz, H-5'), 8.10 (1H, dd, J = 2.31, 8.25 Hz, H-6'), 8.64 (1H, d, J = 2.31 Hz, H-2'), 13.36 (1H, s, 5-OH).

^{13}C NMR (100 MHz in DMSO-d_6) see Table 38.

TABLE 38: ^{13}C NMR data for compounds P1 – 13

Carbon	**P1**	**P2**	**P3**	**P4**	**P5**	**P7**	**P8**	**P9**	**P13**
2	155.3	166.7	156.0	155.2	155.8	155.3	155.3	155.1	147.0
3	137.5	156.8	137.7	137.3	137.2	137.9	137.6	137.4	137.9
4	178.8	182.3	178.5	177.9	178.4	177.8	177.7	178.0	177.3
5	155.3	156.2	155.9	157.1	155.9	157.0	158.1	155.6	157.5
6	106.6	108.5	112.5	107.3	112.6	107.0	106.4	106.7	99.2
7	162.4	161.5	162.0	162.1	162.5	162.9	162.2	159.7	165.4
8	101.5	92.1	108.5	101.9	108.4	90.0	92.6	101.6	94.3
9	151.4	152.4	151.4	154.4	151.3	154.3	153.9	151.5	162.4
10	103.9	101.5	107.0	104.8	107.1	104.7	103.7	104.0	104.4
1'	121.6	143.1	120.6	120.4	120.5	120.7	120.8	120.9	123.8
2'	115.3	121.5	130.1	115.4	115.5	111.9	115.2	129.9	116.6
3'	147.2	116.2	115.7	147.6	135.6	147.4	145.2	115.7	147.7
4'	148.5	156.2	160.3	148.5	148.8	149.7	148.6	160.0	149.6
5'	115.8	116.2	115.7	115.4	115.6	115.5	115.7	115.7	116.6
6'	120.6	121.5	130.1	120.8	120.2	122.1	120.5	129.9	121.0
3-OMe	59.5		59.6	59.2	59.4	59.5	59.6	59.6	
7-OMe	60.2	56.4	60.3		60.3	56.2			
3'-OMe	56.5			55.9		55.7			
6-Me	8.1	7.2	8.0	7.2	8.0	7.1	7.3	8.0	
8-Me	8.5		8.2	8.3	8.2			8.2	
3'-Me					8.7				

4.3. STRUCTURAL ELUCIDATION OF COMPOUNDS P1 – P13

4.3.1 COMPOUND P1 (6, 8-di-C-methylquercetin 3, 7, 3'-trimethyl ether)

Compound **P1** showed a molecular ion peak $(M)^+$ at *m/z* 372.1103 leading to a molecular formula of $C_{20}H_{20}0_7$. The UV spectrum showed two maxima peaks at 355 and 257 nm respectively which are associated with a flavonoid system. The band at 270 nm showed a large bathochromic shift in the presence of sodium acetate and an additional band appeared at 342 nm in the methanol / NaOH spectrum which suggested a flavonol with a 7-OMe group. The ^{1}H NMR spectrum showed that there were two aryl methyl singlets at δ 2.12 and 2.36 and three methoxyl peaks were observed at δ 3.78 (OMe-3), 3.97 (OMe-3') and 4.03 (OMe-7). The spectrum further showed the presence of only three aromatic signals at δ 6.91 (1H, d), 7.60 (1H, dd) and 7.70 (1H, d) suggesting a 3',4'-disubstituted aromatic ring C and a completely substituted aromatic ring A with a correlated typical chemical shift of a chelated 5-OH group at δ 12.92 of a flavone. When the ^{13}C NMR data for **P1** in Table 38 was compared with literature (Roitman and James, 1985; Breitmeier and Voelter, 1990) it was evident from the analysis that the 4′-substituent was an hydroxyl group while that of 3' was a methoxyl group which was confirmed by the chemical shifts of their carbons at δ C-3' (147.2) and C-4' (148.5) which also reflect in the slight variation observed in the neighboring carbons at δ C-2' (115.3) and C-5' (115.8). The methoxyl groups were linked to C-3 (δ 137.5), C-3' (δ 147.2) and to C-7 (δ 162.4) which were in agreement with the HMBC correlations OMe-3/C-3 with OMe-3'/C-3' and OMe-7/C-7 (Table 39). The methyl groups were attached to C-6 and C-8, as highlighted by the HMBC cross peaks OH-5/C-5, C-6, C-4a; Me-6/C-5, C-6, C-7 and that of Me-8/C-7, C-8, C-8a. Based on the above analysis the structure of compound **P1** was confirmed to be 3,7,3'-trimethoxy- 6,8-di-C-methyl -5,4'-dihydroxylflavone (6,8-di-C-methylquercetin 3,7, 3'-trimethyl ether) as shown in Fig. 9.

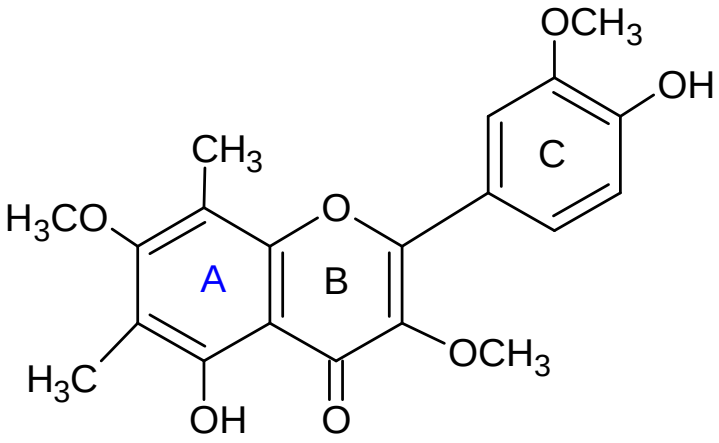


Fig. 9 Structure of compound P1

TABLE 39: NMR spectroscopic data for compound P1

^{13}C Signal (δ)	HMBC	^{1}H (δ, J Hz)	^{13}C Assignment
155.3	H-2', 6'		2
137.5	3-OMe		3
178.8			4
155.3	5-OH, Me-6		5
106.6	5-OH, Me-6		6
162.4	Me-6, Me-8, 7-OMe		7
101.5	Me-8		8
151.4	Me-8		4a
103.9	5-OH		8a
121.6	H- 5'		1'
115.3		7.70 (1H, d, 2.3)	2'
147.2	H-2', H-5'		3'
148.5	H-2', H-6'		4'
115.8	H-6'	6.91 (1H, d, 8.5)	5'
120.6	H-2'	7.60 (1H, dd, 2.3 and 8.4)	6'
8.1		2.12 (3H, s)	6-Me
8.5		2.36 (3H, s)	8-Me
56.5		3.97 (3H, s)	3'-OMe
59.5		3.78 (3H, s)	3-OMe
60.2		4.03 (3H, s)	7-OMe

4.3.2 COMPOUND P2 (Piliostigmol)

The mass spectrum of **P2** showed a molecular ion base peak $(M)^{+\cdot}$ at *m/z* 330.0681 and this together with the mass spectral fragmentation suggested a molecular formula of $C_{17}H_{14}0_7$. The UV spectrum exhibited two peak maxima at 232 and 287 nm, which were in close agreement with those obtained for capillarisin, a closely related 2-O-(*p*-hydroxyphenyl) chromone, which was isolated from *Artemisia capillaris* (Komiya et al., 1975), thus indicating the possible presence of a similar chromophore in **P2**. The ^{1}H NMR spectrum showed a characteristic pattern for an A_2B_2 system, as would be expected from a para-disubstituted aromatic system of the phenolic ring C, with doublets at δ 6.85 and 7.19 (J= 8.8 Hz), as a well as a singlet at δ 6.72 which could be attributed to H-8 in the A ring (Komiya et al., 1975; Hashidoko et al., 1991; Huang et al., 1993). Other signals of note were a chelated 5-OH at δ 12.95, a singlet at 11.65 due to the 3-OH, a methyl singlet at δ 2.01 and a methoxyl singlet at δ 3.90. In the ^{13}C NMR spectrum (Table 38), the signal due to C-3 appeared at δ 156.8 while that of C-8 was observed at δ 92.1 and this was corroborated by HMQC data (Table 40). The observed HMBC correlations for **P2** (Fig 11) confirmed that the single ring A proton was located at position 8 (cross peaks H-8/C-4, C-6, C-7, C-4a and C-8a) and that the single aryl methyl group was attached at C-6 (cross peaks OH-5/C- 5, C-4a) and Me-6/C-5, C-6, C-7) whereas the methoxyl group was linked to C-7 (cross peak OMe/C-7). No HMBC correlations were observed between rings B and C, thus confirming the presence of the oxygen bridge C1'-O-C 2 (Table 40). All the above data is in agreement with the structure of **P2** to have as the structure of 2-*O*-(*p*-

hydroxyphenyl)-3,5- dihydroxy-6-*C*- methyl-7-methoxychromone, and is hereby given the trivial name piliostigmol (Fig. 10).

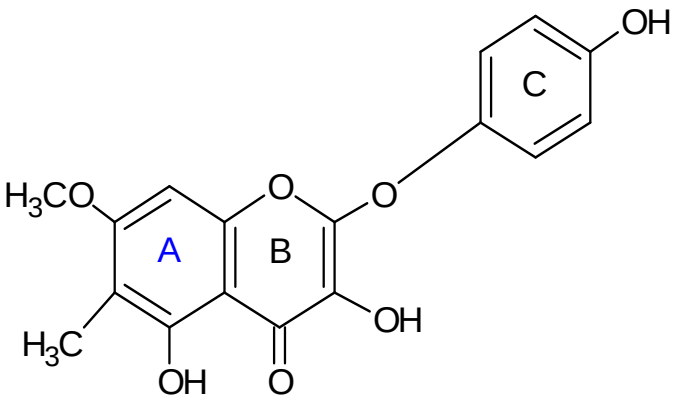


Figure 10 – Structure of Piliostigmol (P2)

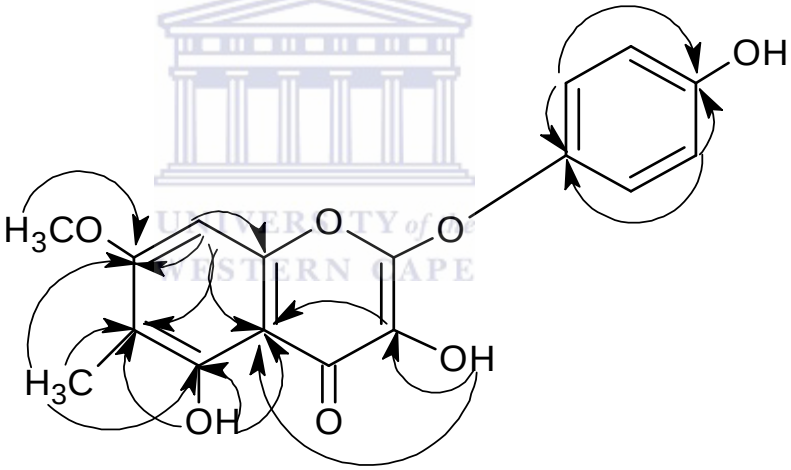


Figure 11 - P2 HMBC correlations for Piliostigmol

TABLE 40: NMR spectroscopic data for compound P2

^{13}C Signal (δ)	HMQC correlation (δ)	HMBC	^{13}C Assignment
166.7			2
156.8	11.65 s (3-OH)	3-OH	3
182.3			4
156.2		5-OH, Me-6	5
108.5		5-OH, Me-6, H-8	6
161.5		Me-6, 7-OMe, H-8	7
92.1	6.72 s (H-8)		8
152.4		H-8	8a
101.5		5-OH, 3-OH, H-8	4a
143.1		H-2', 6'; H-3', 5'	1'
121.5	7.19 d (H-2', H-6') (2H, J = 8.8 Hz)		2', 6'
116.2	6.85 d (H-3', H-5') (2H, J = 8.8 Hz)		3', 5'
156.2		H-2', 6'; H-3', 5'	4'
7.3	2.01 s (Me-6)		6-Me
56.4	3.90 s (7-OMe)		7-OMe

2-O-(*p*-hydroxyphenyl) chromones are relatively rare in nature, and Piliostigmol is the first phenoxychromonol reported. The first 2-phenoxychromone to be isolated was capillarisin from *Artemisiae capillaries* (Compositae) (Komiya et al., 1975). Subsequently, Hashidoko et al., (1991) isolated two 2-phenoxychromones from the leaves of *Rosa rugosa* (Rosaceae), while Huang et al., (1993) reported three 2-phenoxychromones from *Epimedium sagittatum* (Berberidaceae) including an 8-prenylated derivative. In 1996, Piliostigmin, another 2-phenoxychromone was isolated from the leaves of *Piliostigma thonningii* (Ibewuike et al., 1996). The reported biological activities were found more in *P.thonningii* and it was discovered that the 2-phenoxychromones in capillarisin has a biliary excretion stimulating effect

(Komiya et al., 1975) whereas it was observed that Piliostigmin isolated from *P.thonningii* did not exhibit any significant antibacterial or anti-inflammatory (PG synthesis inhibitory) activity. On the contrary Piliostigmol was found to possess high activity both as an antimicrobial and antioxidant (Babajide et al., 2008). It was assumed that the activity observed may be due to the positioning of the 3-OH group in the ring B. Although Nakanishi et al., (1983) proposed that 2-phenoxychromones may be biosynthesized from the corresponding flavones by a Baeyer- Villiger type of oxidation or via an epoxy quinone intermediate in which the main activity reside on carbon -3 (Hashidoko et al., 1991).

4.3.3 Compound P3 (6, 8-di-C-methylkaempferol 3, 7-dimethl ether)

Compound **P3,** a bright yellow crystalline solid, gave a positive reaction with ferric chloride, and a bright yellow colour with vanillin-H_2SO_4 spray reagent. The infra-red spectrum showed a carbonyl absorption band at 1642 cm-^{1} and –CH aliphatic stretch at 3000-2800 cm^{-1}. There was also an OH band at 3182 cm^{-1}. These results are consistent with compound **P3** being a flavonoid. The ultraviolet spectrum showed two maxima at 332.0 nm (Band I) and275.5 nm (Band II). These values are typical of a 3-O-substituted flavonol (Wollenweber, 1982). A Band I bathochromic shift of +66.5 nm in the presence of NaOH, and the absence of any shift in the sodium acetate spectrum indicated the presence of a free 4'-OH group, as well as the absence of a free 7-OH group, respectively. Compound **P3** is therefore a flavonol in which the 3-OH and 7-OH groups are substituted, and with a free 4'-OH group. The ^{1}H NMR spectrum of **P3** showed two methyl singlets (δ 2.13 and 2.30) and two methoxyl singlets (δ 3.73 and 3.80). The presence of an AA'CC' system due to ring C at δ 6.97 and 8.00 (J = 8.9 H_Z) implied that the free 4'-OH group indicated by the NaOH shift was the only substituent on this ring. Also, the presence of a free 5-OH group (indicated by the H-bonded proton singlet at δ 12.8) further implied that the two methoxyl and two methyl substituens could only be at positions 3, 7, 6 and 8 respectively. Compound **P3** must therefore be 6, 8-di-C-methylkaempferol 3,7-dimethyl ether (5,4'-dihydroxy-3,7-dimethoxy-6,8-dimethylflavone) (Figure 12). This

structure was confirmed by the mass spectrum which showed an $(M+H)^+$ ion at m/z 343, consistent with the molecular formula $C_{19}H_{18}O_6$. The ^{13}C NMR spectrum (Table 38) also exhibited 17 signals as expected, corresponding to a C_{19} -flavoniod in which C-2', 6' and C-3', 5' are respectively isochronous. The HMBC spectrum of **P3** was used to assign the individual carbon resonance (Table 41). While the methyl and methoxyl signals were assigned by comparison with literature (Tanrisever *et al*., 1987).

Compound **P3** had previously been reported from *Alluaudia dumosa* (Rasamoelisendra *et al*., 1989), and the ^1H NMR and UV spectra are in agreement with those published. The complete ^{13}C spectral assignment (Table 41) is reported for the first time for this plant.

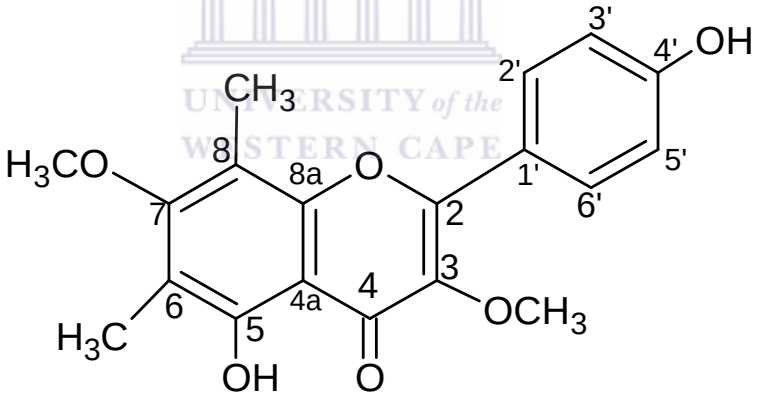


Fig. 12-Structure of compound P3

TABLE 41: NMR spectroscopic data for compound P3

13C Signal (δ)	DEPT	1H Signal (δ)	HMBC	^{13}C Assignment
156.0	C		H-2', 6'	2
137.7	C		3-OMe	3
178.5	C			4
155.9	C		5-OH, Me-6	5
112.5	C		5-OH, Me-6	6
162.0	C		7-OMe, Me-6, Me-8	7
108.5	C		Me-8	8
151.4	C		Me-8	8a
107.0	C		5-OH	4a
120.6	C		H-3', 5'	1'
130.1	CH	8.00d (2H, J = 8.9 Hz)	H-2', 6'	2', 6'
115.7	CH	6.97d (2H, J = 8.9 Hz)	H-3', 5'	3', 5'
160.3	C		H-2', 6'; H-3', 5'	4'
8.0	CH_3	2.13s		6-Me
8.2	CH_3	2.30s		8-Me
59.6	CH_3	3.80s		3-OMe
60.3	CH_3	3.73s		7-OMe

4.3.4 COMPOUND P4 (6, 8-di- C- methylquercetin 3,3'- dimethyl ether)

The molecular formula of **P 4** was assigned as $C_{19}H_{18}0_7$ from the molecular ion peak at *m/z* 360.0954. The UV and IR spectra obtained were also analyzed and compared with that of literature which showed a similar trend in the UV spectrum at λmax 354 and 262 nm respectively, but which suggested a flavonol with a free 7-OH group in this case. The ^{1}H NMR spectrum exhibited the signals of two aryl methyl groups at δ 2.11 (Me-6) and 2.41 (Me-8); and two methoxyl groups at δ 3.89 (OMe-3) and 3.98 (OMe-3'), The ring C aromatic signals were typical of a 3',4'-substituted pattern similar to that of **P1** viz., δ 7.03 (1H, d), 7.62 (1H, dd) and 7.75 (1H, d). The signals of three -OH groups were observable at δ 10.23 (4'-OH), 12.95 (7-OH) and 13.05 (5-OH). The ^{13}C spectrum (Table 38) showed a similar trend to that of compound **P1**, in which the tri-substituted C ring has an hydroxyl group at C-4' and a methoxyl group at C-3'. The HMBC data (Table 42) for ring A clearly demonstrated a slight difference from that of **P1** at the C-7 position to which the OH has been assigned. The cross peaks observed for OH-5/C-5, C-6, C-4a and Me-6/C-5, C-6, C-7, Me-8/C-7, C-8, C-8a all of which indicated that the two aryl methyl groups were located at C-6 and C-8. The two methoxyl groups were located at C-3 and C-3' as corroborated by their HMBC correlations with C-3 and C-3' respectively. **P4** was consequently assigned the structure 3,3'-dimethoxy-6,8-di-C-methyl- 5,7,4'-trihydroxyflavone (6,8-di- C- methylquercetin 3,3'- dimethyl ether) as shown in Fig. 13

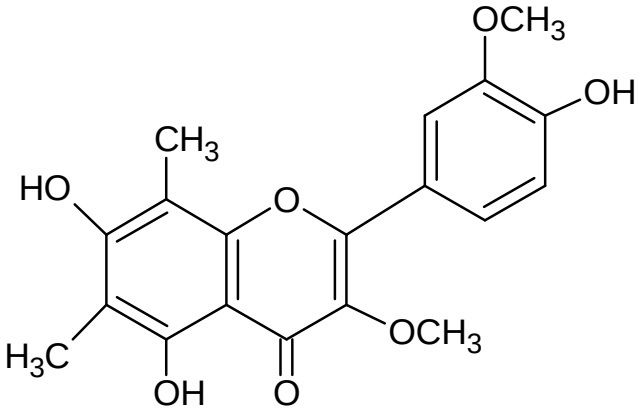


Fig. 13 – Structure of compound P4

TABLE 42: NMR spectroscopic data for compound P4

^{13}C Signal (δ)	HMBC	^{1}H (δ, J Hz)	^{13}C Assignment
155.2	H-2', 6'		2
137.3	3-OMe		3
177.9			4
157.1	5-OH, Me-6		5
107.3	5-OH, Me-6, Me-8		6
162.1	Me-6, Me-8		7
101.9			8
154.4	Me-8		8a
104.8	5-OH, Me-8		4a
120.4	H- 5'		1'
115.4	H-6'	7.75 (1H, d, 2.3)	2'
147.6	H-2', H-5'		3'
148.5	H-2', H-5', H-6'		4'
115.4		7.03 (1H, d, 8.6)	5'
120.8	H-2'	7.62 (1H, dd, 2.3 and 8.6)	6'
7.2		2.11 (3H, s)	6-Me
8.3		2.41 (3H, s)	8-Me
55.9		3.98 (3H, s)	3'-OMe
59.2		3.89 (3H, s)	3-OMe

4.3.5 COMPOUND P5 (6, 8, 3'-tri-C-methylquercetin 3, 7-dimethyl ether)

The EIMS of **P5** showed the molecular ion peak $(M)^+$ at *m/z* 356.3821 corresponding to a molecular formula of $C_{20}H_{20}0_{6.}$ The UV and IR spectra obtained were also analysed and compared with that of literature which showed similar trend to that of **P1** in the UV spectrum at λ_{max} 363 and 263 nm respectively, suggesting a flavonol with a 7-OMe group in this case. The ^{1}H NMR spectrum exhibited signals due to three aryl methyl groups at δ 2.16 (Me-6), 2.29 (Me-3') and 2.42 (Me-8). It also showed two methoxyl signals at δ 3.84 (OMe-3) and 3.92 (OMe-7). The ring C aromatic signals again resembled a typical 3',4'-disubstituted pattern similar to that experienced in **P1** viz., δ 7.02 (1H, d), 7.62 (1H, dd) and 7.74 (1H, d). Peaks of two OH groups were observed at δ 10.13 (4'-OH) and 13.02 (5-OH). The ^{1}H NMR and ^{13}C NMR signals of the C ring were similar to that of **P1** except for the peak located at δ 2.29 due to the 3′-Me group confirmed in the ^{13}C NMR spectrum for C-3' at δ135.6 and 3'-Me at δ 8.7 (Table 38).

Further analysis of the ^{1}H NMR spectrum showed that ring A was fully substituted and posses an hydroxyl group at C-5, since due to the typical singlet at δ 13.02 and a further signal at δ 10.13 was assigned to the 4'-OH on ring C. There were 3 aryl methyl groups; one at δ 2.16 assigned to C-6 , one at δ 2.42 assigned to C-8 and the third on ring C at δ 2.29 assigned to C-3'. These were further confirmed by the HMBC cross peak correlations (Table 43) which appeared very similar to those observed for **P1** and **P4.** From the forgoing, **P5** has been identified as 3,7-dimethoxy -6,8,3' - tri - C- methyl- 5,4' - dihydroxyflavone (6,8,3'-tri-C-methylquercetin 3,7-dimethyl ether) Fig. 14.

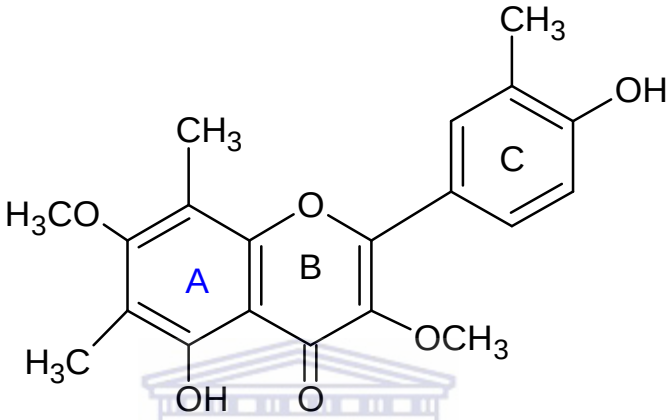


Figure 14 – Structure of compound P5

TABLE 43: The NMR spectroscopic data for compound P5

^{13}C Signal (δ)	HMBC	^{1}H (δ, J Hz)	^{13}C Assignment
155.8	H-2', 6'		2
137.2	3-OMe		3
178.4			4
155.9	5-OH, Me-6		5
112.6	5-OH, Me-6		6
162.5	Me-6, Me-8, 7-OMe		7
108.4	Me-8		8
151.3	Me-8		8a
107.1	5-OH		4a
120.5	H- 5'		1'
115.5		7.74 (1H, d, 2.3)	2'
135.6	Me-3', H-5'		3'
148.8	H-2', H-6',		4'
115.6	H-6'	7.02 (1H, d, 8.6)	5'
120.2	H-2'	7.62 (1H, dd, 2.3 and 8.6)	6'
8.0		2.16 (3H, s)	6-Me
8.2		2.42 (3H, s)	8-Me
8.7		2.29 (3H, s)	3'-Me
59.4		3.84 (3H, s)	3-OMe
60.3		3.92 (3H, s)	7-OMe

4.3.6 Compound P7 (6-C- methyquercetin 3, 7, 3'-trimethyl ether)

The IR spectrum of compound **P7** exhibited characteristic carbonyl absorption at 1651cm^{-1} and a strong O-H absorption at 3400cm^{-1}. The UV spectrum of the compound showed a Band I absorbance at 346 nm, and a Band II absorbance at 256.5 nm. These absorption bands are consistent with a 3-O-substituted flavonol (Wollenweber, 1982). The bathochromic shift of Band I in the presence of NaOH (+64 nm) indicated the presence of a free 4'-OH group, while the absence of a similar shift in the presence of sodium acetate also showed the absence of a free 7-OH group. The ^{13}C NMR and DEPT spectra of compound **P7** showed 19 carbon resonances consisting of four methyl groups which included three oxygenated methyl groups, four methine and eleven quaternary signals. The mass spectrum showed a [MH]+ ion at *m/z* 358.4133. These data are consistent with a molecular formula of $C_{19}H_{18}O_7$ for compound **P7**.

The ^{1}H NMR spectrum of compound **P7** showed an aryl methyl singlet at δ 2.08, as well as three methoxyl singlets at δ3.91, 3.98, and 4.01. In the aromatic region, the presence of one proton singlet at δ6.90 indicated a pentasubstituted ring A, while the doublets at δ7.07 and 7.77 as well the doublet of a doublet at δ7.72 confirmed the presence of a 1,3,4-trisubstituted ring C. Compound **P7** is therefore a quercetin derivative having the hydroxyl groups at C-3 and C-7 methylated.

The electron impact (EI) mass spectrum of compound **P7** exhibited prominent ions at m/z 358 ($M^{+\cdot}$, 100%), 357 $[M-H]^+$, 343 $[M-Me]^+$ and 315 $[M-Me-CO]^+$, all consistent with a 3-methoxyflavonol (Kingston, 1971). The characteristic RDA fragment ions m/z 181 $[A+H]^+$ and 151 $[C]^+$ indicated the presence of both a methyl group and a methoxyl substituent on ring A, and a methoxyl group on rign C (Figure 16). Since the UV spectrum showed no bathchromich shift in the presence of NaOAc, the ring A methoxyl substituent must located at C-7, while the ring C methoxyl must be located at C-3' supported in the ^{1}H NMR spectrum which indicated a 1', 3', 4'-trisubstituted pattern, and in addition the NaOH bathochromic shift of Band I suggested the presence of a free 4'-OH group. Compound **P7** is therefore assigned as 3, 7, 3'-tri-O-methyllquercetin possessing a methyl group at C- 6 or C-8. The position

of the aryl methyl at C-6 was confirmed by the ^{1}H-^{13}C COSY and HMBC spectra. The COSY spectrum was used to assign the proton signals to their respective carbon resonances (Table 44). The presence of the ring A methyl group at C-6 was confirmed by the observed HMBC correlations Me-6/C-5, C-6, C-7 and also by the H-8/C-6, C-7, C-8a and C-4a cross-peaks. This allowed the unambiguous assignment of compound **P7** as 6-C-methyquercetin 3, 7, 3'-trimethyl ether (6-C-methyl-5,4'-dihydroxy-3,7,3'-trimethoxyflavone) (Figure 15). It has previously been isolated from *Alluaudia humbertii* Choux (Didiereaceae) (Rabesa and Voirin, 1985). The spectra (UV, MS) are in excellent agreement with those published in literature (Rabesa and Voirin, 1985), while the 13C NMR spectrum and the complete assignment (Table 44) are reported here for the first time.

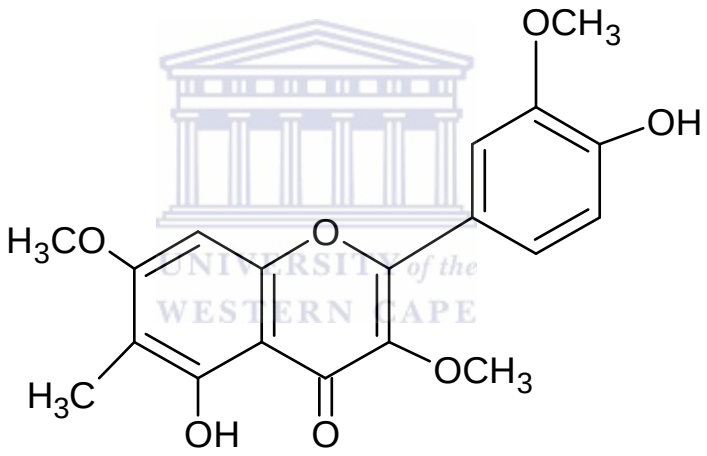


Figure 15 – Structure of compound P7

TABLE 44: NMR Spectroscopic Data for Compound P7

13C Signal (δ)	**DEPT**	**1H – 13C COSY**	**HMBC**	**^{13}C Assignment**
155.3	C		H-6'	2
137.9	C		3-OMe	3
177.8	C			4
157.0	C		5-OH, Me-6	5
107.0	C		5-OH, Me-6	6
162.9	C		7-OMe	7
90.0	CH	6.90s	C-6, C-7, C-9, C-10	8
154.3	C		H-8	8a
104.7	C		5-OH	4a
120.7	C		H- 5'	1'
111.9	CH	7.77d (J = 2.3 Hz)	H-6'	2'
147.4	C		3'-OMe, H-5'	3'
149.7	C		H-5', H-6'	4'
115.5	CH	7.07d (J = 8.6 Hz)		5'
122.1	CH	7.72dd (J = 2.3 and 8.6 Hz)	H-2'	6'
7.1	CH_3	2.08s	C-5, C-6, C-7	6-Me
55.7	CH_3	3.98s	C-3'	3'-OMe
56.2	CH_3	4.01s	C-7	7-OMe
59.5	CH_3	3.91s	C-3	3-OMe

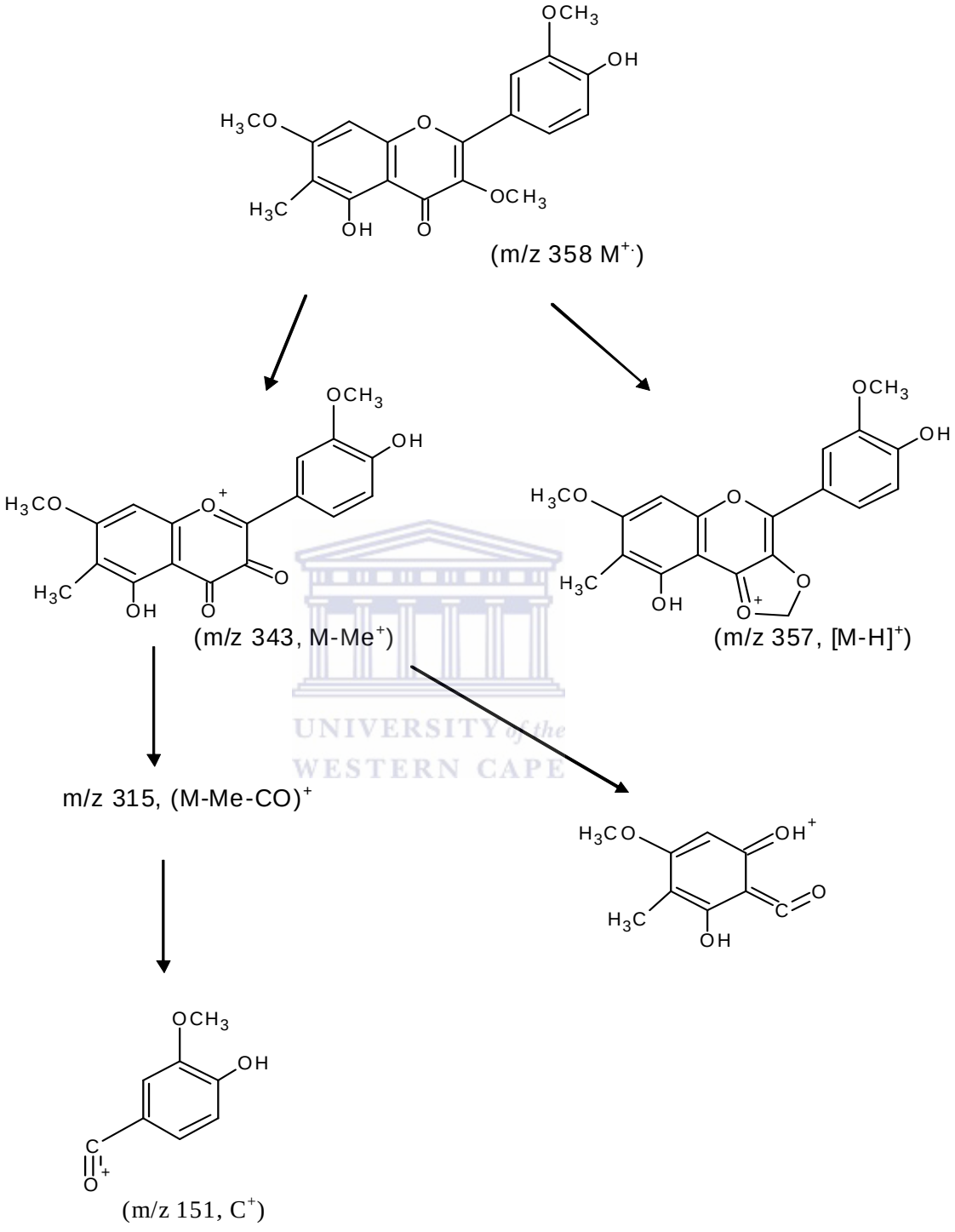


Figure 16: Mass spectral fragmentation pattern for compound P7

4.3.7 Compound P8 (6-C-methylquercetin 3-methyl ether)

Compound **P8** exhibited a $[MH]^+$ peak in the CI mass spectrum at m/z 331, corresponding to a molecular formula $C_{17}H_{14}O_7$. The UV spectrum was typical of a 3-methoxyflavonol with free 7-OH and 4 '-OH groups (Bands I and II at 359.5 and 260.5, MeOH + NaOH: + 50nm; MeOH+ NaCAc: +17.5nm).

The ^{1}H NMR spectrum of P8 displayed an aryl methyl singlet at δ 2.17 and a methoxyl singlet at δ 3.92. The aromatic region included a singlet at δ 6.66 as well as the signals of a C ring ABC substitution pattern viz., δ 7.10 (1H, d, J = 8.6 Hz); 7.64 (1H, dd, J = 2.3 and 8.6 Hz) and 7.76 (1H, d, J = 2.3 Hz). The downfield signal at δ13.10 was assigned to the strongly H-bonded 5-OH, while the signal at δ 10.23 was assigned to 4'-OH. Together with the UV data **P8** has been assigned as a 3-methoxyflavonol with a 3', 4'-disubstituted ring C, and a penta-substituted ring A bearing the free 5-OH and 7-OH groups.

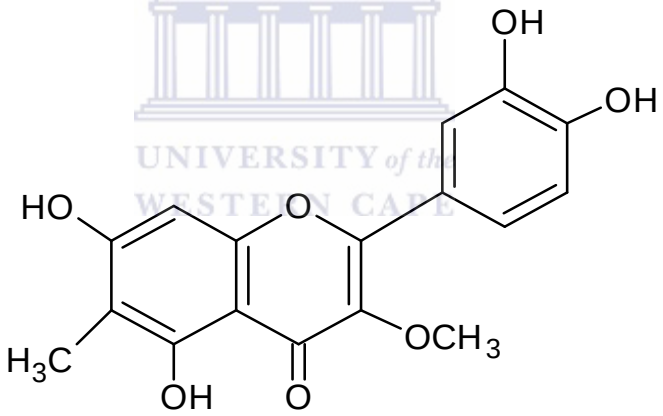


Figure 17 – Structure of compound P8

The ^{13}C NMR spectrum showed the expected 17 resonance lines (Table 38), and was nearly similar to that of **P2**. Since the UV spectrum of **P8** indicated the presence of a 3',4'- dihydroxy aryl substituent supported by the ^{13}C chemical shifts of C-2' and C-5' (Roitman and James, 1985), a free 7-OH and a 3-methoxy group, in our view the aryl methyl group (δ 2.17), could only be assigned at either C-6 or C-8. Confirmation that its position was at C-6 was derived from the HMBC spectrum (Table 45) which showed correlations between 5-OH/C-5, C-6, C-4a, Me-6/C-5, C-6, C-7 and H-8/C-6,

C-7, C-8a, C-4a. The location of the methoxyl group at C-3 (δ 137.60) was confirmed by the cross-peak OMe-3/C-3.

TABLE 45: The NMR spectroscopic data for compound P8

^{13}C Signal (δ)	**HMBC**	**^{1}H (δ, J Hz)**	**^{13}C Assignment**
155.3	H-2', 6'		2
137.6	3-OMe		3
177.7			4
158.1	5-OH, Me-6		5
106.4	5-OH, Me-6, H-8		6
162.2	Me-6, H-8		7
92.6		6.66 (1H, s)	8
153.9	H-8		8a
103.7	5-OH, H-8		4a
120.8	H- 5'		1'
115.2	H-6'	7.76 (1H, d, 2.3)	2'
145.2	H-2', H-5'		3'
148.6	H-2', H-5', H-6'		4'
115.7		7.10 (1H, d, 8.6)	5'
120.5	H-2'	7.64 (1H, dd, 2.3 and 8.6)	6'
7.3		2.17 (3H, s)	6-Me
59.6		3.92 (3H, s)	3-OMe

The structure of Compound **P8** was therefore established as 3-methoxy-6-C-methyl-5, 7, 3', 4'-tetrahydroxyflavone (6-C-methylquercetin 3-methyl ether) (Figure 17). It had previously been isolated from *Vellozia phalocarpa* (Velloziaceae) by Harborne *et al.*, (1994), and the UV and mass spectral data are in close agreement with those reported therein. The complete proton and carbon-13 NMR spectral assignments (Table 45) are reported here for the first time for this plant.

4.3.8 Compound P9 (6, 8-di-C-methylkaempferol 3-methyl ether)

Compound **P9**, a yellow waxy amorphous powder and melting at 250 – 253^{0}C, gave a $[MH]^+$ peak at *m/z* 329 in the mass spectrum, consistent with a molecular formula $C_{18}H_{16}O_6$. The Band I and Band II absorbances at 275.5 and 334.5 nm respectively indicated a 3-O-substituted flavonol, while the large bathochromic shift in the presence of NaOH (+76.5nm, Band I) and the appearance of a new band at 332.0 nm indicated the presence of a free 7-OH and 4'-OH group.

Compound **P9** had a very simple proton NMR spectrum, with two aryl methyl singlets at δ2.15 and 2.35, a methoxyl singlet at δ3.88, and AA'BB' doublets at δ7.10 and 8.05 (J = 9Hz), as well as a chelated 5-OH signal at δ13.00. This, in conjunction with the UV data, indicated that **P9** possessed a 4'-OH mono-substituted ring C as well as a fully substituted ring A with a 7-OH and 5-OH group. The methoxyl and two methyl groups could therefore only be at the three remaining free positions viz., C-3, C-6 and C-8. The UV spectrum was consistent with that of a 3-methoxyflavonol, thus implying that the two methyl groups were at C-6 and C-8. This was confirmed by the HMBC spectrum (Table 46) which clearly demonstrated the expected correlations: 5-OH/C-5, C-6, C-4a; OMe-3/C-3; Me-6/C-5, C-6, C-7 and Me-8/C-7, C-8, C-8a. Compound **P9** was therefore assigned as 3-methoxy-6,8-di-C-methyl-5,7,4'-trihydroxyflavone (6,8-di-C-methylkaempferol-3-methyl ether) (Figure 18). Previously compound **P9** had been isolated and reported (Voirin, 1983) and our UV

data are in agreement with those published therein. The ^{1}H and ^{13}C NMR spectra which are completely assigned in Table 46 are reported here for the first time.

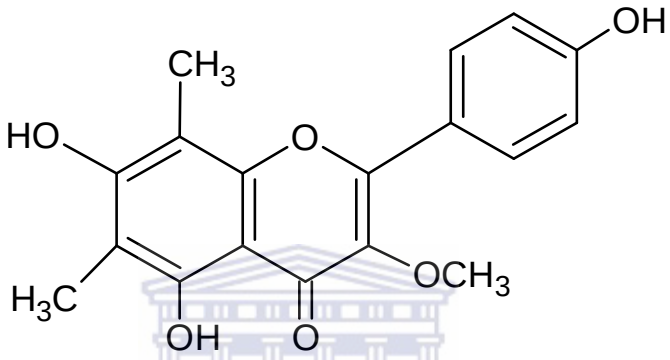


Figure 18 – Structure of compound P9

TABLE 46: NMR spectroscopic data for compound P9

^{13}C Signal (δ)	HMBC	^{1}H (δ, J Hz)	^{13}C Assignment
155.1	H-2', 6'		2
137.4	3-OMe		3
178.0			4
155.6	5-OH, Me-6		5
106.7	5-OH, Me-6		6
160.0	Me-6, Me-8		7
101.6	Me-8		8
151.5	Me-8		8a
104.0	5-OH		4a
120.9	H-3', 5'		1'
129.9	H-2', 6'	8.05 (2H, d, 9)	2', 6'
115.7	H-3', 5'	7.10 (2H, d, 9)	3', 5'
159.8	H-2', 6'; H-3', 5'		4'
8.0		2.15 (3H, s)	6-Me
8.2		2.35 (3H, s)	8-Me
59.6		3.88 (3H, s)	3-OMe

4.3.9 Compound P13 (Quercetin)

Compound **P13** showed IR absorbtion bands at 1672 cm^{-1} (C=O), and 3400 cm^{-1} (OH). It also exhibited UV Bands I and II at 374.0 and 256.0 nm respectively, characteristic of flavonols (Wollenweber, 1982). There were also large bathochromic shifts in the presence of $AlCl_3$ (+62 nm), NaOAc (+13.5 nm) and NaOH (+51 nm), with a new band appearing at 322 nm in the MeOH + NaOH spectrum. These UV data are consistent with those of a flavonol possessing free 3, 7, 3' and 4' hydroxyl groups (Wollenweber, 1982; Voirin, 1983).

The ^{1}H NMR spectrum of compound **P13** exhibited only aromatic signals, indicating the absence of aliphatic substituents on the flavonoid nucleus. The spectrum exhibited the characteristic 5-OH signal at δ 13.36, as well as the *m*-coupled doublets of an AB system at δ 6.74 and 6.78 (J = 1.98 Hz) consistent with a 5,7-disubstituted ring A. An ABC system corresponding to a 3', 4'-disubstituted ring C was also evident from the three aromatic signals at δ 7.40 (1H, d, J = 8.25 Hz), 8.14 (1H, dd, J = 2.31 and 8.25 Hz) and 8.64 (1H, d, J = 2.31 Hz). The ^{13}C NMR spectrum of **P13** also exhibited only aromatic signals, with the typical C-3 signal of a flavonol at δ 137.9. In the spectrum (Table 38), the signals for C-2' and C-5' were coincident at δ 116.6, pointing to these two carbons being *ortho* to hydroxyl groups (Roitman and James, 1985). These observations, in conjunction with the UV data and the proton spectrum, indicated that **P13** was a flavonol with free 3, 5, 7, 3', 4'-pentahydroxy groups i.e. quercetin (Figure 19). This was confirmed by co-TLC with authentic quercetin (Sigma) and by comparison of its ^{13}C NMR spectrum with the published spectrum of quercetin (Markham and Ternai, 1976; Markham and Chari, 1982).

Quercetin is a flavonoid which is widely distributed in the plant kingdom, occurring as the free aglycone or more commonly as glycosides (Wollenweber and Dietz, 1981). It had previously been isolated from *Piliostigma thonningii* (Bomardelli *et al*., 1973), a report which has now been reconfirmed in this plant *P. reticulatum*.

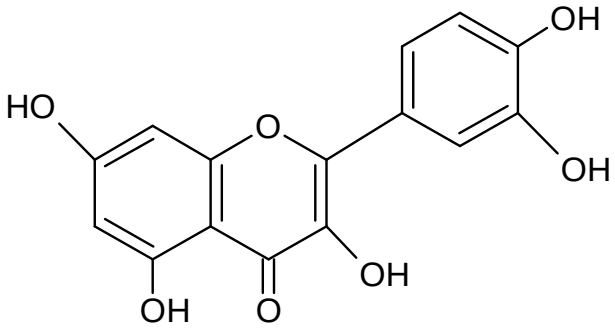


Fig. 19 – Structure of compound P13

In conclusion the products **P3, P7**, **P8, P9** and **P13** are compounds that were very similar to those already reported in literatures and can be found in the references (Rasamoelisendra et al., 1989; Rabesa and Voirin, 1985; Harborne et al., 1994; Voirin, 1983 and Bombardelli et al., 1973) respectively. The UV and IR data were in agreement with that of the literature, as well as the ^{1}H NMR data for the compounds after critical comparison. The ^{13}C NMR and HMBC spectra completely supported the structures of these C- methylflavonols in the same way it supported the new isolates.

Thus 4 new flavonoids **P1, P2, P4** and **P5** have been isolated and fully characterized from *Piliostigma reticulatum* while a further 5 known flavonoids, **P3, P7, P8, P9** and **P13** were isolated and are reported for first time to be present in the extracts of this plant to the best of our knowledge.

4.4. BIOLOGICAL EVALUATION

4.4.1 Brine shrimp lethality bioassay

High doses of bioactive compounds have on occasion been found to be toxic and hence the pharmacology of bioactive compounds can be preliminarily tested or detected from their toxicology results. Thus the *in vivo* lethality in a simple zoologic organism, the brine shrimp larvae (nauplii), has been used as a convenient monitor for the screening of bioactive natural products (Meyer et al., 1982).

The brine shrimp nauplii have been used for a number of bioassay systems in which natural product extracts, fractions or pure compounds are tested at three concentrations viz., 10, 100 and 1000ppm or μg/ml in vials with ten nauplii in

triplicate for each concentration. The number of survivors counted after 24hours were used in estimating the LC_{50} with a 95% confidence using the Finney probit analysis computer programme. The brine shrimp toxicity bioassay was conducted on ten samples, namely, the three extracts hexane**,** ethyl acetate and methanol and 7 isolates **P1, P2, P4, P5, P7, P8** and **P9**. The results (Table 47) showed that all the extracts and nearly all the isolates were active (AC) (LC_{50} < 1000 µg/ml), except **P5** and **P7** which were inactive (IA). Compound **P2** (LC_{50} = 0.43) showed the highest activity (very active, VA) being active at all the concentration levels tested. Since the lethality of a test substance to brine shrimp nauplii has been linked to the possible ability of such substances to kill cancer cells (antitumor activity), as well as pesticidal and antibacterial activities (Meyer et al., 1982). It may be deduced that all the VA and AC samples should be good candidates for such applications. Some of the compounds merely slow down the activity of the nauplii as shown in the hexane extract and **P1** (Table 48) in which such compounds are usually suspected to have an adverse effect on the Central Nervous System (CNS) (Meyer et al., 1982; Aderogba et al., 2006).

Table 47: LC_{50} and average dead nauplii after 24hours for *P. reticulatum*

The results were classified based on the regressional finney probit analysis programme of the LC_{50} as Very active **(VA)**, Active **(AC)** and Inactive **(IA)** at the three test concentrations.

Test material	1000 µg/ml	100 µg/ml	10 µg/ml	LC_{50}	Remarks
Hex	9	7	3	33.39	AC
EtOAc	10	8	6	*	AC
MeOH	10	10	7	3.38	VA
P1	9	7	4	23.15	AC
P2	10	10	9	0.44	VA
P4	10	8	6	6.12	AC
P5	5	3	1	989.29	IA
P7	4	1	0	1756.39	IA
P8	10	8	7	2.41	VA
P9	10	10	6	6.25	AC

* Data did not converge and therefore could not be regressed by the finnery probit analysis programme.

Table 48: Average number of survived nauplii after 24hours for *P. reticulatum*

Test mat	Vial at 1000µg/ml	% at 1000µg/ml	vial at 100µg/ml	% at 100µg/ml	vial at 10µg/ml	% at 10 µg/ml	vial for control expt %
Hex	0.67	6.67	3.33	33.33	7.0	70.0	100
EtOAc	0.33	3.33	2.33	23.33	3.67	36.67	100
MeOH	0	0	0.33	3.33	2.67	26.67	100
P1	0.67	6.67	3.33	33.33	6.0	60.0	100
P2	0	0	0	0	1.33	13.33	100
P4	0	0	1.67	16.67	4.33	43.33	100
P5	5	50.0	7	70.0	8.67	86.67	100
P7	6.0	60.0	8.67	86.67	10.0	100	100
P8	0.33	3.33	1.67	16.67	3.33	33.33	100
P9	0	0	0.33	3.33	4.0	40.0	100

Hex: hexane extract, **EtOAc**: ethyl acetate extract, **MeOH**: methanol extract.

4.4.2 Antimicrobial screening

Antimicrobial analysis was carried out by using microdilution titre techniques as shown in section 3.10.3.2 (Drummond and Waigh, 2000; Rahman and Gray, 2005) in which 96 well plates were used for the analysis with two Gram-positive bacteria (*Staphylococcus aureus* NCTC 6571 and *Bacillus subtilis* NCTC 8236) and two Gram-negative bacteria (*Escherchia coli* NCTC 10418 and *Proteus vulgaris* NCTC 4175). The two fungi used as microorganism for this analysis were *Aspergillus niger* ATCC 10578 and *Candida albicans* ATCC 10231.

MIC were calculated using the excel spread sheet. The results of the antimicrobial activities were considered for compounds **P1 – P9** and the 3 extracts as shown in Table 49. Generally all the extracts and isolates showed some level of activity except compounds **P5** and **P7**. It was also evident from the table that the trend observed resemble that reported for the cytotoxicity tests, where Piliostigmol **P2** showed the highest activity against *E. coli* (MIC = 2.57 μg/ml ; 0.006 μmol) which interestingly enough 3 times more active than Amoxicillin whereas **P5** and **P7** showed the least activity in which no activity was observed for **P7** against Gram negative bacteria and the fungus *A. niger*.

In molar concentration, the order of activity against the Gram-positive bacteria, *S. aureus* was **P2** > **P4** > **P8** >**P 9** >**P1** >**P5** >**P7** while *B. subtilis* gave the order P4 > **P8** >**P 1** >**P5** which followed the same trend except that no activity was observed for **P7** in this case**.** The sequence of relative potencies against the Gram-negative *E. coli* was: **P2 > P1 > P9 > P4 > P8> P5 > P7** and that for *P.vulgaris* was: **P2 > P8 > P4 > P9 > P1> P5 > P7.** The activity order against the fungi *C. albicans* was: **P2**> **P9** > **P4** > **P1** > **P8** > **P5** > **P7** while that for A. *niger* was: **P1 > P9 > P2 > P4> P8 > P7**.

Generally, flavonoids and oxychromones are known to exhibit a wide range of activities including anti-inflammatory, antithrombotic, antiviral and hepatoprotection which may, in some measure, we and others believe to be due to their ability to scavenge free-radicals (Aderogba et al., 2006; Akdemir et al., 2001; Saija et al., 1995). Indeed some specific flavonoids were reported to be potent inhibitors of indole-3-acetic acid oxidase activity (Ferrer et al., 1992) while a selection of quercetins have also been reported to have strong lipid peroxidation inhibitory effects and cytotoxicity (Cos et al., 2001) when tested against oral microorganisms.

Thus, the present findings on the antimicrobial activities of the C-methylflavonols and oxychromonol isolated from *P. reticulatum* (Babajide et. al., 2008; Ibewuike et al., 1997) have further strengthened the previous and present findings about the effectiveness of certain flavonoids and isoflavanoids as vectors against microbial infections and thus makes a degree of sense regarding the folk medicinal uses of this plant for the treatment of viral infections, oral sores and inflammations (Iinuma et al., 1994; Kirtikar et al., 1993; Yusuf et al., 1994; Liu et al., 1990; Babajide, 2008; Shai et al., 2008; Mukhlesur et al., 2007; Ibewuike et al., 1997).

Table 49: Minimum inhibitory concentrations (MIC) of the constituents of *Piliostigma reticulatum*

Compounds	*S. aureus*		*B. subtilis*		*E. coli*		*P. vulgaris*		*A. niger*		*C. albicans*	
	µg/ml	µmol	µg/ml	µmol	µg/ml	µmol	µg/ml	µmol	µg/ml	µmol	µg/ml	µmol
Hex	06.50	0.018	12.50	0.032	25.00	0.083	10.50	0.031	25.00	0.081	12.50	0.027
EtOAc	12.50	0.035	25.00	0.102	50.00	0.203	12.50	0.051	25.00	0.102	50.00	0.203
MeOH	12.50	0.025	20.00	0.067	25.00	0.074	12.50	0.035	25.00	0.072	10.50	0.038
P1	10.50	0.037	20.00	0.058	12.50	0.033	20.00	0.076	12.50	0.028	25.00	0.102
P2	03.25	0.008	NoT	NoT	02.57	0.006	06.50	0.018	20.00	0.063	10.50	0.033
P4	06.50	0.015	10.50	0.034	25.00	0.075	12.50	0.038	25.00	0.075	20.00	0.058
P5	50.00	0.156	25.00	0.075	200.0	0.443	100.0	0.321	NoT	NoT	200.0	0.413
P7	300.0	0.479	-	-	-	-	-	-	-	-	300.0	0.483
P8	10.50	0.034	12.50	0.037	25.00	0.076	12.50	0.033	25.00	0.088	50.00	0.161
P9	12.50	0.036	NoT	NoT	12.50	0.035	25.00	0.072	20.00	0.061	12.50	0.037
Am	03.25	0.008	06.50	0.018	10.50	0.032	06.50	0.016	–	–	–	–
Flu	–	–	–	–	–	–	–	–	–	–	20.00	0.062

Hex: hexane extract , **EtOAc**: ethyl acetate extract, **MeOH**: methanol extract
P1 – **P9** are the isolated compounds **1** – **9**
Am: Amoxicillin standard, **Flu**: Fluconazole, NoT: Not Tested

Test Organisms are:-
Gram-positive bacteria: *Staphylococcus aureus (S. aureus)* and *Bacillus subtilis (B. subtilis)*
Gram-negative bacteria: *Escherichia coli (E. coli) and Proteus vulgaris (P. vulgaris)*
Fungi : *Aspergillus niger (A. niger) and Candida albicans (C. albicans)*

4.5 CHEMICAL EVALUATION

4.5.1 The antioxidant assay

Total antioxidant evaluation was carried out on the *P. reticulatum* extracts and isolates by first evaluating the antioxidant content present in the extracts i.e (polyphenols, flavonols, flavanols, anthocyanins and flavanones). This was followed by evaluating antioxidant capacity in terms of ORAC (Oxygen radical absorption capacity), FRAP (Ferric reducing antioxidant power) and TEAC (Trolox equivalent antioxidant capacity)

4.5.2 Polyphenolic content of P. reticulatum

The presence and amount of polyphenols in the extracts was determined by using gallic acid as a standard because polyphenols are generally a diverse class of compounds produced by plants that are characterized by the presence of more than one phenol ring in which gallic acid is known to be one of them. The class is subdivided into acetophenones, benzofurans, chromones, coumarins, flavonoids, phenolic acids, phenylacetic acids, phenylpropanoids, quinones, stilbenes and xanthones. Polyphenols found in vegetables, fruits, and teas are known to prevent degenerative diseases including cancers through antioxidative action and the modulation of several protein functions. The number of natural polyphenols has been estimated to number over one million; because they generally occur as glycosides which show great variety. However, the bioactivity is attributed mainly to the aglycon moieties and not the sugar moieties. The antioxidative potency is mainly ascribed to the catechin molecule of the aglycons and these can be classified into polycyclic types such as flavonoids, anthraquinones and others simple polyphenols. The amount of polyphenols present was evaluated as shown in 3.11.2 using the Folin Ciocalteu reagent with gallic acid as the standard. The results illustrated that the quantity of polyphenols present in the extracts varied as shown in Figure 20A in which there is appreciable amount of polyphenols in all the three extracts with the highest value recorded for ethyl acetate while the lowest was recorded for hexane extract. This means the ethyl acetate and methanol extracts are better sources of polyphenolics antioxidant.

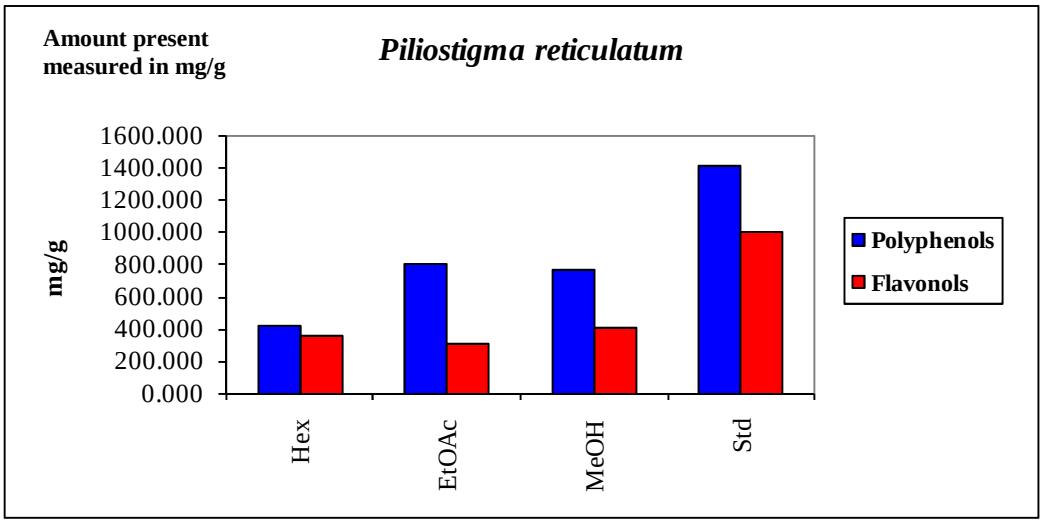


Figure 20A – Bar chart illustrating and comparing the amount of polyphenols and flavonols present in the various extracts of *P. reticulatum.*

4.5.3 Flavonoid content

A similar trend for the distribution of flavonoids consisting of flavonols, flavanols and flavanones was observed. Flavonoids are found in most dietary intakes because of their potential beneficial cardiovascular effects and also for their prophylactic effects against hypertension. A wide variety of dietary flavonoid sources includes apples, cranberries, purple grapes, red wine, teas, cocoas and chocolates. Evaluation of flavonol content was made using quercetin as the standard as shown in 3.11.3 while flavanols make use of catechin as listed in 3.11.4 and flavanone makes use of Naringenin as standard (3.11.6).

The results are illustrated in Figures 20A, 20B and 20C Graphs where it was evident that there was the presence of flavonols and some flavanol but that flavanones were found in either trace amounts or not at all when compared with the standard. This finding confirmed the present project viz., that no flavanone was detected nor isolated from the plant extracts.

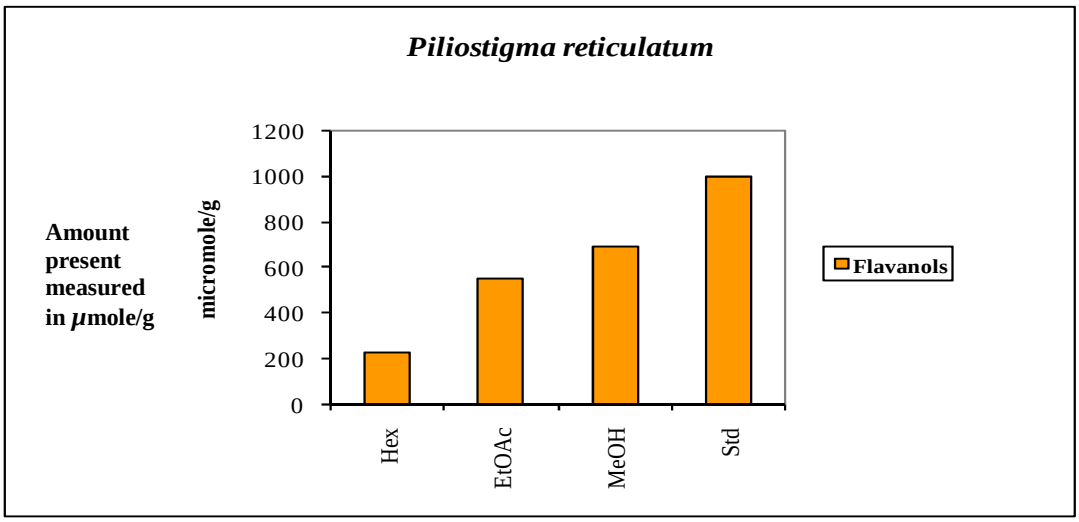


Figure 20B – Bar chart showing the amount of flavanols present in the various extracts of *P. reticulatum.*

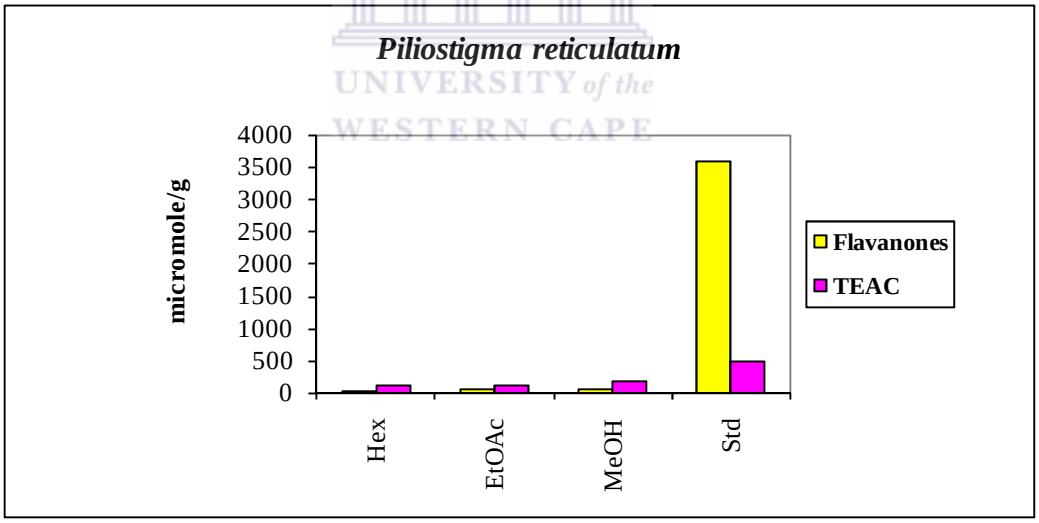


Figure 20C – Bar chart illustrating and comparing the amount of flavanones and the Trolox equivalent antioxidant capacity present in the various extracts of *P. reticulatum.*

4.5.4 Anthocyanin content

The anthocyanin content was evaluated by measuring the amount of pigments present. These pigments are known to be responsible for the attractive red to purple to blue colours of many fruits and vegetables. The pigments are now known to play an important role in the health sector because of their nutraceutical health benefits. They play a vital role in the reduction of coronary heart disease and increased visual acuity, and also have antioxidant and anticancer properties as explained in 1.6.4.

The total anthocyanin content in the plant extracts were determined by measuring absorptivity of the solution at a wavelength between 490 to 550 nm by differential measurement of the absorbance at two different pH values as shown in 3.11.5.

The result obtained is illustrated in Figure 20D in which it is evident that anthocyanins were absent in the plant except for the traces observed in the EtOAc extract.

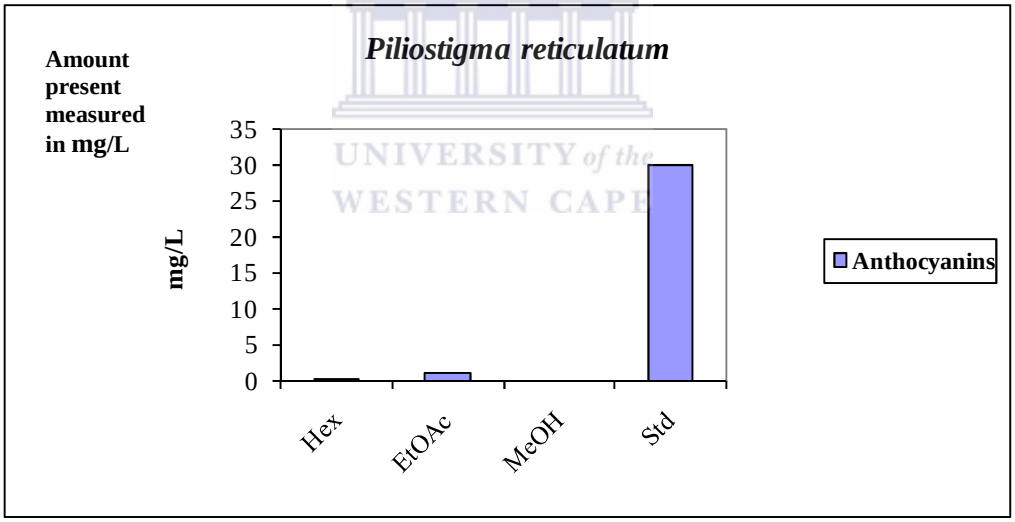


Figure 20D – Bar chart showing the amount of anthocyanins present in the various extracts of *P. reticulatum.*

4.5.5 The Oxygen radical absorbance capacity assay (ORAC)

The ORAC is another method for quantification of the amount of antioxidant in a material. This fluorescence-based method utilizes the fact that the fluorescence of

phycoerythrin (PE) changes with respect to time upon damage caused by peroxyl or hydroxyl radical attack (Prior et al., 1995; 2001). The ORAC method is a simple, sensitive, and reliable way to measure the peroxyl radical absorbing capacity (with AAPH) of antioxidants and has successfully been performed using the ORAC method with H_2O_2-Cu^{2+}. The ORAC method was performed using a fluorescence spectrophotometer until zero fluorescence occurs. The results are reported as the ORAC value, which refers to the net protection area under the quenching curve of β-PE (fluorescein) in the presence of an antioxidant. The ORAC value was calculated by dividing the area under the sample curve by the area under the Trolox curve with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as being the net protection area provided by 1 μM Trolox in final concentration. When the area under the curve for the sample is compared to the area under the curve for Trolox, the result is given in Trolox equivalents as shown in 3.11.7. The results showed that *P. reticulatum* has high antioxidant values and this can be attributed to the high flavonoid content in them as shown in Figure 20E where **P2** recorded the highest value and **P5** and **P8** having the lowest antioxidant properties. The implication is that in formulating a good antioxidant agent, the methanolic extract and Piliostigmol (**P2**) are good candidates to choose from.

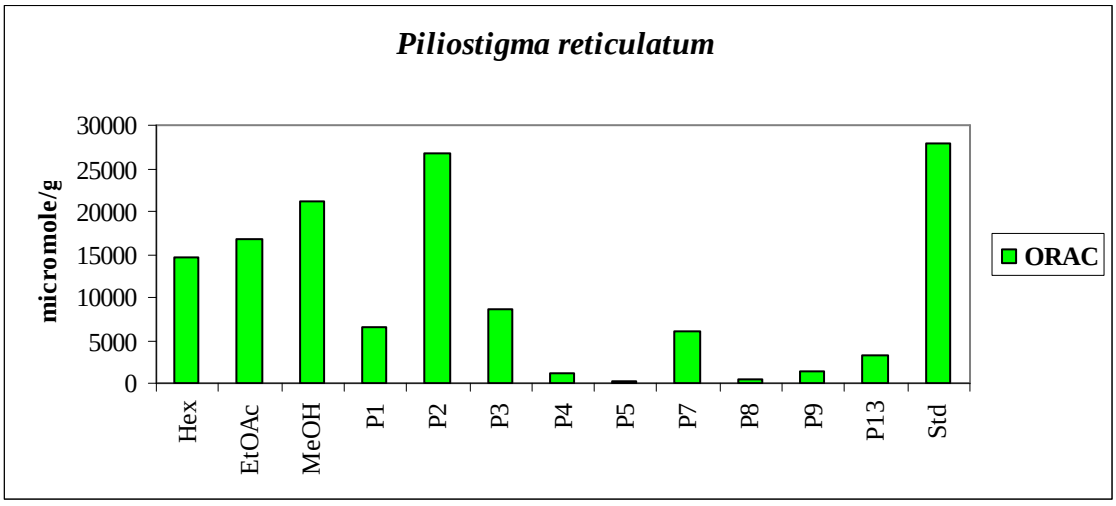


Figure 20E – Bar chart showing the ORAC values (The antioxidant capacity) of some extracts and isolates present in *P. reticulatum.*

4.5.6 The Ferric reducing antioxidant power (FRAP) assay

The FRAP assay uses an oxidation/reduction potential to measure the ability of a sample to reduce Fe^{III} to Fe^{II}. Antioxidants are known to donate electrons in the same manner as a reductant in an oxidation/ reduction reaction does and thus it may be assumed that the FRAP assay is a good method for evaluating antioxidant capacity. However, it does not directly measure the antioxidant capacity of a potential antioxidant. In addition, since there are no free radicals introduced into the system, there is no way of comparing the antioxidant capacity towards different kinds of radicals. The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method by employing an easily reduced oxidant present in stoichiometric excess. At low pH, reduction of a ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous form, which has an intense blue colour, was monitored by measuring the change in absorption at 593nm with time as explained in 3.11.8. The change in absorbance was directly related to the reducing power of the electron donating antioxidants present in the reaction mixture.

It should be noted that the FRAP assay cannot measure the antioxidant capacity of certain antioxidants accurately such as ascorbic acid, which can react with Fe^{II} and SH group-containing antioxidants (glutathione) and also the FRAP assay does not take into account the quantity of inhibition and thus leaves out an important component of the total antioxidant capacity but it easy to generate the capacitating power of the antioxidant factor in the sample through FRAP than ORAC. The result of this investigation is as shown in Figure 20F where **P5** and **P8** recorded very low activity. The FRAP assay at a glance reconfirmed the consistency in the antioxidant capacity of the extracts and isolates as observed in ORAC in which the same high values were recorded for both the methanolic extract and **P2** whereas **P5** and **P8** still show the same trend of low antioxidant power.

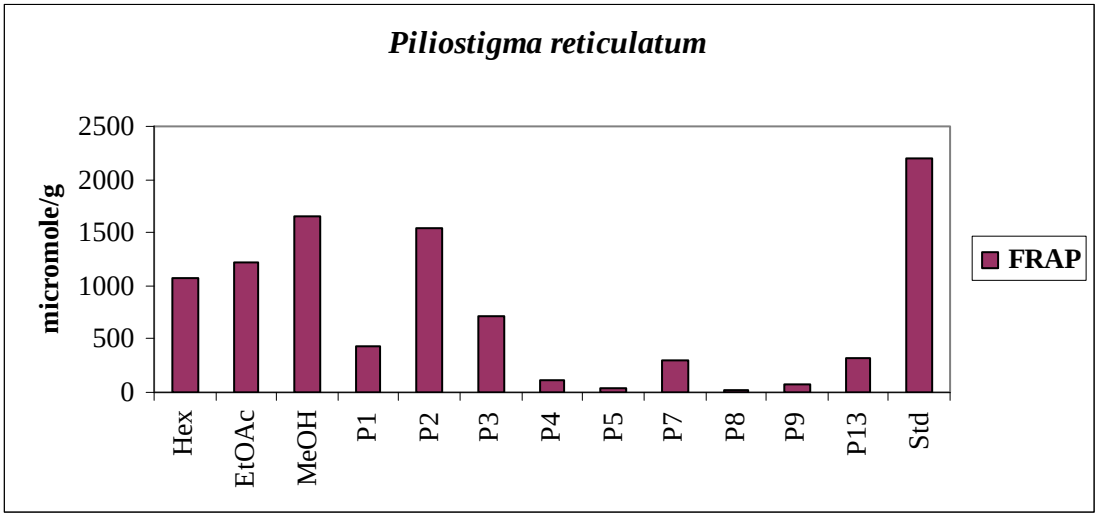


Figure 20F – Bar chart showing the FRAP values (Ferric reducing antioxidant power) of some extracts and isolates present in *P. reticulatum.*

4.5.7 The Abts Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was carried out using the ABTS (2,2'-azino-di-3-ethylbenzthialozine sulphonate) radical cation scavenging power which has the ability to determine the total antioxidant status (TAS) of a sample. The radical cation was monitored by measurement of its characteristic absorption maxima at 640, 734 and 820nm. Antioxidants added to the system can either scavenge the $ABTS^{\bullet+}$ formed or interfere in the radical generating process. Measurement of the absorbance at a specific time after addition of the sample enables the calculation of a percentage scavenging power (3.11.9). The results as illustrated in Figure 20C demonstrate a fair scavenging power and similar profile as observed in ORAC.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 *CISSAMPELOS CAPENSIS*

The aerial shoot (3.5kg) and roots (2.6kg) of *C. capensis* were used in this project. The extractions and isolations were specifically targeted towards the alkaloids and flavonoids present in the plant as shown by the preliminary phytochemical screening results below. The total tertiary alkaloid (TTA) was extracted from the aerial shoot (500g) of the plant. This afforded TTA (1.85g, 0.37%). The residual aqueous fraction generated a BTA (48.7g, 9.74%) after concentration as shown in section 3.7.1.0.

5.1.2 Sequential extraction of the aerial shoot and root

In a further isolation, 1kg of the aerial shoot was sequentially extracted with hexane, dichloromethane, ethyl acetate, methanol and water. This afforded the hexane (17.35g, 1.74%), dichloromethane (26.33g 2.63%), ethyl acetate (32.84g, 3.28%), methanol (56.24g, 5.62%) and water extracts (25.71g, 2.57%) (3.7.2.0). Again, 1kg of the root powder was sequentially extracted as described above to afford the hexane (7.38g, 0.74%), dichloromethane (12.65g, 1.27%), ethyl acetate (27.19g, 2.72%), methanol (53.05g, 5.31%) and water extracts (26.44g, 2.64%) (3.7.3.0).

All the extracts were **phytochemically screened** for detection of secondary plant metabolites using the Wagner TLC method (Wagner and Bladt, 2001). The result in Table 50 showed clearly the distribution profile of the plant metabolite in the various extracts in which all the extracts showed the presence of flavonoids and phenolics while anthraquinones and essential oils were absent except the presence of essential oils in CCAH and CCAD while flavonoid being absent in TTA. Tannins and saponin were absent in the TTA, BTA, hexane and DCM extracts while the TTA, EtOAc, MeOH and water extracts showed the presence of alkaloid as shown in Table 50.

Table 50:- Phytochemical screening result of the *C. capensis* extracts

Test materials	**Tann**	**Phen**	**Glyc**	**Sapo**	**Flav**	**Alka**	**Anth**	**Esse**
CCRH	N-ve	P+ve	N-ve	N-ve	P+ve	N-ve	N-ve	N-ve
CCRD	N-ve	P+ve	N-ve	N-ve	P+ve	N-ve	N-ve	N-ve
CCRE	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCRM	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCRW	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCAH	N-ve	P+ve	N-ve	N-ve	P+ve	N-ve	N-ve	P+ve
CCAD	N-ve	P+ve	N-ve	N-ve	P+ve	N-ve	N-ve	P+ve
CCAE	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCAM	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCAW	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
CCET	P+ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve
TTA	N-ve	P+ve	N-ve	N-ve	N-ve	P+ve	N-ve	N-ve
BTA	N-ve	P+ve	N-ve	P+ve	P+ve	P+ve	N-ve	N-ve

CCRH = *C. capensis* root hexane extract; **CCRD** = *C. capensis* root DCM extract; **CCRE** = *C. capensis* root EtOAc extract; **CCRM** = *C. capensis* root MeOH extract; **CCRW** = *C. capensis* root H_2O extract; **CCAH** = *C. capensis* aerial shoot Hexane extract; **CCAD** = *C. capensis* aerial shoot DCM extract; **CCAE** = *C. capensis* aerial shoot EtOAc extract; **CCAM** = *C. capensis* aerial shoot MeOH extract; **CCAW** = *C. capensis* aerial shoot H_2O extract

CCET = *C. capensis* aerial shoot 80% EtOH extract; **TTA** = *C. capensis* aerial shoot total tertiary alkaloid; **BTA** = *C. capensis* aerial shoot neutralized aqueous residual fraction

Tannins (**Tann**), Phenolics (**Phen**), Glycosides (**Glyc**), Saponins (**Sapo**), Flavonoids (**Flav**), Alkaloids (**Alka**), Anthraquinones (**Anth**) and Essential oils (**Esse**)

5.1.3. Isolation of the constituents

5.13.1 Isolation of constituents in TTA

Column gradient chromatography (CGC) of the TTA (1.6g) gave 7 fractions coded A – G as shown in section 3.7.1.1 and Table 12 on page 77 with their LC_{50} value in the brine shrimp lethality assay which was used in monitoring the fractionation of the plant extracts. From Table 12, fraction E shows the highest activity VA (very active) although it is not in the highest yield as used in the case of *P. reticulatum.* Three fractions A, C and G were found to be inactive (IA) as shown in Table 12 in section 3.7.1.1. Based on the activity observed in fraction E, it was further chromatographed yielding five subfractions coded TI – TV as shown in Table 13. Using similar activity guided methods as described earlier, fractions TIII and TIV were repeatedly and separately chromatographed on PTLC to afford compounds **C1** (0.058 g) and **C2** (0.021g) as shown in sections 3.7.1.1 – 3.7.1.4.

5.1.3.2 Isolation of the major constituents in the methanol extract of the aerial shoot

The CGC of the methanol extract (25.6g) gave 10 fractions coded A – J as shown in Table 14 on page 79. Using the observed cytotoxicity results in Table 14, fraction F was rechromatographed which finally yielded **C3** (0.04 g), **C4** (0.028 g), **C5** (0.026 g) and **C6** (0.024 g) as shown in section 3.7.2.1 – 3.7.2.6.

5.1.3.3 Isolation of the major constituents in the methanol extract of the root

The methanol extract (30.0g) also yielded 5 Fractions coded K – O as shown in Table 16 on page 81. Based on their observed activity pattern for the cytotoxicity test fractions M and N were rechromatographed to afford C7 (0.078 g), **C8** (0.065 g), **C9**, (0.088 g) for fraction M while fraction N afforded **C10** (0.068 g) and **C11** (0.0163 g) as shown in the experimental procedure in section 3.7.3.1 – 3.7.3.9.

Critical examination and comparisons in terms of colour, texture, R_f value and melting points of compounds **C1** – **C11** resulted in the following assessment. **C1**, **C4** and **C7** displayed similar properties; **C2** and **C8** displayed similar properties; **C6** and **C9** displayed similar properties and finally **C5** and **C10**

displayed similar properties. After combining these fractions it thus resulted in 6 compounds labeled as **C1**, **C2**, **C3**, **C5**, **C6 and C11.**

TLC investigations of these 6 compounds revealed **C1**, **C2** and **C3** to be alkaloids as demonstrated by their positive test to Dragendorff using berberine as plate marker while **C5**, **C6** and **C11** were flavonoids due to their positive test to vanillin in sulphuric acid using quercetin as a marker.

5.2 PHYSICO-CHEMICAL DATA OF ISOLATED COMPOUNDS

5.2.1 C1 (dihydromorphinandienone alkaloid)

A reddish yellow powder with m.p 85 – 87^{0}C.

TLC: Hex: $CHCl_3$: MeOH (3:10:2), **C1** R_f (Solvent): 0.58

UV λ_{max} nm (log ε): 222, 260, 304.

IR $\boldsymbol{\nu}_{max}$ cm^{-1} pronounced peaks: 3485, 2940, 1685, 1615, 1500.

EIMS: molecular ion peak $(M)^{+\cdot}$ *m/z* 375.0216 (calculated mol. formula $C_{20}H_{25}O_6N$).

The ^{1}H NMR and ^{13}C NMR see Table 51

5.2.2 C2 (9, 10 – dimethoxyaporphine alkaloid)

A reddish-brown fluffy powder with m.p 221 – 223^{0}C,

TLC in Hex: $CHCl_3$: MeOH (3:10:2), **C2** R_f (Solvent): 0.67

UV λ_{max} nm (log ε): 225, 243, 277, 300 (shoulder).

IR $\boldsymbol{\nu}_{max}$ cm^{-1} pronounced peaks: 3420, 3050, 2925, 1636, 1458, 1387, 1060.

EIMS: molecular ion peak $(M)^{+\cdot}$ *m/z* 341.0112. (calculated mol. formula $C_{19}H_{19}O_5N$).

The ^{1}H NMR and ^{13}C NMR see Table 52

5.2.3 C3 (8, 14-dihydromorphinandienone alkaloid)

A golden brown powder with m.p 77 – 80 °C.

TLC: Hex: $CHCl_3$: MeOH (3:10:2), **C3** R_f (Solvent): 0.42

UV λ_{max} nm (log ε): 226, 261,302

IR (ν cm^{-1}) pronounced peaks: 3517, 2952, 1685, 1616, and 1510.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 329.2133 (calculated frag. gave $C_{19}H_{23}O_4N$).

The ^1H NMR and ^{13}C NMR see Table 53

5.2.4 C5 (6 -C-methylquercetin 3, 3',7, 8 -tetramethyl ether)

A Yellow amorphous powder with m.p. 234 – 236°C.

R_f (Solvent): 0.28 (A), 0.60 (B)

UV λ_{max} nm (log ε): 255 (4.64), 357 (4.62); +NaOAc: 271, 365; +NaOH: 276, 343, 410. +$AlCl_3$: 279, 440.

IR (ν cm^{-1}) pronounced peaks: 3401, 2932, 1653, 1612, 1556

EIMS: molecular ion peak $(M)^{+\cdot}$ *m/z* 388.0103. (calculated mol. formula $C_{20}H_{20}O_8$).

^1H NMR: δ 2.14 (3H, s, Me-6), 3.74 (3H, s, OMe-3), 3.96 (3H, s, OMe-3'), 4.01 (3H, s, OMe-7); 4.56 (3H, s, OMe-8), 6.91 (1H, d, J= 8.5 Hz, H-5'), 7.60 (1H, *dd*, J = 2.3, 8.4 Hz, H-6'), 7.70 (1H, d, J= 2.3 Hz, H-2'), 10.90 (1H, s, 4'-OH), 12.92 (1H, s, 5-OH). ^{13}C NMR: see Table 54

5.2.5 C6 (methylenedioxyisoflavanone)

A yellow waxy powder with m.p. 185 – 187^0C

TLC Hex : EtOAc : MeOH (2:7:1) **C6** R_f (Solvent) 0.48

UV $\boldsymbol{\lambda}_{max}$ nm (log ε): 214(4.10), 218 (4.12), 288 (4.00), 333 (sh) (3.25).

IR $\boldsymbol{\nu}_{max}$ cm^{-1} pronounced peaks at : 3421, 2914, 1634, 1472, 1386, 1358, 1257, 1224, 1161, 1100, 1068, 925;

EIMS : molecular ion peak $(M)^{+\cdot}$ *m/z* 378.0625 (calcd for $C_{18}H_{18}O_9$, 378.0623).

^1H NMR, ^{13}C NMR and HMBC are as shown in Tables 55 and 56 respectively.

5.2.6 C11 (6-C-methylquercetin 3-methyl ether)

A creamy yellow powder with m.p. 217 – 219 °C.

R_f (Solvent): 0.63 (A), 0.40 (B)

UV λ_{max} nm (log ε): 260 (4.62), 351 (4.60); +NaOAc: 260, 355; +NaOH: 270, 405; +A1$C1_3$: 277, 435.

IR (ν cm^{-1}) pronounced peaks: 3500 – 3400, 1652, 1613, 1568, 1556

EIMS: molecular ion peak $(M)^{+\cdot}$ *m/z* 346.0752. (calculated mol. formula $C_{17}H_{14}O_8$).

^{1}H NMR: δ 2.11 (3H, s, Me-6), 3.87 (3H, s, OMe-3), 7.03 (1H, d, J=8.6Hz, H-5'), 7.62 (1H, dd, J= 2.3, 8.6 Hz, H-6'), 7.75 (1H, d, J= 2.3Hz, H-2'), 10.23 (1H, s, 4'-OH), 11.15 (1H, s, 3'-OH).12.05 (1H, s, 8-OH), 12.90 (1H, s, 7-OH), 13.05 (1H, s, 5-OH).

^{13}C NMR: see Table 57.

5.3 STRUCTURAL ELUCIDATION OF COMPOUNDS C1 – C11

5.3.1 C1 (dihydromorphinandienone alkaloid)

Compound **C1,** a combination of **C1**, **C4** and **C7,** is a reddish yellow powder with average melting point of 86^0C. The UV spectrum has a λ_{max} values at 222 nm which is known to be associated with an α,β -unsaturated carbonyl chromophore (Stuart, K.L., 1971) and that at 260 nm should be for a typical aromatic ring system. The shoulder observed at 304 nm is a general characteristic of 8,14-dihydromorphinandienone alkaloids (Freitas et al., 1995). The IR spectrum showed pronounced bands at 3485 cm^{-1} (hydroxyl group), 2940 (C-H of an olefinic group), 1685 cm^{-1} (α, β -unsaturated carbonyl), 1615 cm^{-1} (C=C olefinic) and 1500 cm^{-1} (C=C aromatic). The El mass spectrum indicated the molecular formula to be $C_{20}H_{25}NO_6$. From the base peak of M^+ [375], the following fragments were observed: 360 $[M- CH_3]^+$, 332 $[360 - CO]^+$, 238 $[M – C_8H_90_2]^+$ and 42 $[H_2C=C=O]^+$, being characteristic of the morphinandienone alkaloids (Wheeler et. al 1967). The complete analysis of ^{1}H NMR, ^{13}C NMR, HMBC, COSY and NOESY were used in assigning the structure of **C1**. The ^{1}H NMR (400 MHz, pyridine- d_5) showed only one aromatic signal at δ6.91 which implies that the aromatic ring is penta-substituted. The four singlets which integrated for three protons each were observed at δ 2.26 for (N-Me), with the other three at δ3.69 for MeO-6, 3.76 for MeO-3 and 3.83 for MeO-1. The two single proton signals at 10.75 and 11.50 were attributable to HO-4 and HO-5 respectively.

The ^{13}CNMR (100MHz, pyridine-d_5) similarly showed a signal at δ194.7 (s, C-7) for the α,β-unsaturated carbonyl, δ43.1 (s, N-Me), δ55.3 (s, C-6-OMe), δ56.5 (s, C-3-OMe) and δ57.2 (s, C-1-OMe). The complete assignments of all the protons and carbons are given in Table 51. This was compared with literature and found to be correct (Blasko and Cordell 1988, and Vecchietti et al., 1981). The spectroscopic analysis above confirmed compound **C1** to be 5,6-dehydro-4,5-dihydroxy-1,3,6-trimethoxy- 17-methylmorphinan-7-one (Figure 21).

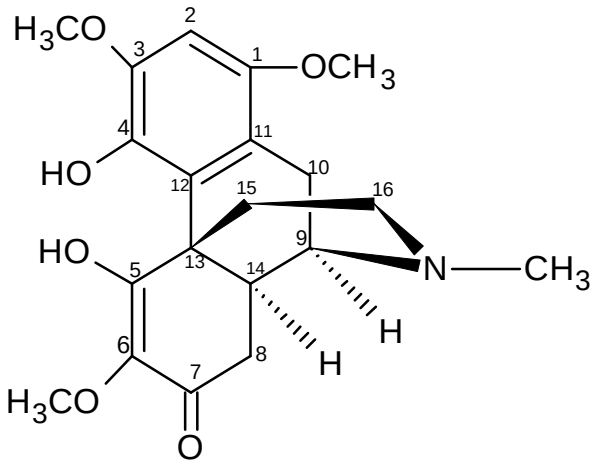


Fig. 21-Structure of compound C1

(C1) 5, 6-dehydro-4,5-dihydroxy-1,3,6-trimethoxy- 17-methylmorphinan-7-one.

Table 51: ^{1}H NMR (400 MHz) data of C1 and ^{13}C NMR (100 MHz) data of C1 in C_5D_5N

	C	^{1}H
1	146.4	--
2	111.2	6.91 s (1H)
3	148.3	--
4	145.6	--
5	125.3	--
6	152.2	--
7	194.7	--
8	40.3	ax 3.63 dd (J = 14.2, 17.4 Hz)
		eq. 2.64 dd (J = 17.4, 4.6 Hz)
9	56.9	2.83 *br* d (J = 5.6 Hz)
10	28.5	ax 3.12 d (J = 17.0 Hz)
		eq 2.74 dd (J = 17.0, 5.6 Hz)
11	131.2	--
12	128.7	--
13	38.5	--
14	42.7	2.40 m
15	32.3	2.21 - 2.11 m (2H)
16	46.5	2.40 m (1H)/2.30 m (1H)
MeO-1	57.2	3.83 s (3H)
MeO-3	56.5	3.76 s (3H)
MeO-6	55.3	3.69 s (3H)
Me-N	43.1	2.26 s (3H)
HO-4	--	10.75 br s (1H)
HO-5	--	11.50 br s (1H)

5.3.2 C2 (methoxyaporphine alkaloid)

The TTA (**C2**) and (**C8**) of the methanolic extract of the root of *C. capensis* generated a compound represented by **C2**, a reddish-brown fluffy powder with average mp of 222^0C. Its UV spectrum showed absorptions at 225, 243, 277 and 300 (shoulder) nm which are characteristic of 1, 2, 3-trisubstituted aporphinoid systems (Guinaudeau et al., 1979 and Guinaudeau and Bruneton, 1993). The IR spectrum showed bands at 3420, 3050, 2925, 1636, 1458, 1387 and 1060 cm^{-1} confirming the aporphinic skeleton of **C2**. The [EIMS] showed an $[M]^+$ at (341.0112) which suggested the molecular formula of $C_{19}H_{19}O_5N$. An intense analysis of the ^{1}HNMR, ^{13}CNMR, HMBC, COSY and NOESY spectra were used in assigning the structure. The ^{1}H NMR (400 MHz, C_5D_5N) spectrum showed an AB set of doublets at δ5.94 and 6.13 (J = 1.2 Hz), characteristic of a methylenedioxy group at positions C-1 and C-2 in aporphines (Guinaudeau and Bruneton, 1993). The presence of an ABCD tetra substituted ring system with signals at δ 7.31 (br s) and 8.32 (br s) is a strong indication that ring D is disubstituted. The ^{13}C NMR (100 MHz, C_5D_5N) spectrum showed a signal at δ101.3 (s, C-1, 2 methylenedioxy). In the ^{1}H - ^{1}H NOESY spectrum one of the methylenedioxy protons (δ 6.13) showed a correlation with H-11 (δ8.32) while H-8 (δ7.31) correlated with the H-7 methylene protons at (δ2.95-3.10). Complete assignments of all protons and carbon atoms are listed in Table 52. The spectroscopic analysis above established the structure of **C2** as 1, 2-methylenedioxy-3- hydroxy -9, 10-dimethoxyaporphine (Figure 22)

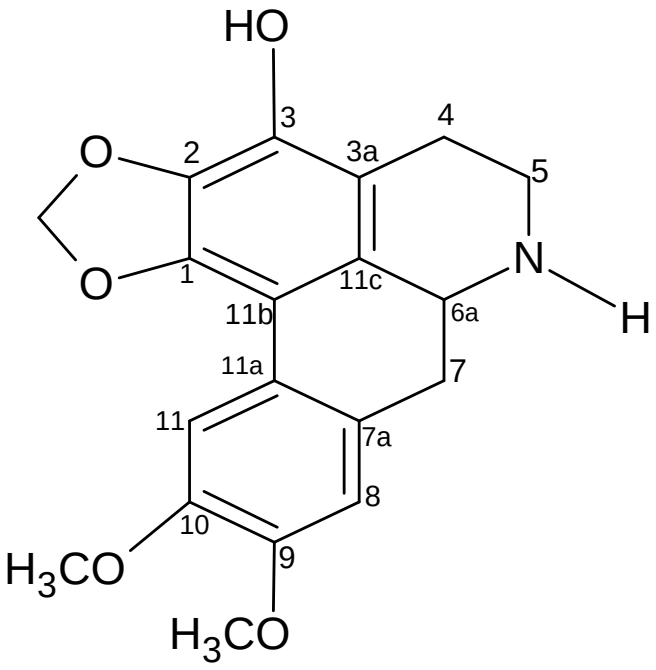


Fig. 22 – Structure of compound C2

1,2-methylenedioxy-3- hydroxy -9,10-dimethoxyaporphine

Table 52: ^{1}H NMR (400 MHz) data of C2 and ^{13}C NMR (100 MHz) data of C2 in C_5D_5N

C	δ ^{13}C	^{1}H
1	144.8	-
2	134.3	-
3	140.2	-
3a	118.0	-
4	25.3	2.92-3.12 2H m
5	44.1	2.92-3.12 1Hm, 3.45 1H m
6a	54.7	4.06 1H dd, (J = 5.2, 12.4 Hz)
7	37.1	2.95-3.10 2H m
7a	135.6	-
8	128.9	7.31 1H (br s)
9	160.2	-
10	161.9	-
11	127.1	8.32 1H (br s)
11a	133..7	-
11b	107.1	-
11c	130.2	-
O-CH_2-O	101.3	5.94, 6.13 2H A-B *qJ* = 1.2 Hz
MeO-9	56.5	3.76 s (3H)
MeO-10	55.3	3.69 s (3H)
HO-3	-	10.75 *br s* (1H)

5.3.3 C3 (8, 14-dihydromorphinandienone alkaloid)

Compound **C3**, a golden brown powder with m.p.77 – 80 °C was isolated from the methanolic extracts of the aerial shoot of *C. capensis* which showed a single spot at R_f : 0.42 in Hex: $CHCl_3$: MeOH (3:10:2). This spot showed a characteristic orange colour of an alkaloid that is closely similar to the berberine standard TLC plate marker used when sprayed with dragendorff. The IR spectrum showed pronounced bands at 3517 cm^{-1} due to the presence of hydroxyl groups while that at 2952 cm^{-1} was assigned to C-H of an olefinic system. The band at 1685 cm^{-1} is characteristic of an α,β-unsaturated carbonyl system as observed in **C1**, the band at1616 cm^{-1} is due to the C=C while that at 1510 cm^{-1} is due to the C=C of an aromatic ring. Its UV spectrum showed absorptions at 226 nm which is known to be characteristic of an α,β-unsaturated carbonyl chromophore and at 261 nm due to an aromatic ring while the band at 302 nm is characteristic of the 8,14-dihydromorphinandienone alkaloids as was noticed in **C1** (Stuart, K.L., 1971). The El mass spectrum of 329.2133 provided the molecular formula as $C_{19}H_{23}O_4N$ in which the following fragments were observed:- (1) 314 $[M- CH_3]^+$, (2) 286 $[314 – CO]^+$, (3) 192 $[M – C_8H_9O_2]^+$ and (4) 42 $[H_2C=C=O]^+$ confirming the characteristics of a morphinandienone alkaloid as observed in **C1** (Wheeler et. al 1967). The ^1H NMR spectrum showed an AB doublet of doublets at δ 6.90 and 6.78 (J = 8.4 Hz) while the three 3-proton singlets at δ 2.28 for N-Me, δ 3.62 for MeO-6 and δ 3.74 for MeO-3 were assigned as indicated. The singlet at δ 10.72 was attributable to HO-4. The ^{13}C NMR spectrum showed 19 carbon signals which supported the calculated molecular formula of $C_{19}H_{23}O_4N$. The carbons were assigned as shown in Table 53 where δ 43.3 is for N-Me, δ 55.2 for C-6-OMe, δ 56.6 for C-3-OMe and δ 194.5 for the C-7-C=O. Complete assignments of all the protons and carbons are given in Table 53.

The structure of compound **C3** was therefore established to be 5, 6-didehydro-4-hydroxy-3, 6-dimethoxy-17-methylmorphinan-7-one (8, 14 dihydromorhinandienone alkaloid) as shown in Figure 23. Literature shows that **C3** has previously been isolated from *Croton* species (Vecchietti et al., 1981) and some *Cissampelos* such as *C. sympodialis* (Freitas et al., 1995) and *C. pareira*

(Amresh et al., 2007). This is however the first time the alkaloid has been reported to be found in *C. capensis* to the best of our knowledge.

Table 53: **^{1}H NMR (400 MHz) data of C3 and ^{13}C NMR (100 MHz) data of C3 in C_5D_5N**

	C	^{1}H	NOESY	HMBC
1	119.2	6.78 br d (J = 8.4 Hz)		C10, C12, C3
2	112.0	6.90 d (J = 8.4 Hz)	C1	C11, C4
3	147.2	--		
4	145.6	--		
5	125.3	8.30 s	C6	C14, C12, C7
6	152.2	--		
7	194.5	--		
8	40.5	ax 3.64 dd (J = 14.0, 17.6 Hz)	C14, C7	C13
		eq. 2.60 dd (J = 17.6, 4.4 Hz)		
9	57.4	2.80 *br* d (J = 5.8 Hz)	C10, C14	C13, C14, C16, C11
10	28.4	ax 3.12 d (J = 17.2 Hz)	C9, C11	C14, C1, C12
		eq 2.75 dd (J = 17.2, 5.8 Hz)		
11	132.2	--		
12	128.2	--		
13	38.5	--		
14	42.0	2.40 m		
15	33.2	2.22 - 2.12 m (2H)	C16	C12
16	47.6	2.42 m (1H)/2.30 m (1H)	C15	C13, C9
MeO-3	56.6	3.74 s (3H)		C3
MeO-6	55.2	3.62 s (3H)		C6
Me-N	43.3	2.28 s (3H)		C16, C9
HO-4		10.72 br s (1H)		

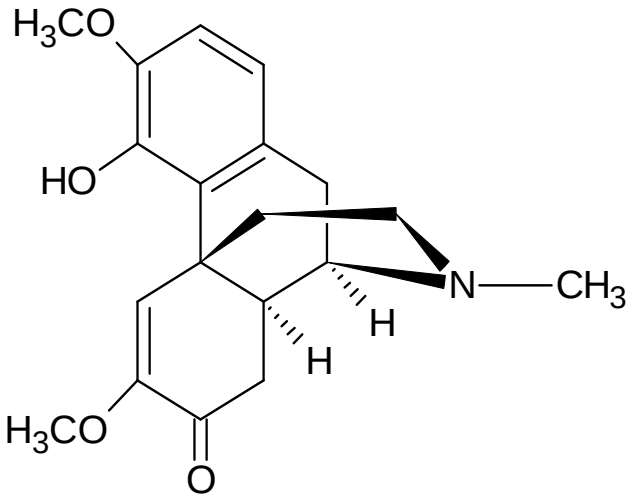


Figure 23 – Structure of compound C3

5, 6-didehydro-4-hydroxy-3, 6-dimethoxy-17-methylmorphinan-7-one

5.3.4 C5 (6 -C-methylquercetin 3, 3', 7, 8 -tetramethyl ether)

Compound **C5,** representing the combination of **C5** and **C10**, is a yellow amorphous powder with an average m.p of 235^0C. It had a molecular ion base peak $(M)^{+\cdot}$ at *m/z* 388.0103 leading to a molecular formula of $C_{20}H_{20}0_8$. The IR spectrum showed pronounced bands at 3401 cm^{-1} (hydroxyl group), 2932 cm^{-1} (C-H olefinic), 1653 cm^{-1} (carbonyl group), 1612 cm^{-1} (C=C olefinic) and 1556 cm^{-1} (C=C aromatic). The UV spectrum showed two peak maxima at 357 and 255 nm respectively. The band at 271 nm showed a large bathochromic shift in the presence of sodium acetate, and an additional band appeared at 343 nm in the methanol / NaOH spectrum which suggested a flavonol with a 7-OMe group. The ^{1}H NMR spectrum showed an aryl methyl singlet at δ 2.14 and four distinct methoxyl singlet peaks at δ 3.74 (OMe-3), 3.96 (OMe-3'), 4.01 (OMe-7) and 4.56 (OMe-8). The spectrum further showed the presence of only three 1-proton aromatic signals at δ 6.91 (d, J = 2.3 Hz), 7.60 (dd, J = 8.5 and 2.3 Hz) and 7.70 (d, J = 8.5 Hz) suggesting a 3',4'-disubstituted aromatic ring C, and a fully substituted aromatic ring A. The characteristically chelated 5-OH group was observed at δ 12.92 while that of 4'-OH was observed δ 10.90. By carefull comparison of the ^{13}C NMR data of **C5** with existing literature (Roitman and

James, 1985 and Breitmeier and Voelter, 1990), the 4'-substituent was assigned to a hydroxyl group while that at the 3' position was assigned as a methoxyl group. Corroboration of this was evident from chemical shifts of the substituted carbons, viz., C-3' at δ 147.2 and C-4' at 148.5. The other methoxyl groups were attached at C-3 (δ 137.5), C-7 (δ 145.4) and to C-8 (δ at 149.5 ppm which was in agreement with the HMBC correlations OMe-3/C-3 and OMe-7/C-7 and OMe-8/C-8.

TABLE 54: NMR spectroscopic data for compound C5

13C Signal (δ)	**DEPT**	**1H – 13C COSY**	**HMBC**	**^{13}C Assignment**
155.5	C		H-2', H-6'	2
137.5	C		3-OMe	3
178.8	C			4
156.3	C		5-OH, Me-6	5
106.6	C		5-OH, Me-6	6
145.4	C		7-OMe	7
149.5	C		C-7, C-9, C-10	8
151.4	C		8-OMe	9
103.9	C		5-OH	10
121.6	C		H- 5'	1'
115.3	CH	7.70d (J = 2.3 Hz)	H-6'	2'
147.2	C		3'-OMe, H-5'	3'
148.5	C		H-5', H-6'	4'
115.8	CH	6.91d (J = 8.5 Hz)		5'
120.6	CH	7.60dd (J = 2.3 and 8.4 Hz)	H-2'	6'
59.5	CH_3	3.74 s	C-5, C-6	3-OMe
60.2	CH_3	4.01 s	C-7	7-OMe
63.5	CH_3	4.56 s	C-8	8-OMe
56.5	CH_3	3.96 s	C-3'	3'-OMe
8.1	CH_3	2.14 s		6-Me

The only methyl group present was assigned as being attached to C-6 at 106.6 ppm as demonstrated by the HMBC cross peaks OH-5/C-5, C-6, C-10; Me-6/C-5, C-6, C-7. Other carbon peaks were equally assigned as shown in Table 54. Based on the above information the structure of **C5** was suggested to be 3,7,8 3'-tetramethoxy- 6 - *C*-methyl- 5,4'-dihydroxyflavone (6 -*C*-methylquercetin 3, 3',7, 8 -tetramethyl ether) as shown in Fig. 24.

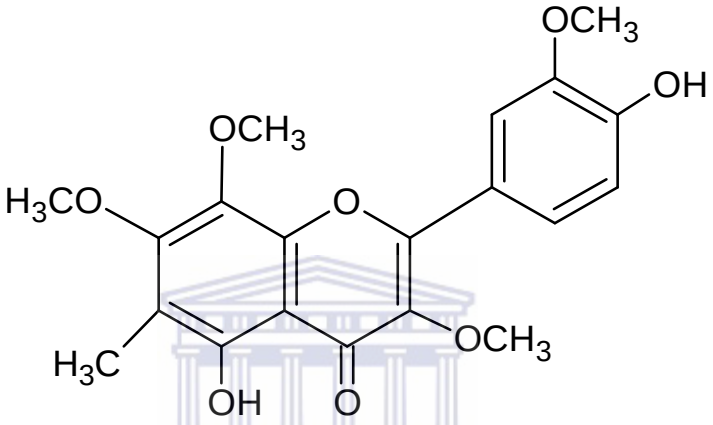


Figure 24 - Structure of compound C5

3,7,8 3'-tetramethoxy- 6 - *C*-methyl- 5,4'-dihydroxyflavone (6 –*C* methylquercetin 3, 3',7, 8 -tetramethyl ether)

5.3.5 C6 (Isoflavanone)

Compound **C6** obtained from the methanolic extract of the aerial shoot and the methanolic extract of the root (**C9**) were combined to provide **C6**, a yellow waxy powder with m.p.185 – 187^{0}C as a single spot with R_f of 0.48 in Hex : EtOAc : MeOH (2:7:1). The molecular formula of **C6** was assigned as $C_{18}H_{18}O_9$ based on the molecular ion peak obtained at *m*/*z* 378.0625 and the fragmentation pattern in the EIMS. An exhaustive comparative study of the data obtained, revealed the general features of an isoflavanone nucleus (Mabry et al., 1970; Markham and Chari, 1982 and Rahman et al., 2007). The UV absorption spectrum with maxima at 333 and 288 nm suggested an isoflavonoid. The IR spectrum had a strong absorbance at 3421 cm^{-1} due to an O-H group present, 2914 cm^{-1} for the C=C-H group, and 1634 cm^{-1} for the C=O system. The ^{1}H NMR spectrum showed an

ABC pattern of 1-proton singlets viz., δ 4.69 (*t*, *J* = 11.0 Hz), δ 4.50 (*dd*, *J* = 10.5, 5.6 Hz) and δ 4.43 (*dd*, *J* = 11.0, 5.6 Hz) in which the H-2 and H-3 protons were similar to that observed in Markham and Chari's work in 1982 showing an isoflavanone system. The ^{1}H NMR spectrum as shown inTable 55 also exhibited three H-bonded groups viz., C-5 hydroxyl at δ 12.89, C-7 hydoxyl at 12.98 and C-8 hydroxyl at δ 13.02. Two 1-proton singlets at δ 6.52 and δ 6.80 were assigned to H-6 of ring A and H-6′ of ring C of the isoflavanone. The presence of 2 methoxyl groups as well as a methylenedioxy group were apparent from their signals at δ 3.96 (2'-OMe), 3.87 (5'-OMe) and δ 5.90 (–OCH_2O–) respectively. The ^{13}C NMR spectrum in Table 55 demonstrated that compound **C6** contained a total of 18 carbons including a carbonyl group as indicated by the IR absorption peak at 1634 cm^{-1} and the EIMS for $C{=}O^{+}$ fragment. The assignment of all carbons and the placement of the 2 methoxyl and a methylenedioxy group within the molecule were achieved by 2D analysis which was further confirmed by the HMBC. In Table 56 the HMBC for the C-5 hydroxyl group showed correlation to a methine at δ 97.7 and a quaternary carbon at δ 103.3 which were assigned for C-6 and C-4a respectively. The signals at δ 168.6 was assigned to C-7, δ 164.7 to C-9 and that at δ 169.3 to C-8 as derived from its correlation with H-6 and its direct correlation from an HMQC data. Correlation of the methoxyl protons with the carbons at δ 142.5 and 145.6 in the HMBC data proved the presence of C-2′ and C-5′ methoxy groups in these positions. The methylenedioxy group exhibited 3J correlation with δ values at 137.8 and 149.8 while the 3J correlation of H-6′ was also to δ 149.8 thereby placing the methylenedioxy between C-3′ and C-4′. The assignment of C-6′ at δ 125.1 was also confirmed by the direct coupling observed in the HMQC in which It was seen that in ring C a ^{3}J for H-6′ to the methine carbon at δ 48.5 proved the latter's identity as C-3. Furthermore, C-2 was assigned to the peak at δ 71.1 as it showed a 2J correlation to H-3 in the HMBC and HMQC spectra with direct coupling with H-2 in HMQC. The COSY spectrum showed correlation between H-3 to the two non-equivalent protons at C-2. The large diaxial coupling (*J* = 11.0 Hz) observed between H-3 and H-2 placed the aryl substituent at C-3 to be *pseudo*-equatorial Based on the above information the structure of compound **C6** was therefore suggested to be 5, 7, 8 -

trihydroxy-2′, 5′-dimethoxy-3′,4′- methylenedioxyisoflavanone (Figure 25) and appears to be a new compound since we have not been able to identify it from the literature.

Table 55: ^{1}H NMR (400 MHz) data of C6 and ^{13}C NMR (100 MHz) data of C6 in C_5D_5N

Position	**^{1}H**	**^{13}C**
2	4.69, *t*, *J* = 11.0 Hz	71.1
	4.50, *dd*, *J* = 10.5, 5.6 Hz	-
3	4.43, *dd*, *J* = 11.0, 5.6 Hz	48.5
4	–	197.6
5	–	166.0
6	6.52, s	97.7
7	–	168.6
8	–	169.3
8a	–	164.7
4a	–	103.3
1′	–	121.4
2′	–	142.5
3′	–	137.8
4′	–	149.8
5′	–	145.6

Position	**^{1}H**	**^{13}C**
6′	6.80, s	125.1
5-OH	12.89, *br. s*	–
7-OH	12.98, *br. s*	–
8-OH	13.02, *br. s*	–
2′-OMe	3.96, *s*	59.6
5′-OMe	3.87, s	60.2
–OCH_2O–	5.90, *s*	102.0

Table 56: HMBC data (400 MHz, C_5D_5N) of **C6**

Protons	**C6 (^{1}H - ^{13}C)**	
	J_2	**J_3**
H-2	C-3	C-9, C-4, C-1′
H-3	C-2, C-4, C-1′	C-2′, C-6′
H-6	C-5, C-7	C-10, C-8
H-6′	C-5′	C-3, C-2′, C-4′
HO-5	C-5	C-6, C-10
HO-7	C-7	C-6, C-8
HO-8	C-8	C-7, C-9
MeO-2′	–	C-2′
MeO-5′	–	C-5′
–OCH_2O–	–	C-3′, C-4′

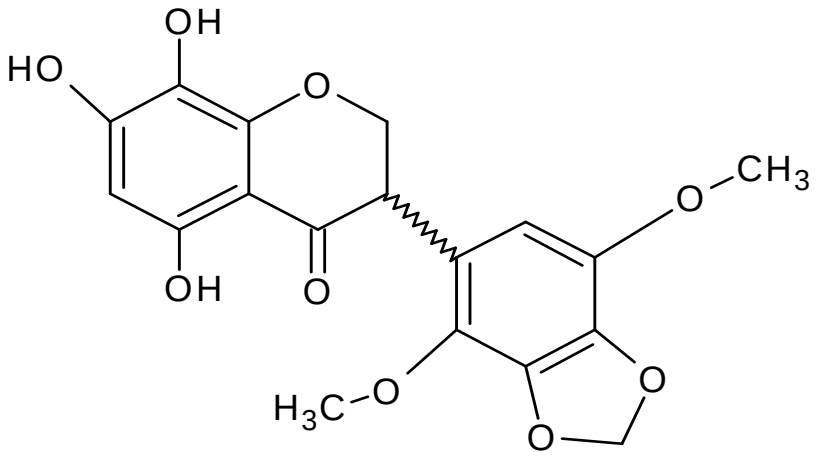


Fig 25- 5, 7, 8- trihydroxy-2′5′-methoxy-3′, 4′- methylenedioxyisoflavanone (C6)

5.3.6 C11 (6-C-methylquercetin 3-methyl ether)

Solvent A: toluene – ethyl acetate (19: 1);

Solvent B: toluene – ethyl acetate – methanol (10: 7: 3)

Compound **C11**, a creamy yellow powder with m.p 217-219 °C was isolated from the methanolic extracts of the root which showed a single spot at R_f : 0.63 and 0.40 when ran in solvent system A and B respectively. These spots showed an intense yellow colour that is closely similar to that of pure Quercetin (Sigma) standard TLC plate marker used when sprayed with 1% vanillin–H_2SO_4 followed by heating at 110 ^{0}C for 4min.

The molecular formula of **C11** was assigned as $C_{17}H_{14}0_8$ on the basis of the molecular ion peak at *m/z* 346.0752. The UV spectrum with maxima at 351 and 260 nm suggested a flavonol with a 7-OH group. A deep broad band observed in the IR spectrum at 3500 – 3400 cm^{-1} is indicative of the presence of a hydrogen-bonded hydroxyl groups. The ^{1}H NMR spectrum exhibited two 3-proton singlets assignable to one aryl methyl group at δ 2.11 (Me-6) very similar to that of **C5** and one methoxyl group at δ 3.87 due to (OMe-3). The aromatic region showed three 1-proton signals typical of a 3',4'-disubstituted ring C similar to that observed for **C5** viz., δ 7.03 (d, J = 2.3 Hz), δ 7.62 (dd, J = 8.6 and 2.3 Hz) and δ 7.75 (d, J = 2.3 Hz). A broad clustered of 1-proton siglets at δ 10.23 (4'-OH),

11.15 (3'-OH).12.05 (8-OH), 12.90 (7-OH) and 13.05 (5-OH) are due to five OH groups. The ^{13}C spectrum (Table 57) demonstrated a pattern similar to that of compound **C5**, in which the C ring is di-substituted, having two hydroxyl groups at C-3'(163.6) and C-4'(164.5). The HMBC data for ring A was, as expected and slightly different from that of **C5,** with the C-7(159.2) position showing the presence of an OH group. The HMBC cross peaks observed for OH-5/C-5, C-6, C-4a and Me-6/C-5, C-6, C-7; OH-8/C-7, C-8, C-8a indicated that the OH groups were located at C-7 and C-8 due to their respective coupling effects. The location of the single available methoxyl group and methyl group at C-3 and C-6 respectively, were further verified by their HMBC correlations with C-3 and C-6 respectively. Consequently the structure of **C11** was assigned as 3 -methoxy-6 -*C*-methyl-3',4',5,7,8 -pentahydroxyflavone (6 -*C*- methylquercetin -3-methyl ether) (Fig. 26).

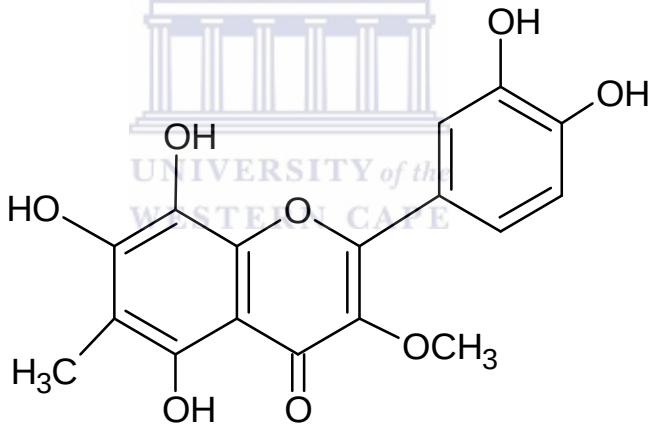


Figure 26 –Structure of compound C11

3 -methoxy-6 -*C*-methyl-3',4',5,7,8 -pentahydroxyflavone (6 -*C*-methylquercetin -3-methyl ether)

TABLE 57: The NMR spectroscopic data for compound C11

^{13}C Signal (δ)	HMBC	^{1}H (δ, J Hz)	^{13}C Assignment
155.0	H-2', 6'		2
137.3	3-OMe		3
177.9			4
157.1	5-OH, Me-6		5
107.3	5-OH, Me-6		6
159.2	Me-6, 8-OH		7
162.8	7-OH, Me-6		8
154.4	8-OH		8a
104.8	5-OH		4a
120.4	H- 5'		1'
115.4	H-6'	7.75 (1H, d, 2.3)	2'
163.6	H-2', H-5'		3'
164.5	H-2', H-5', H-6'		4'
115.4		7.03 (1H, d, 8.6)	5'
120.8	H-2'	7.62 (1H, dd, 2.3 and 8.6)	6'
7.2		2.11 (3H, s)	6-Me
59.2		3.87 (3H, s)	3-OMe

The whole process in this case confirm the isolation and characterization of 6 compounds **C1, C2, C3, C5, C6** and **C11** from the TTA and methanolic extracts of *Cissampelos capensis* aerial shoot and root in which only **C3** was found to be already isolated from some few Cissampelos (Vecchietti et al., 1981; Freitas et al., 1995 and Amresh et al., 2007) while the remaining 5 compounds seems new to the best of our knowledge. It should be noted that there were several extracts, fractions and subfractions that were not analysed due to insufficient tests and information.

5.4 BIOLOGICAL EVALUATION

5.4.1 Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was performed on all the extracts, fractions and some of the isolates as shown in sections 3.10.2.0 and 4.5.3 for *P. reticulatum*. Using the same procedure, the LC_{50} with 95% confidence were estimated for the different extracts used in the phytochemical screening and the 6 isolates viz., **C1, C2, C3, C5, C6** and **C11**.

The distribution pattern of the plant metabolites in the extracts were clearly evident in their brine shrimp cytotoxicity behaviour (Tables 58, 59 and 60), where three test bands were used viz., Inactive ($LC_{50} > 700$), Active ($LC_{50} < 700$) and Very active ($LC_{50} < 10$). The results demonstrated that all the hexane extracts showed no activity. Clearly evident was the finding that the highest bioactivity is found in the methanolic extracts, both in the root and the aerial shoot of this plant. The total tertiary alkaloid (TTA) showed a very high active value of 0.32 (Table 60) indicating that is much better than the BTA with a value of 64.87. This was further confirmed by the highly active compound generated from the TTA as shown in compound **C1**. It should be noted that all the extracts and isolates (**C1**, **C3** and **C6**) that are very active should be considered as good candidates for antimicrobial and antiviral agents since the lethality of a test substance to brine shrimp nauplii has been linked to the possible ability of such substances to kill cancer cells (antitumor activity) and possess anti-inflammatory properties (Mc Laughlin et al., 1991; Babajide et al., 2008; Farthing M.J., 2000). However, this does not mean that the inactive compounds (**C11** and hexane extract) would not be useful for some other purposes such as good antioxidant agents.

Table 58: **Average number of dead nauplii after 24hours for *C. capensis***

Test materials	Vials at 1000 µg/ml			Vials at 100 µg/ml			Vials at 10 µg/ml			Vials for control expt.
	1	2	3	1	2	3	1	2	3	
CCRH	9	9	10	10	10	10	10	10	10	10
CCRD	2	2	1	4	4	6	7	9	7	10
CCRE	2	3	3	5	5	6	7	6	8	10
CCRM	0	0	0	0	0	0	1	1	1	10
CCRW	2	3	2	4	4	4	6	7	8	10
CCAH	6	7	5	9	6	6	9	10	9	10
CCAD	2	1	2	5	4	4	7	6	7	10
CCAE	1	3	2	4	3	4	6	6	7	10
CCAM	0	0	0	0	1	0	2	3	2	10
CCAW	6	6	7	8	10	8	10	10	10	10
CCET	0	0	0	1	2	2	4	3	4	10
TTA	0	0	0	0	0	0	1	0	1	10
BTA	3	2	3	5	4	5	7	6	7	10
C1	0	0	0	0	0	0	0	1	0	10
C2	1	1	1	3	4	4	7	6	7	10
C3	0	0	0	0	1	1	3	3	4	10
C5	2	2	2	4	3	4	6	7	8	10
C6	0	1	0	1	1	0	3	4	4	10

Table 59: Average number of survived nauplii after 24hours for *C. capensis*

Test materials	Vials at 1000 µg/ml		Vials at 100 µg/ml		Vials at 10 µg/ml		Vials for control expt.
	Ave. surv.	% surv.	Ave. surv.	% surv.	Ave. surv.	% surv.	% surv.
CCRH	9.33	93.33	10	100	10	100	100
CCRD	1.67	16.67	4.67	46.67	7.67	76.67	100
CCRE	2.67	26.67	5.33	53.33	7	70	100
CCRM	0	0	0	0	1	10	100
CCRW	2.33	23.33	4	40	7	70	100
CCAH	6	60	7	70	9.33	93.33	100
CCAD	1.67	16.67	4.33	43.33	6.67	66.67	100
CCAE	2	20	3.67	36.67	6.33	63.33	100
CCAM	0	0	0.33	3.33	2.33	23.33	100
CCAW	6.33	63.33	8.67	86.67	10	100	100
CCET	0	0	1.67	16.67	3.67	36.67	100
TTA	0	0	0	0	0.67	6.67	100
BTA	2.67	26.67	4.67	46.67	6.67	66.67	100
C1	0	0	0	0	0.33	3.33	100
C2	1	10	3.67	36.67	6.67	66.67	100
C3	0	0	0.67	6.67	3.33	33.33	100
C5	2	20	3.67	36.67	7	70	100
C6	0.33	3.33	0.67	6.67	3.67	36.67	100
C11	6	60	8.67	86.67	10	100	100

Table 60: LC_{50} and average number of dead nauplii after 24hours for *C. capensis*

Test materials	Vials at 1000 µg/ml	Vials at 100 µg/ml	Vials at 10 µg/ml	LC_{50}	General remarks
CCRH	1	0	0	3526.22	Inactive
CCRD	8	5	2	120.48	Active
CCRE	7	5	3	71.33	Active
CCRM	10	10	9	**3.75**	**Very active**
CCRW	8	6	3	100.03	Active
CCAH	4	3	1	*	Inactive
CCAD	8	6	3	145.86	Active
CCAE	8	6	4	58.64	Active
CCAM	10	10	8	**6.15**	**Very active**
CCAW	4	1	0	2446.33	Inactive
CCET	10	8	6	*	**Very active**
TTA	10	10	9	**0.32**	**Very active**
BTA	7	5	3	64.87	Active
C1	10	10	10	**0.82**	**Very active**
C2	9	6	3	320.66	Active
C3	10	9	7	**6.20**	**Very active**
C5	8	6	3	199.55	Active
C6	10	9	6	**3.23**	**Very active**
C11	4	1	0	1159.62	Inactive

***** Data did not converge and therefore could not be regressed by the finnery probit analysis programme.

5.5. ANTIMICROBIAL ACTIVITY EVALUATIONS

The antimicrobial evaluation was carried out using the diffusion method in which three organisms were used in each case. For Gram-negative bacteria: *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli* Sero type 1 were used while for Gram-positive, *Bacillus subtilis*, *Staphylococcus aureus* and *Bacillus licheniformis* were used. The fungal species used were *Candida albicans*, *Candida eropiralis* and *Aspergillus niger*. The assay was set up as described in section 3.10.3.1 and the inhibition zone for both the bacteria and the fungi were measured in triplicate for all the extracts and the 6 identified isolates as was done in the brine shrimp cytotoxicity assay above. The average was determined and compared to the positive controls used (Amoxicillin; 40µg/ml for bacteria and Fluconazole; 120µg/ml for fungi). The results, which were generated based on the average inhibition zones of the microbial growth (mm), are presented in Table 61. Similar patterns were observed in the microbial analysis except in very few cases as shown in Table 61. The three (3) test organisms used in each case was to allow for a wider spectrum of antimicrobial activities of the extracts and isolates. *S. aureus*, a pyrogenic bacterium known to play a significant role in invasive skin diseases was selected as part of the test organism while *C. albicans* was chosen for this study since it causes serious systemic infections, including an opportunistic infection in patients infected with HIV.

Table 61: Antimicrobial profile of *Cissampelos capensis* (The inhibition zones)

	Psa	Prv	Esc	Bas	Sta	Bal	Caa	Cae	Asn
CCRH	0	0	0	0	0	0	0	0	0
CCRD	0	12	11	13	18	15	0	0	0
CCRE	14	0	15	17	33	19	17	14	20
CCRM	32	28	28	43	35	27	30	36	23
CCRW	0	0	0	11	17	12	25	26	25
CCAH	0	0	0	0	0	0	0	0	0
CCAD	15	18	16	11	13	11	0	0	0
CCAE	14	12	12	17	18	0	0	0	0
CCAM	25	21	20	35	40	44	23	22	19
CCAW	11	12	11	10	24	11	17	15	29
CCET	24	22	31	37	35	28	36	33	34
TTA	36	38	37	45	40	36	38	33	35
BTA	15	17	17	23	28	24	30	32	29
C1	41	34	29	32	30	33	0	0	0
C2	16	19	19	22	19	14	19	15	17
C3	18	0	26	19	0	18	10	11	11
C5	10	13	11	12	10	11	0	0	14
C6	15	13	19	22	28	30	35	29	27
C11	10	11	15	0	0	21	20	26	29
Amx	61	44	49	42	53	47	0	0	0
Flu	0	0	0	0	0	0	46	53	39

*Gram –ve bacteria: - *Pseudomonas aeruginosa* (**Psa**) , *Proteus vulgaris* (**Prv**) and *Escherichia coli* (**Esc**) Gram +ve bacteria: - *Bacillus subtilis* (**Bas**), *Staphylococcus aureus* (**Sta**) and *Bacillus licheniformis* (**Bal**) Fungi: - *Candida albicans* (**Caa**), *Candida eropiralis* (**Cae**) and *Aspergillus niger* (**Asn**) Ref. standards:- **Amx**:- Ammoxicilin and **Flu**:- Fluconazole

The results in Table 61 showed that different levels of activity were observed for both the extracts and the isolates. Any value > 20 mm is assumed to be moderately active. There was more antibacterial than antifungal activity. The highest activity was recorded for the Gram +ve bacteria while that of Gram –ve bacteria is more than that of the fungi. The maximum zone (45 mm) of antibacterial effect was observed in the TTA fraction against the Gram +ve organism *B. subtilis* which may be due to the strong antimicrobial and antiviral activities of the alkaloid in the plant which is well known for treatment of malaria, fungal infections, inflammations and cancer (Kaur et al., 2009 and Mc Gaw et al., 2000). No activity was recorded for the hexane extracts against all nine organisms. High values of activity were recorded for the drug resistance breed of bacteria *P. aeuruginosa*, where 36mm was recorded for TTA (36mm), CCAM (25mm) and CCRM (32mm).

Some preferential activity is observed in cases where activity was preferentially selected on the organisms (Babajide et al., 2008 and Shai et al., 2008). For example, it can be noted that CCRD was only active against Gram +ve and –ve bacteria. A similar observation can be found in CCAE which is only active against 5 out of the 9 organisms with no antifungal effect on all 3 fungi used. Similar observation can be found for CCRW which is only active against 6 of the organisms with no Gram –ve activities.

The above observed biological activities may be due to the behavior of both alkaloids and flavonoids which are known to exhibit a wide range of activities which includes anti inflammatory, antithrombotic and antiviral due to their ability to scavenge free-radicals (Babajide et al., 2008; Shai et al., 2008; Rahman et al., 2007; Ibewuike et al., 1997 and Liu et al., 1990).

5.6 CHEMICAL EVALUATION

5.6.1 The antioxidant assay

Total antioxidant evaluation was conducted on the *C. capensis* extracts and isolates by evaluating the antioxidant content in the extracts and the antioxidant capacity in both the extracts and isolates as did in *P.reticulatum* and described in section in 3.11.1 – 3.11.9.

5.6.2 Polyphenolic content

The polyphenolic content was determined by using gallic acid as a standard and Folin Ciocalteu reagent as the antioxidant marker as shown in 3.11.2. The results showed that the amount of polyphenols present in the extracts were in various concentrations as shown in Figure 27A in which the 80% ethanolic extract (CCET) has the highest content while CCAE and the TTA has the lowest. This result futher confirmed the enrich nature of the methanolic extracts for both the aerial shoot and the root as a good source of polyphenols because of their high values as shown in Figure 27A.

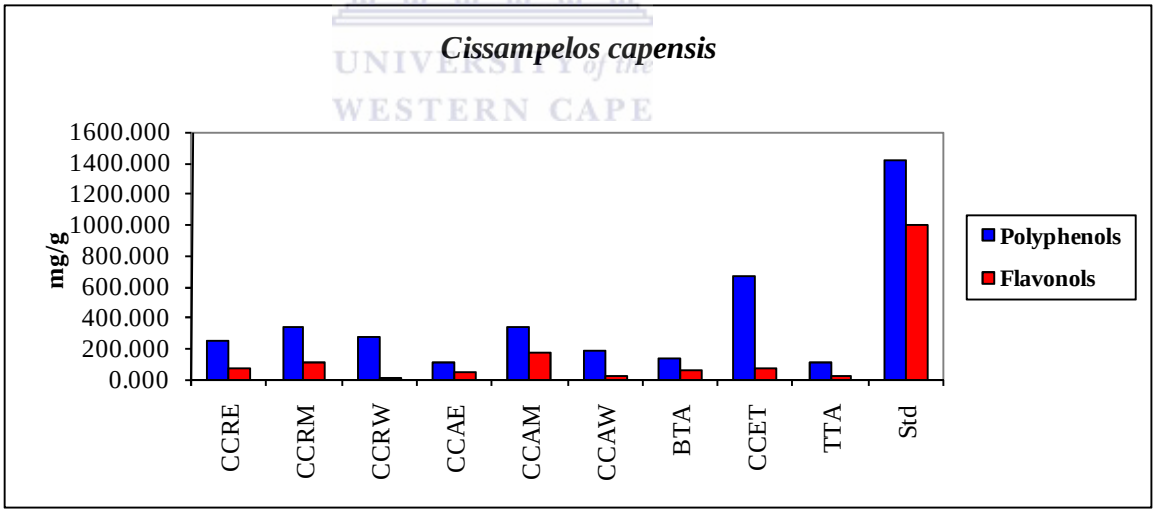


Figure 27A – Bar chart illustrating and comparing the amount of polyphenols and flavonols present in the various extracts of *C. capensis*

5.6.3 Flavonoid content

Closely related profiles were observed for flavonoids which consist of flavonols, flavanols and flavanones. Evaluation of flavonol content was made using quercetin as the standard as shown in 3.11.3 while the flavanol content made use of catechin as listed in 3.11.4 and flavanone content made use of the Naringenin standard (3.11.6).

Results are shown in Figures 27A, 27B and 27C From these it is evident that the presence of flavonols, some flavanols and traces of flavanones are present when compared with the standard and this confirms the actual isolation of a flavanone (**C6)**. The results further show the consistency of the methanolic extract in both the root and the aerial shoot with high values as shown in Figures 27A and 27B.

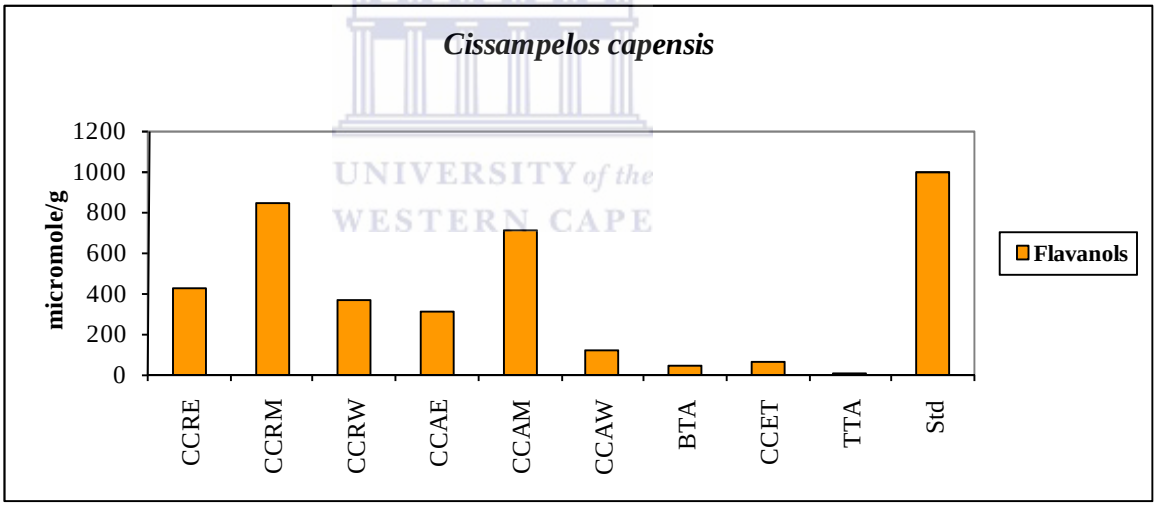


Figure 27B – Bar chart showing the amount of flavanols present in the various extracts of *C. capensis*

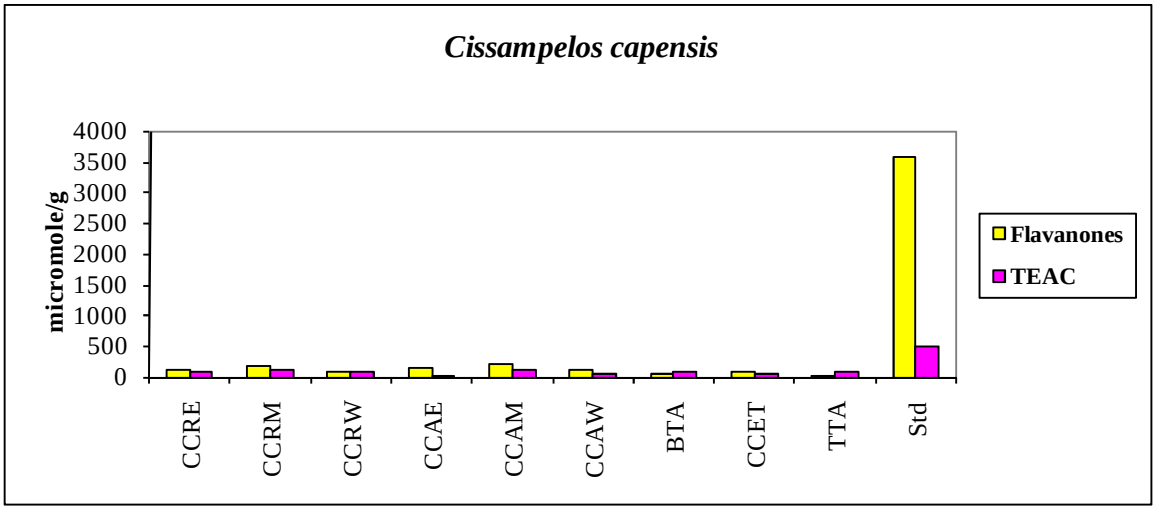


Figure 27C – Bar chart illustrating and comparing the amount of flavanones and the Trolox equivalent antioxidant capacity present in the various extracts of *C. capensis.*

5.6.4 Anthocyanins contents

The anthocyanin content was also evaluated by measuring the amount of pigments present as shown in 3.11.5.The result obtain is as shown in Figure 27D in which it was observed that anthocyanins were absent in the plant except for the traces observed in CCAE, CCAW, TTA and CCET.

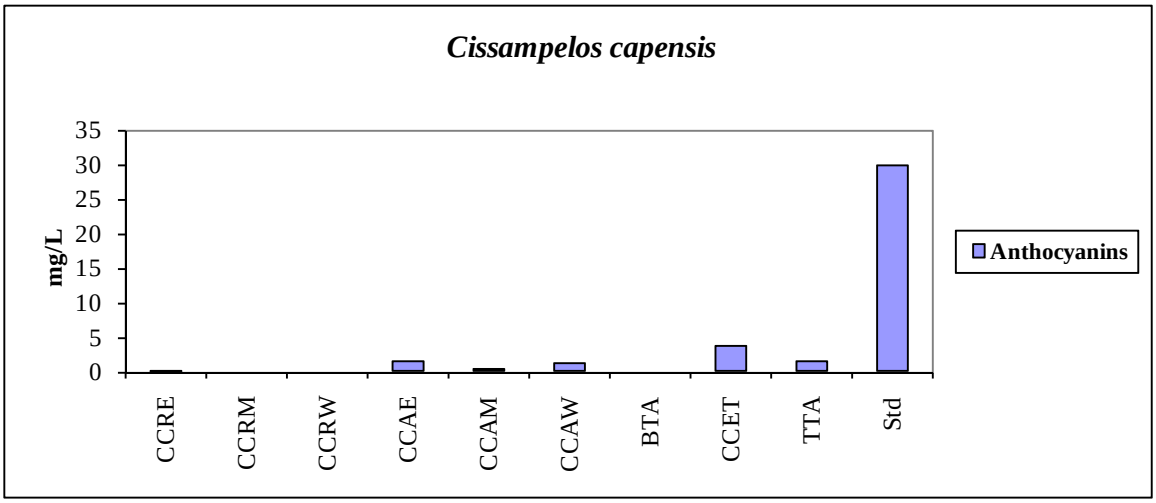


Figure 27D – Bar chart showing the amount of anthocyanins present in the various extracts of *C. capensis*

5.6.5 The Oxygen radical absorbance capacity assay (ORAC)

The ORAC (3.11.7) results showed that *C. capensis* also have high antioxidant values and this can be attributed to the flavonoids found in them as shown in Figure 27E. This is clearly evident in **C11**. It can also be observed that the methanolic extracts of both the root and aerial shoot possesses high antioxidant capacity as shown by Figure 27E

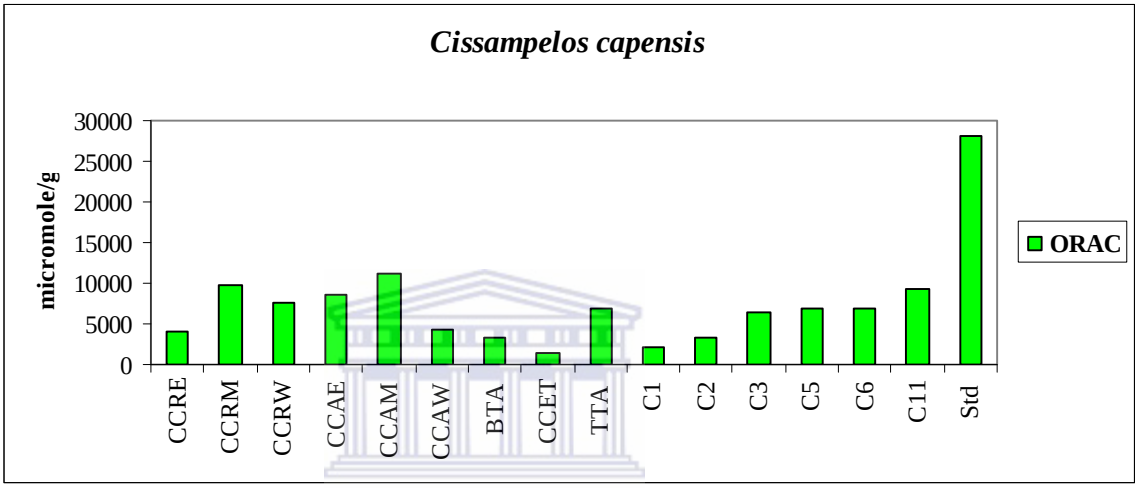


Figure 27E – Bar chart showing the ORAC values (The antioxidant capacity) of some extracts and isolates present in *C. capensis.*

5.6.6 The Ferric reducing antioxidant power assay (FRAP)

The FRAP redox potential was clearly exhibited by **C5** and the methanolic extracts as shown in Figure 27F where **C5** recorded the highest value for the isolates and CCAM for the extracts.

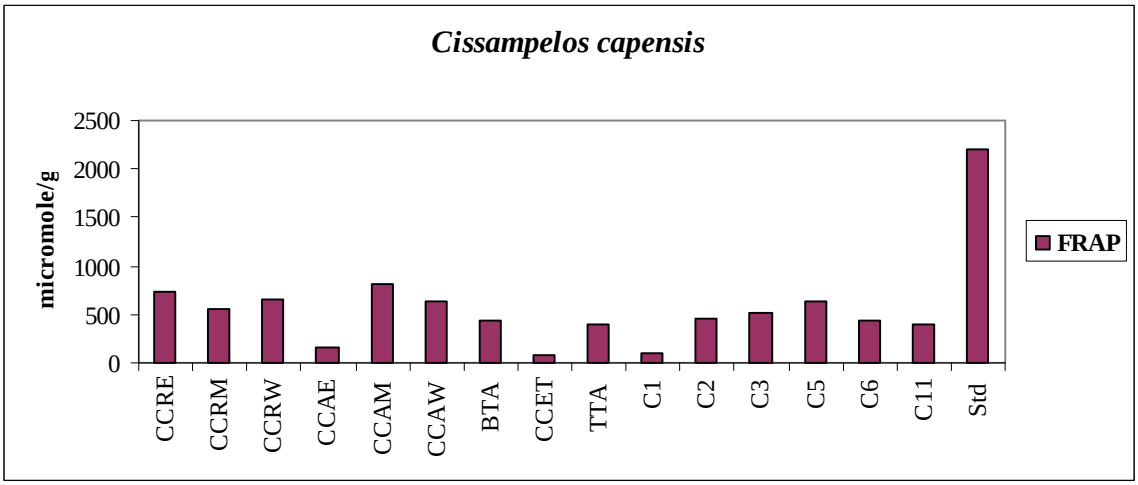


Figure 27F – Bar chart showing the FRAP values (Ferric reducing antioxidant power) of some extracts and isolates present in *C. capensis*

5.6.7 The Abts Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay (3.11.9) results can also be found in Figure27C which showed a fair scavenging power as observed in CCRM and CCAM which is an indication that the methanolic extracts is a better choice for formulating a viable and active antioxidant.

CHAPTER 6

RESULTS AND DISCUSSION

6.1 *GERANIUM INCANUM*

The whole plant of *G. incanum* was collected at two different periods, one in winter and the other in summer. The plant was washed, dried and milled separately as shown in section 3.3.0. This afforded 3.98kg for the winter and 4.0kg for the summer collection. A similar extraction procedure as described earlier was followed for the summer collection (2kg) to afford a hexane (29.45 g, 1.47%), dichloromethane (37.69 g, 1.89%), ethyl acetate (125.28 g, 6.26%), methanol (183.25g, 9.16%) and water extract (86.11g, 4.31%) (Section 3.8.0). For the isolation of constituents in the ethyl acetate fraction of the summer collection, 25.0 g of the extract was chromatographed to afford compounds **G1**, **G2** and **G3** as shown in Section 3.8.1.0 – 3.8.1.4. The appearance of the fractions was as follows: **G1**; a bright yellow powder (0.049 g); **G2**: an off-white powder (0.088 g) and **G3**, a creamy off-white powder (0.044 g). Similarly, 20.0g of the methanol fraction of the summer collection was chromatographed. This afforded compounds **G4** and **G5** as shown in Section 3.8.2.0 – 3.8.2.3. In this case **G4** was a white powder (0.048 g) and **G5** turned out to be a light yellow amorphous powder (0.038 g).

The winter collection (2kg) gave a hexane (48.35 g, 2.42%), dichloromethane (34.43 g, 1.72%), ethyl acetate (107.26 g, 5.36%), methanol (211.77g, 10.59%) and water extract (79.32g, 3.97%). CGC of the ethyl acetate extract afforded compounds **G6**, **G7** and **G8** as shown in Section 3.8.3.0 – 3.8.3.6. **G6** was obtained as a brownish yellow powder (0.038 g); **G7** as an off-white powder (0.055 g) and **G8** as a bright yellow amorphous powder (0.077 g). The chromatographic profile of the methanolic fraction of the winter collection afforded compounds **G9**, **G10**, **G11**and **G12** as shown in Section 3.8.4.0 – 3.8.4.6. In this case **G9** was obtained as a bright yellow powder (0.067g); **G10** as an off-white powder (0.0532 g); **G11 i**s a light yellow amorphous powder (0.098 g) and **G12** as a creamy yellow powder (0.0658g).

All extracts were subjected to preliminary phytochemical screening for the presence of secondary plant metabolites as shown in Section 3.5.0. Results of this

analysis showed that *G. incanum* collected during summer was phytochemically different from that collected during winter as shown in Table 62. The reason for this variation is presumed to be due to the high nutrient absorption rates and water intake by the plant during winter which is greater than that available during summer in South Africa (Clark et al., 2009; Alireza et al., 2009). Phytochemical screening for the detection of natural plant products in the extracts were specifically targeted for tannins, phenolics, glycosides, saponins, flavonoids, alkaloids, anthraquinones and essential oils which were performed according to the method used by Wagner (Wagner and Bladt, 2001).

Results indicate the presence of saponins, tannins, flavonoids, phenolics as well as essential oils found only in the hexane and DCM extracts of the summer collection. Cardiac glycosides, alkaloids and anthraquinones were evidently absent in all the extracts as shown in Table 62.

These observations confirmed the phytochemical profile recorded in the literature for this plant (De Wet and Van Wyk, 2008; Dic. of Nat. Prod., 1996; VanWyk, 2008; Amabeoku, 2009).

Table 62:- Phytochemical screening result of the *G. incanum* extracts

Test materials	**Tann**	**Phen**	**Glyc**	**Sapo**	**Flav**	**Alka**	**Anth**	**Esse**
GISH	P+ve	P+ve	N-ve	N-ve	N-ve	N-ve	N-ve	P+ve
GISD	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	P+ve
GISE	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve
GISM	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve
GISW	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve
GIWH	P+ve	P+ve	N-ve	N-ve	N-ve	N-ve	N-ve	N-ve
GIWD	P+ve	P+ve	N-ve	N-ve	N-ve	N-ve	N-ve	N-ve
GIWE	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve
GIWM	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve
GIWW	P+ve	P+ve	N-ve	P+ve	P+ve	N-ve	N-ve	N-ve

GISH = *G. incanum* summer collection Hexane extract; **GISD** = *G. incanum* summer collection DCM extract; **GISE** = *G. incanum* summer collection EtOAc extract; **GISM** = *G. incanum* summer collection MeOH extract, **GISW** = *G. incanum* summer collection H_2O extract; **GIWH** = *G. incanum* winter collection Hexane extract; **GIWD** = *G. incanum* winter collection DCM extract; **GIWE** = *G. incanum* winter collection EtOAc extract; **GIWM** = *G. incanum* winter collection MeOH extract; **GIWW** = *G. incanum* winter collection H_2O extract; While Tannins (**Tann**), Phenolics (**Phen**), Glycosides (**Glyc**), Saponins (**Sapo**), Flavonoids (**Flav**), Alkaloids (**Alka**), Anthraquinones (**Anth**) and Essential oils (**Esse**)

A total of 12 compounds were successfully isolated from both the summer and winter collections of the *G. incanum* plant material viz., (**G1** – **G12**), with only **G5**, **G10** and **G12** being fully analysed and reported in this thesis while the remainder cannot be conclusively identified due to insufficient spectral data and other physicochemical parameters needed to completely justify the nature and structure of the compounds. Some data available is given here. **G2** is an off white powder with m.p. 196-198°C. R_f value of 0.35 and 0.60 in solvent system A and B respectively. The UV λ_{max} nm (log ε): 217 (5.14), 267 (4.77), 238, 263, 285 while in the EIMS the molecular ion base peak $(M)^+$ *m/z* is 972.1017. (calculated frag. gave $C_{41}H_{30}O_{27}$).

G3 is a creamy off white crystal with m.p. 147 -149^{0}C. TLC in Hex: EtOAc: MeOH (3:7:1) gave a R_f of 0.45. The UV $\boldsymbol{\lambda}_{max}$ (log ε): 241, 286 and 400 and the IR: $\boldsymbol{\nu}_{max}$ cm^{-1} pronounced peaks were found at 3430 (b) (OH), 1645 (s) (C=O), 1617 (s), 1492, 1380, 1320, 1284, 1249, 1213, 1176 and 1084. The EIMS: molecular ion peak $(M)^{+\cdot}$ *M/z* 274.0102. (calculated mol. formula $C_{15}H_{14}O_5$).

G2 and **G3** were strongly suspected to be tannins because they were both positive to feric chloride test.

Results of **G5, G10** and **G12** are hereby presented.

6.2. PHYSICO-CHEMICAL DATA OF ISOLATED COMPOUNDS

6.2.1 G5 (Quercetin)

A light yellow amorphous powder with m.p. 314 - 316˚C, R_f (Solvent): 0.79 (A), 0.47 (B).

IR (ν cm^{-1}) pronounced peaks: 3420, 3271, 1675, 1624, and 1511.

UV λ_{max} nm (log ε): 255 (4.76), 376 (4.80); +NaOAc: 261, 388; +AlCl3: 271, 437; +NaOH: 282, 426.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 302.0113 (calculated frag. gave $C_{15}H_{10}O_7$).

^{1}H NMR (400 MHz in pyridine-d5): δ 6.75 (1H, d, J = 1.98 Hz, H-8), 6.78 (1H, d, J= 1.98 Hz, H-6), 7.41 (1H, d, J = 8.25 Hz, H-5'), 8.20 (1H, dd, J = 2.31, 8.25 Hz,

H-6'), 8.65 (1H, d, J = 2.31 Hz, H-2'), 10.40 (1H, s, 4'-OH), 10.95 (1H, s, 3'-OH) 11.65 (1H, s, 3-OH). 12.75 (1H, s, 7-OH), 13.35 (1H, s, 5-OH).

^{13}C NMR ran at (100 MHz in DMSO-d_6) : see Table 63.

6.2.2 G10 (16α-hydroxy-(-)-kauran-18-oic acid)

An off white powder, m.p. 224 - 226˚C, R_f (Solvent): 0.63 (A), 0.42 (B), 0.21 (C).

IR (ν cm^{-1}) pronounced peaks: 3383, 2928, and 1692.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 321.2113 (calculated frag. gave $C_{15}H_{10}O_7$).

^{1}H NMR (600 MHz, pyridine-d5): δ 1.90 (1H, bd, J = 12.8 Hz, H-1α), 1.02 (1H, ddd, J = 13.5, 12.8, 3.2 Hz, H-1β), 1.82 (1H, m, H-2α), 1.62 (1H, m, H-2β), 1.87 (1H, m, H-3α), 2.25 (1H, ddd, J = 13.6, 12.7, 4.8 Hz, H-3β), 2.16 (1H,bd, J = 12.0 Hz, H-5), 1.70 (1H, m, H-6α),1.62 (1H, dt, J = 14.4 Hz, H-6β), 1.75 (1H, m, H-7α), 1.65 (1H, m, H-7β), 1.24 (1H, d, J = 6.4 Hz, H-9), 1.70 (1H, m, H-11α), 1.67 (1H, m, H-11β), 1.67 (1H, m, H-12α), 1.75 (1H, m, H-12β), 2.30 (1H, bs, H-13), 2.16 (1H, d, J = 12.0 Hz, H-14a), 2.10 (1H,d, J=12.0 Hz, H-14b), 2.05 (1H, d, J = 14.3 Hz, H-15α), 1.78 (1H, d, J=14.3 Hz, H-15β), 1.69 (3H, s, Me-17), 1.54 (3H, s, Me-19), 1.20 (3H, s, Me-19), 1.20 (3H, s, Me-20). ^{13}C-NMR: Table 64.

6.2.3 G12 (6, 8-di-C-methylquercetin 3, 7-dimethyl ether)

A creamy yellow powder with m.p. 201 - 203˚C, R_f (Solvent): 0.44 (B), 0.26 (C).

UV λ_{max} nm (log ε): 262 (4.67), 364 (4.63); +NaOAc: 263, 366; +$AlCl_3$: 280, 446; +NaOH: 268, 413.

IR (ν cm^{-1}) pronounced peaks: 3400, 2931, 1656, 1615 and 1556.

EIMS: the molecular ion base peak $(M)^+$ *m/z* 359.1103. (calculated frag. gave $C_{19}H_{18}O_7$).

^{1}H NMR (400 MHz in DMSO-d_6): δ 2.21 (3H, s, Me-6), 2.42 (3H, s, Me-8), 3.86 (3H, s, OMe-3), 3.91 (1H, s, OMe-7), 7.03 (1H, d, J = 8.6 Hz, H-5'), 7.63 (1H, dd, J = 2.31, 8.6 Hz, H-6'), 7.75 (1H, d, J = 2.31 Hz, H-2'), 10.56 (1H, s, 4'-OH), 10.92 (1H, s, 3'-OH), 13.00 (1H, s, 5-OH). ^{13}C-NMR: see Table 63.

TABLE 63: ^{13}C NMR data for compounds G5 and G12

Carbon	G5	G12
2	147.0	155.9
3	137.7	137.8
4	177.3	178.5
5	157.5	155.9
6	99.2	112.5
7	165.4	162.0
8	94.3	108.4
8a	162.4	151.3
4a	104.4	107.0
1’	123.8	120.8
2’	115.2	115.2
3’	147.7	145.3
4’	149.6	148.9
5’	115.2	115.8
6’	121.0	120.6
3-OMe		59.5
7-OMe		60.3
3’-OMe		
6-Me		8.0
8-Me		8.2

6.3 STRUCTURAL ELUCIDATION OF COMPOUNDS G5, G10 AND G12

6.3.1 G5 (Quercetin)

Compound **G5** showed IR absorbtion bands at 1675 cm^{-1} (C=O), and 3420 cm^{-1} (OH). It also exhibited UV Bands I and II at 376.0 and 255.0 nm respectively, maxima characteristic typical of flavonols (Wollenweber, 1982). Large bathochromic shifts in the presence of $AlCl_3$ (+62nm). NaOAc (+13.5nm) and NaOH (+51nm), with a new band appearing at 282nm in the MeOH + NaOH spectrum were also apparent. These UV data are consistent with those of a flavonol possessing free 3, 7, 3', and 4' hydroxyl groups (Wollenweber, 1982; Voirin, 1983).

The proton NMR spectrum of compound **G5** showed only aromatic signals, indicating the absence of aliphatic substituents on the flavonoid nucleus. The spectrum exhibited the characteristic 5-OH proton signal at δ 13.35 and four singlets at δ 10.40 (4'-OH); 10.95 (3'-OH); 11.65 (3-OH) and 12.75 (7-OH). *Meta-coupled* doublets of an AB system were observed at δ 6.75 and 6.78 (J = 1.98 Hz) which are consistent with a 5,7-disubstituted ring A. An aryl ABC trisubstituted system corresponding to a 3', 4'- disubstituted ring C was also evident from the three 1-proton aromatic signals at δ 7.41 (d, J = 8.25 Hz), 8.20 (dd, J = 2.31 and 8.25 Hz) and 8.65 (d, J = 2.31 Hz). The ^{13}C NMR spectrum of **G5** also exhibited only aromatic signals, with the typical C-3 signal of a flavonol at δ 137.7. In the pyridine- d5 spectrum (Table 63), the signals for C-2' and C-5' were coincident at δ 115.2, indicating that these two carbons were *ortho* to hydroxyl groups (Roitman and James, 1985). These observations, in conjunction with the UV data and the proton spectrum, indicated that compound **G5** was a flavonol with free 3,5,7,3',4'-pentahydroxy groups identical to **P13** which was identified as quercetin. Confirmation was provided by co-TLC with pure quercetin. The complete analysis and spectral comparison shows that **G5** is a quercetin (Figure 28) which to the best of our knowledge is been reported for the first time to be present in this plant.

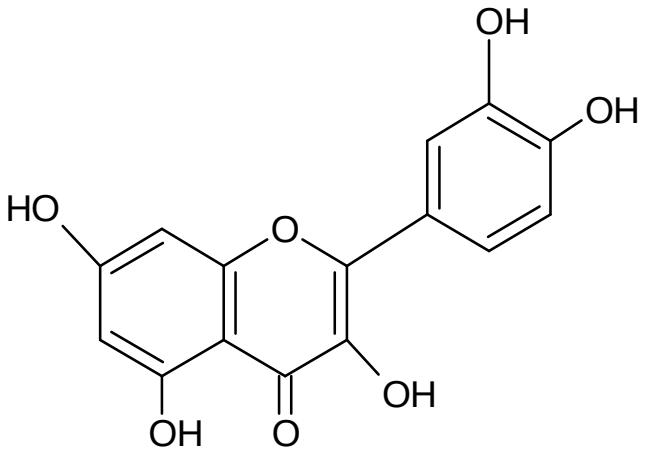


Fig. 28 Structure of compound G5

6.3.2 G10- 16α-hydroxy-(-) kauran-18-oic acid

Compound **G10**, a white amorphous solid with m.p. 224 - 226^{0}C, showed no maxima between 200- 550 nm in its ultraviolet spectrum, pointing to the absence of a chromophoric group. The IR spectrum showed a broad – OH band at 3383 cm^{-1} and a carbonyl resonance at 1692 cm^{-1}, both indicative of a carboxylic acid group. The ^{13}C NMR (Table 64) and DEPT spectra (Table 65) of **G10** showed 20 signals representing: three methyl, nine methylene, three methine and five quaternary carbons, while the EI mass spectrum exhibited an [M+H]$^+$ ion at m/z 321.2113 (Δ – 0.6 mmu). All these data are consistent with a molecular formular of $C_{20}H_{32}O_3$. Based on the IR spectrum, the completely aliphatic ^{1}H NMR spectrum as well as the absence of any C=C bond as indicated by the ^{13}C NMR spectrum (Table 64), the five degrees of unsaturated for **G10** were assigned to a tetracyclic ring system with a carboxylic acid group. The presence in the ^{1}H NMR spectrum (Table 65) of three methyl singlets (δ 1.20, 1.54 and 1.69) as well as the ^{13}C carbonyl resonance at δ 181.5 implies that the tetracyclic ring system is a 16-carbon unit. In addition to this, the presence of an oxygen-bearing quaternary carbon at δ 78.0 as well as the base peak at m/z 303.2317 [MH-H_2O]$^+$ (Δ- 0.7 mmu) in the mass spectrum indicated compound **G10** to most likely be a kaurane diterpene acid possessing a tertiary hydroxyl group at C-16 (Serebryakov et al., 1968; Tan *et al*., 1993).

Using the DEPT and HMQC spectra of compound **G10**, it was possible to identify the carbon resonances as methyl, methylene or quaternary, and to assign their

corresponding proton signals (Table 65). Thus, the methyl singlets at δ 1.20, 1.54 and 1.69 showed one bond correlations with the carbon resonances at δ 18.4, 17.2 and 25.2, respectively. Other important proton/carbon HMQC correlations were CH (δ2.16, bd/51.0), CH_2 (δ 1.78, d; 2.05, d/58.9), CH_2 (δ2.10, d/38.2) and CH_2 (δ 1.87, m; 2.25, ddd/37.7) (Figure 30). The two/ three-bond connectivity information obtained from the HMBC (Figure 31) spectrum was further used to assign the carbon signals of the suggested 16-hydroxykauranioc acid. The methyl singlet at δ 1.54/17.2 showed a cross-peak with the carbonyl resonance at δ 181.5, thus identifying it as Me-19. Similarly, the methyl singlet at δ 1.69/25.2 correlated with the oxygen-bearing quaternary carbon (δ 78.0, C-16) and was assigned as Me-17. Other important HMBC correlations were Me-19/C-3, C-4, C-5;Me-17/C-13, C-15; Me-20/C-1, C-9, C-10;H-5/C-4, C-6, C-19, C-20; CH_2 -15/C-7, C-8, C-16, C-17 and CH_2-14/C-12 (Table 65; Figure 31). The ^{1}H- ^{1}H COSY spectrum of compound G10 (Table 65) was used to confirm the proton and carbon assignments from the HMBC spectra especially as the geminal methylene protons were non-equivalent, and showed large couplings with each other. Thus, the C-15 protons appeared as two distinct doublets at δ 1.78 and 2.05 (J_{gem} = 14.3 H_Z). Other ^{1}H-^{1}H correlations were CH_2-2/H-1, H-3; CH_2-1/CH_2-1, CH_2-2, Me-20; CH_2-15/CH_2-15; CH_2-13/CH_2- 13/CH_2-11, CH_2-14 and CH_2-3, Me-20 (Table 65). All the above confirmed the structure of G10 to be 16α-hydroxykauranoic acid. The stereochemistry at C-4 was clarified by comparison of the ^{13}C NMR spectrum with the published spectra of kauranoic acids (Delgado *et a*l., 1985; Monte *et a*l., 1988; Tan *et al.,* 1993). For kauran-18-oic acids (those in which the – COOH group is β i.e equatorial), the C-5 carbon is shielded, while it is deshielded in kauran-19-ioc acids (in which the-COOH is α i.e axial). Also the C-4 methyl group is strongly shielded (*ca*. 17- 18 ppm) in β-equatorial acids and deshielded (*ca.* 28 ppm) in α- axial acids. The peaks for C-4 at δ 47.9, C-5 at 51.0 and C-19 at 17.2 all confirm the β-equatorial orientation of the carboxylic acid moiety at C-4, making compound **G10** 16α –hydroxykauran-18-oic acid (Figure 29).

The NOESY spectrum (Table 65) exhibited the expected H-5/H-9 cross-peak of the kaurane skeleton, while the Me-20/Me-19 correlation supported the α-axial orientation of Me-19. The 16α-hydroxy stereochemistry had been previously

established for the isomeric 16α-hydroxykauran-19-oic acid reported from the fermentation of *Fusarium moniliforme* Sheld (Serebryakov *et al.*, 1968,) and was unambiguously proven further for compound **G10** by the observed H-15β/ Me-17 NOESY correlation (Table 65). It is also to the best of our knowledge the first kaurane- type diterpene to be isolated from *Geranium incanum.*

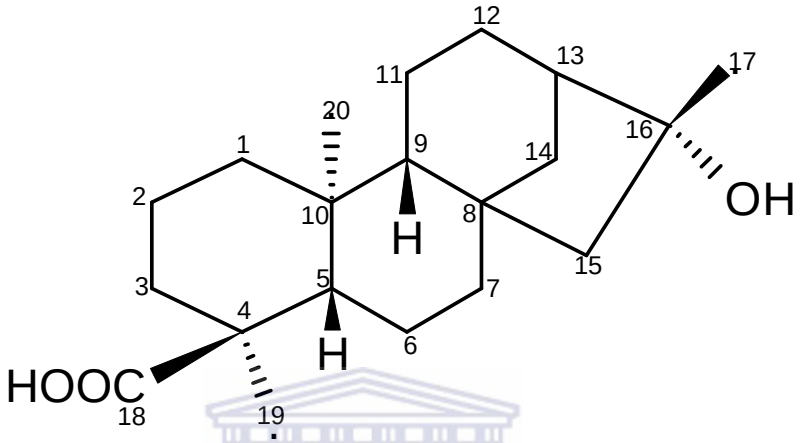


Figure 29: Structure of compound G10

TABLE 64: ^{13}C NMR data for compound G10

Carbon	Chemical Shift (δ)
1	40.1
2	18.4
3	37.7
4	47.9
5	51.0
6	24.0
7	42.3
8	45.9
9	57.6
10	39.1
11	18.4
12	27.4
13	49.6
14	38.2
15	58.9
16	78.0
17	25.2
18	181.5
19	17.2
20	18.4

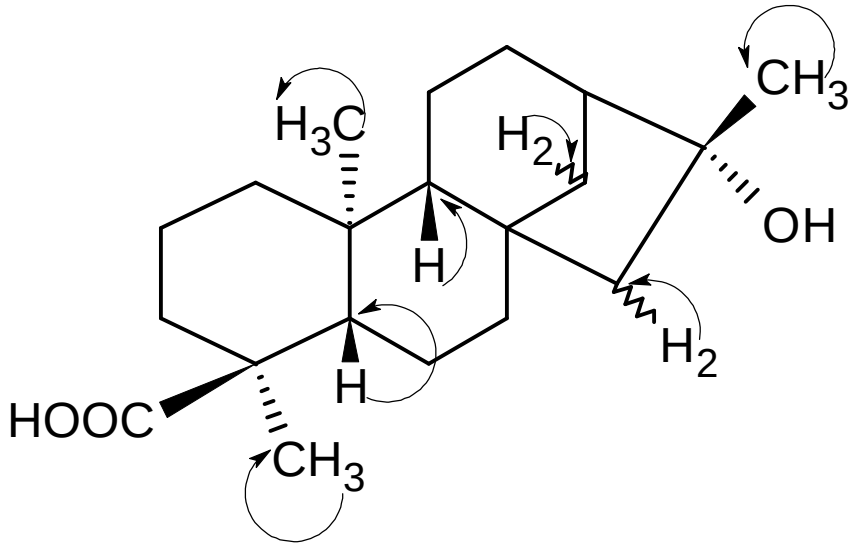


Figure 30-The HMQC correlations for compound G10

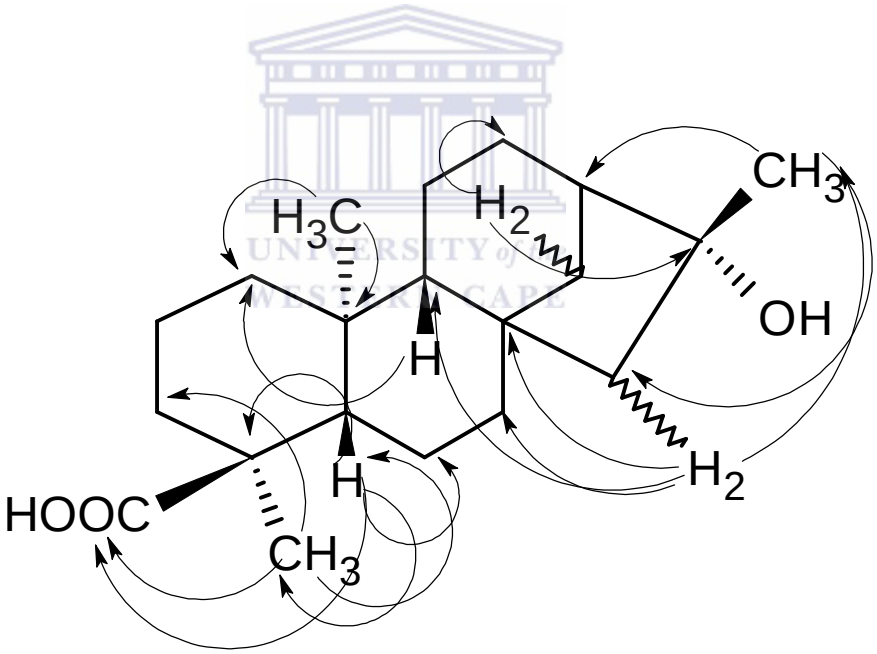


Figure 31-The HMBC correlations for G10

TABLE 65: NMR spectroscopic data for compound G10

^{13}C Signal	DEPT	HMQC (δ, J Hz)	HMBC	^{1}H-^{1}H COSY	NOESY	^{13}C
40.1	CH2	α1.90 bd (12.8)	2,9,10	1β, 2, 20	1β, 11, 20	1
		β 1.02 ddd (13.5, 12.8, 3.2)	2, 10	1α	3β, 5, 9	
18.4	CH2	α 1.82 m			2β, 19, 20	2
		β 1.62 m	10	1, 3	3α	
37.7	CH2	α 1.87 m	5		3β, 19	3
		β 2.25 ddd (13.6, 12.7, 4.8)	2, 4, 18, 19	2, 3, 20	5, 9	
47.9	C					4
51.0	CH	2.16 bd (12.0)	4, 6, 7, 10, 19, 20		6β, 7β, 9	5
24.0	CH2	α 1.70 m			6β, 19, 20	6
		β 1.62 dt (14.4)				
42.3	CH2	α 1.75 m	5		7β, 14a	7
		β 1.65 m	5		9	
45.9	C					8
57.6	CH	1.24 d (6.4)	1,10, 12		12α, 15β	9
39.1	C			11		10
18.4	CH2	α 1.70 m	12		11β	11
		β 1.67 m	12	9		
27.4	CH2	α 1.67 m			12β, 13, 14a	12
					13	
		β 1.75 m			14a, 14b	
49.6	CH	2.30 bs			14b	13
38.2	CH2	a 2.16 d	8, 12, 13	14		14

		(12.0)			20	
		b 2.10 d	8, 12, 13,			
		(12.0)	15, 16		15β	
58.9	CH2	α 2.05 d	7, 8, 9, 13,			15
		(14.3)	16	15β	17	
		β 1.78 d	8, 9, 14, 17			
		(14.3)		15α		
78.0	C					16
25.2	CH3	1.69 s	13, 15, 16			17
181.5	C					18
17.2	CH3	1.54 s	3, 4, 5, 18			19
18.4	CH3	1.20 s	1, 5, 9, 10			20

6.3.3 G12 (6, 8-di-C-methylquercetin 3, 7-dimethyl ether

The UV spectrum of compound **G12** indicated that it was a 3-*O*-substituted flavonol, with Band I and Band II absorbances at 364 and 262 nm, respectively. The absence of a significant NaOAc shift as well as the +49 nm bathochromic shift of Band I in the presence of NaOH indicated a 7-O-substitution and a free 4'-OH group, respectively. The EIMS mass spectrum showed a $[MH]^+$ peak at m/z 359.1141 (Δ 1.0mmu), corresponding to a molecular formular $C_{19}H_{18}O_7$. The ^1H NMR spectrum (Table 66) showed two aryl methyl singlets at δ 2.21 and 2.42, as well as two methoxyl singlets (δ 3.86 and 3.91). The aromatic region still showed the typical pattern of a 3', 4'-disubstituited ring C with three signals at δ 7.03 (1H, d, J=8.6 Hz), 7.63 (1H, dd, J=2.3 and 8.6 Hz) and 7.75 (1H, d, J=2.3 Hz). The absence of any other aromatic signal implied that compound **G12** had a fully substituted ring A, while the singlet at δ 13.0 was also typical of a H–bonded 5-OH. The ^{13}C NMR signals for G12 are as shown in Table 63. Ring A showed the expected substituent effects of an additional C-8 methyl group i.e. large deshielding effects at C-8 and C-6 (ipso, +18.5 ppm; meta, +5.5 ppm) with smaller shielding effects at C-7 (-1.0 ppm, ortho), C-8a (-3.1ppm, ortho) and C-5 (-1.2 ppm, para). All these were further confirmed by the observed HMBC correlations of 5-OH/C-5, C-6, C-4a, Me-6/C-5, C-6, C-7 and Me-8/C-7, C-8, C-8a thus establishing the two methyl groups to be positioned at C-6 and C-8. The methoxyl groups were located at C-3 and C-7 by the OMe-3/C-3 and OMe7/C-7 correlations, respectively.

Compound **G12** was therefore identified as 3, 7-dimethoxy-6, 8-dimethyl-5, 3', 4'-trihydroxyflavone (6, 8-di—C-methylquercetin- 3, 7- dimethyl ether) (Figure 32). The proton and carbon signals in the HMBC spectrum are given in Table 66. Compound **G12** is thus reported for the first time as a new C-methylflavonol isolated from *Geranium incanum*.

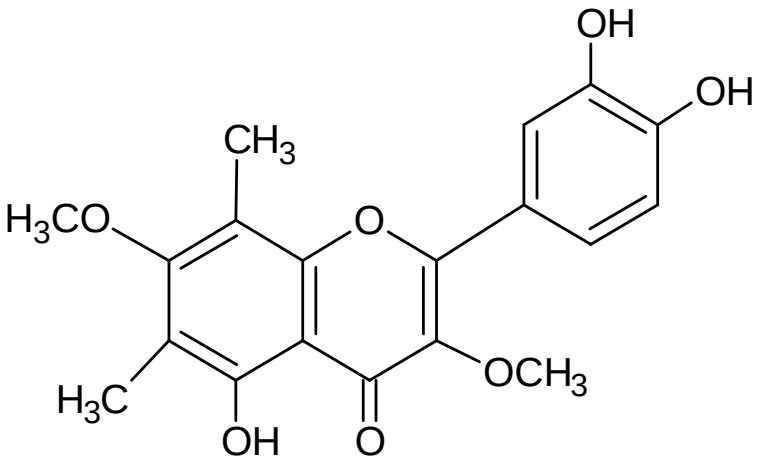


Figure 32 – Structure of compound G12

TABLE 66: NMR spectroscopic data for compound G12

^{13}C Signal (δ)	**HMBC**	**^{1}H (δ, J Hz)**	**^{13}C Assignment**
155.9	H-2', 6'		2
137.8	3-OMe		3
178.5			4
155.9	5-OH, Me-6		5
112.5	5-OH, Me-6		6
162.0	Me-6, Me-8, 7-OMe		7
108.4	Me-8		8
151.3	Me-8		8a
107.0	5-OH		4a
120.8	H-5'		1’
115.2		7.75 (1H, d, 2.31)	2’
145.3	H-2', H-5'		3’
148.9	H-2', H-6'		4’
115.8	H-6',	7.03 (1H, d, 8.6)	5’
120.6	H-2'	7.63 (1H, dd, 2.31 and 8.6)	6’
8.0		2.21 (3H, s)	6-Me

8.2 59.5 60.3		2.42 (3H, s) 3.86 (3H, s) 3.91 (3H, s)	8-Me 3-OMe 7-OMe

6.4 BIOLOGICAL EVALUATION

6.4.1 *Brine shrimp lethality bioassay*

The brine shrimp lethality bioassay was carried out on all the extracts and some selected isolates as shown in section 3.10.2.0. Using identical procedures, the LC_{50} with 95% confidence was estimated for the different extracts used in the phytochemical screening as well as **G2, G3, G5, G10** and **G12**. The distribution pattern of the plant metabolites in the extracts were also clearly shown in their brine shrimp cytotoxicity behaviour as shown in the results (Tables 67, 68 and 69) which demonstrated that both the winter and summer hexane extracts showed no activity. Clearly shown is that the methanolic extracts from both periods were very active. The climatic variation however can be seen in the DCM and EtOAc extracts in which the DCM summer extract is inactive while the winter extract is active. The EtOAc extract of the summer collection is active while that of winter was found to be very active. Compounds **G2** and **G12** were found to be inactive while **G3**, **G5** and **G10** were found to be active and **G10** was the only isolate that was very active (Table 69).

Table 67: Average number of dead nauplii after 24hours for *G. incanum*

Test materials	Vials at 1000 µg/ml			Vials at 100 µg/ml			Vials at 10 µg/ml			Vials for control expt.
	1	2	3	1	2	3	1	2	3	
GISH	6	8	4	9	7	10	10	10	10	10
GISD	10	8	8	8	9	10	10	10	10	10
GISE	2	3	3	5	5	6	8	6	8	10
GISM	0	0	0	1	2	2	4	5	4	10
GISW	1	2	2	4	4	5	9	6	8	10
GIWH	6	7	8	8	10	8	10	9	10	10
GIWD	2	2	1	4	4	6	7	9	7	10
GIWE	0	0	0	1	2	1	4	5	4	10
GIWM	0	0	0	0	0	0	1	2	1	10
GIWW	3	1	1	4	5	5	7	7	7	10
G2	6	7	5	9	6	6	9	10	9	10
G3	2	1	2	5	4	4	7	6	7	10
G5	1	3	2	4	3	4	6	6	7	10
G10	0	0	0	0	1	0	2	3	2	10
G12	6	6	7	8	10	8	10	10	10	10

Table 68: **Average number of survived nauplii after 24hours for *G. incanum***

Test materials	Vials at 1000 µg/ml		Vials at 100 µg/ml		Vials at 10 µg/ml		Vials for control expt.
	Ave. surv.	% surv.	Ave. surv.	% surv.	Ave. surv.	% surv.	% surv.
GISH	6	60	8.67	86.67	10	100	100
GISD	8.67	86.67	9	90	10	100	100
GISE	2.67	26.67	5.33	53.33	7.33	73.33	100
GISM	0	0	1.67	16.67	4.33	43.33	100
GISW	1.67	16.67	4.33	43.33	7.67	76.67	100
GIWH	7	70	8.67	86.67	9.67	96.67	100
GIWD	1.67	16.67	4.67	46.67	7.67	76.67	100
GIWE	0	0	1.33	13.33	4.33	43.33	100
GIWM	0	0	0	0	1.33	13.33	100
GIWW	1.67	16.67	4.67	46.67	7	70	100
G2	6	60	7	70	9.33	93.33	100
G3	1.67	16.67	4.33	43.33	6.67	66.67	100
G5	2	20	3.67	36.67	6.33	63.33	100
G10	0	0	0.33	3.33	2.33	23.33	100
G12	6.33	63.33	8.67	86.67	10	100	100

Table 69: LC_{50} and average number of dead nauplii after 24hours for *G. incanum*

Test materials	Vials at 1000 µg/ml	Vials at 100 µg/ml	Vials at 10 µg/ml	LC_{50}	General remarks
GISH	4	1	0	1759.62	Inactive
GISD	1	1	0	989.66	Inactive
GISE	7	5	3	110.25	Active
GISM	10	8	6	**6.18**	**Very active**
GISW	8	6	2	88.35	Active
GIWH	3	1	0	4156.21	Inactive
GIWD	8	5	2	*****	Active
GIWE	10	9	6	**5.63**	**Very active**
GIWM	10	10	9	**0.44**	**Very active**
GIWW	8	5	3	76.24	Active
G2	4	3	1	*****	Inactive
G3	8	6	3	120.86	Active
G5	8	6	4	50.62	Active
G10	10	10	8	**8.11**	**Very active**
G12	4	1	0	1126.45	Inactive

***** Data did not converge and therefore could not be regressed by the finnery probit analysis programme

6.4.2 Antimicrobial evaluation

The antimicrobial evaluation was carried out using the same diffusion method as used in *Cissampelos capensis*. The assay was set up as described in section 3.10.3.1 and the inhibition zones for both the bacteria and the fungi were also measured.

Table 70: Antimicrobial profile of *Geranium incanum* (The inhibition zones)

	Psa	**Prv**	**Esc**	**Bas**	**Sta**	**Bal**	**Caa**	**Cae**	**Asn**
GISH	0	0	0	0	0	0	0	0	0
GISD	0	0	0	0	0	12	22	0	0
GISE	19	24	18	26	0	28	0	0	0
GISM	27	32	32	38	29	33	30	23	27
GISW	0	0	12	15	13	0	18	21	15
GIWH	0	0	0	0	0	0	0	0	0
GIWD	0	0	0	19	15	22	26	20	23
GIWE	29	26	35	38	30	18	15	17	22
GIWM	34	41	28	36	36	31	30	27	29
GIWW	10	11	15	0	0	20	22	23	28
G2	16	0	15	18	32	19	17	19	23
G3	15	18	16	11	13	11	0	0	0
G5	14	12	12	17	18	0	22	21	28
G10	25	21	20	30	40	37	33	27	29
G12	11	12	11	10	24	11	17	15	38
Amx	61	44	49	42	53	47	0	0	0
Flu	0	0	0	0	0	0	46	53	39

*Gram –ve bacteria: - *Pseudomonas aeruginosa* (**Psa**) , *Proteus vulgaris* (**Prv**) and *Escherichia coli* (**Esc**) Gram +ve bacteria: - *Bacillus subtilis* (**Bas**), *Staphylococcus aureus* (**Sta**) and *Bacillus licheniformis* (**Bal**) Fungi: - *Candida albicans* (**Caa**), *Candida eropiralis* (**Cae**) and *Aspergillus niger* (**Asn**) Amx:- Ammoxicilin and Flu:- Fluconazole

The results in table 70 show different level of activity for both the extracts and the isolates. The highest activity was recorded for the Gram +ve bacteria while that of Gram –ve is greater than that of the fungi. The maximum zone (40 mm) of antibacterial activity was observed for compound **G10** against the Gram-positive organism *Staphylococcus aureus*. No activities however were observed for the hexane extracts against all nine organisms tested. The preferential activity observed in *C. capensis* was also observed in this plant. For example, **G3** was only active against Gram +ve and –ve bacteria. A similar observation is found in GISE which is active against only 5 out of the 9 organisms tested with no antifungal effect. A similar observation can be found in GIWD which is only active against 6 of the 9 organisms tested with no Gram –ve activities as shown in table 70.

6.5. CHEMICAL EVALUATION

6.5.1 The antioxidant assay

Total antioxidant evaluation were also carried out on *G. incanum* extracts and isolates by evaluating both the antioxidant content and the antioxidant capacity as shown in 3.11.1 – 3.11.9.

6.5.2 Polyphenolic content

The polyphenolic content was also evaluated as shown in 3.11.2. The results showed that the amount of polyphenols present in the extracts varied quite a bit as shown in Figure 33A in which **GIWM** has the highest amount while the lowest was recorded for **GIWW** which presumably meant that the methanol extraction process removed a large portion of the polyphenols present.

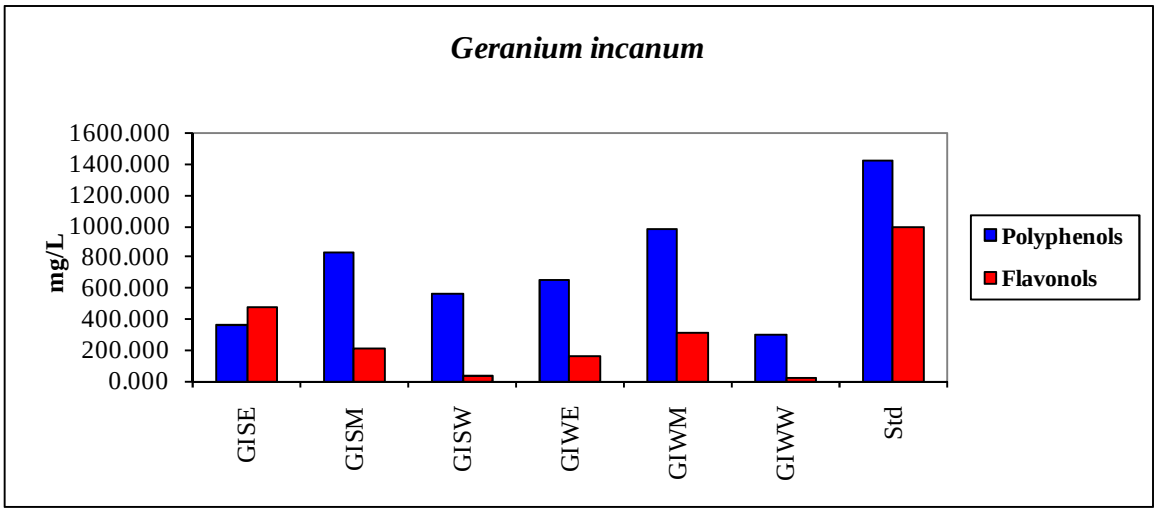


Figure 33A – Bar chart illustrating and comparing the amount of polyphenols and flavonols present in the various extracts of *G. incanum.*

6.5.3 Flavonoid content

Flavonoid which consist of flavonols, flavanols and flavanones were detrmined as described ealier and the results are shown in Figures 33A, 33B and 33C. From these it is evident that more flavonols were present in the ethyl acetate extract of the summer collection and the methanolic extract of the winter collection. The flavanol content is greater in the water extract of the summer than in winter while the methanolic extract of the winter plant recorded a significant presence of flavanols. Flavanones were found in trace amounts only as shown in Figure 33C.

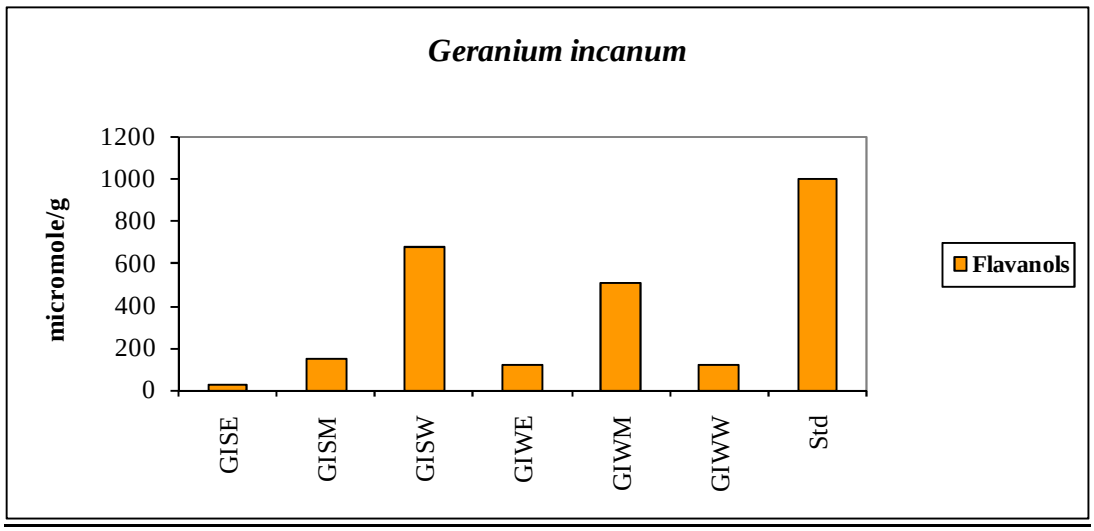


Figure 33B – Bar chart showing the amount of flavanols present in the various extracts of *G. incanum.*

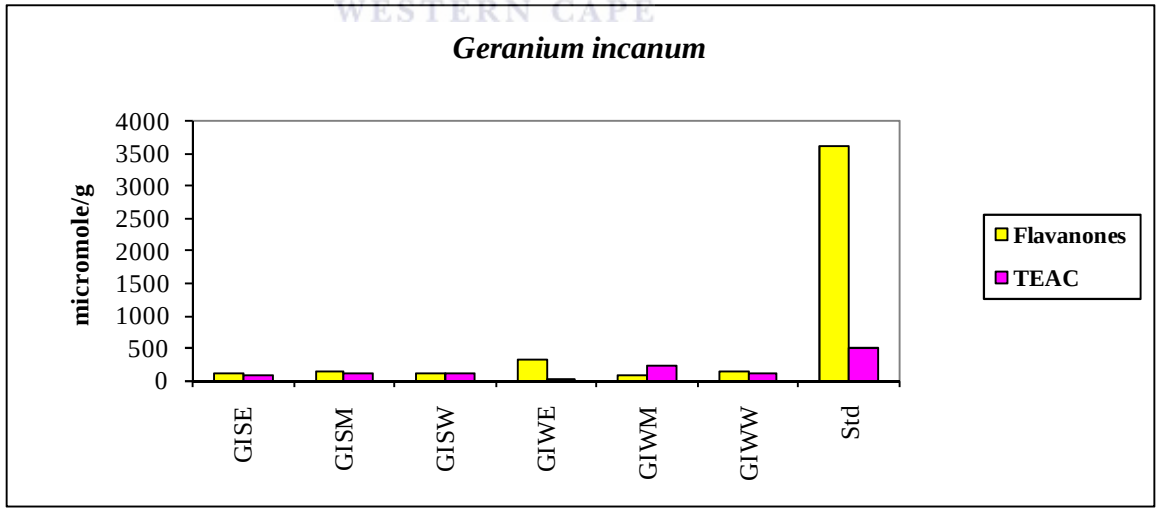


Figure 33C – Bar chart illustrating and comparing the amount of flavanones and the Trolox equivalent antioxidant capacity present in the various extracts of *G. incanum.*

6.5.4 Anthocyanin content

Results obtained (Figure 33D) clearly showed the absence of anthocyanins. Traces observed during winter in the methanol and water extracts were the only ones.

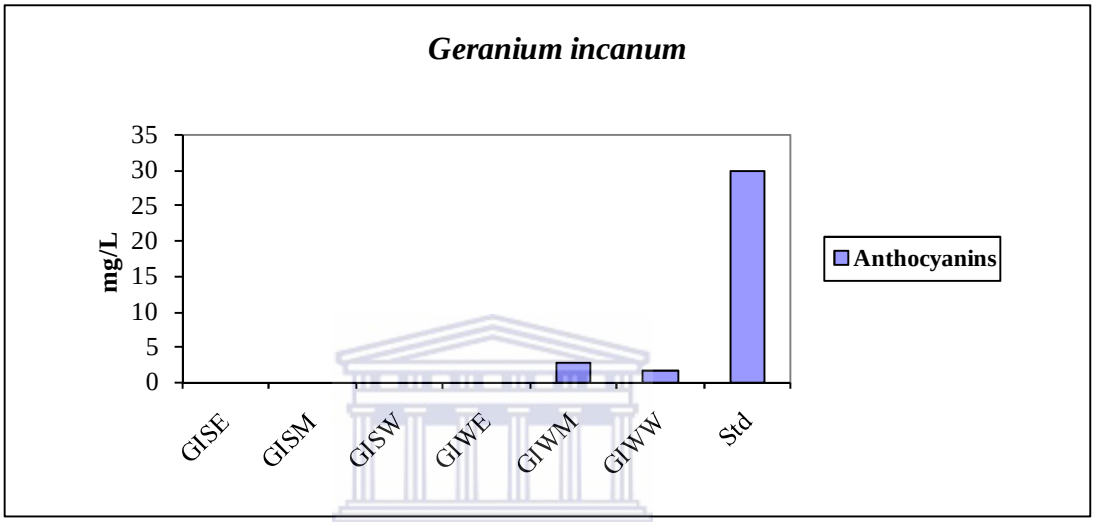
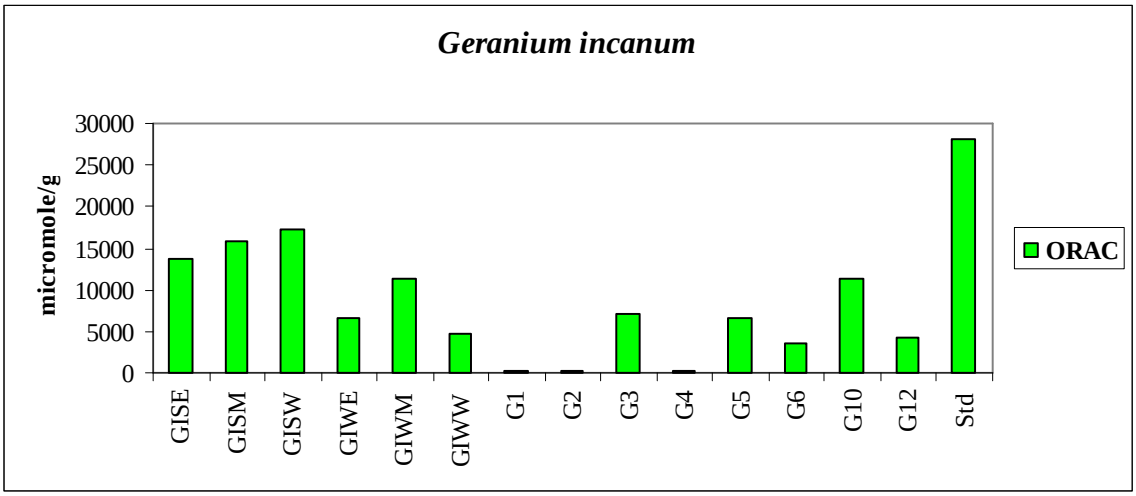


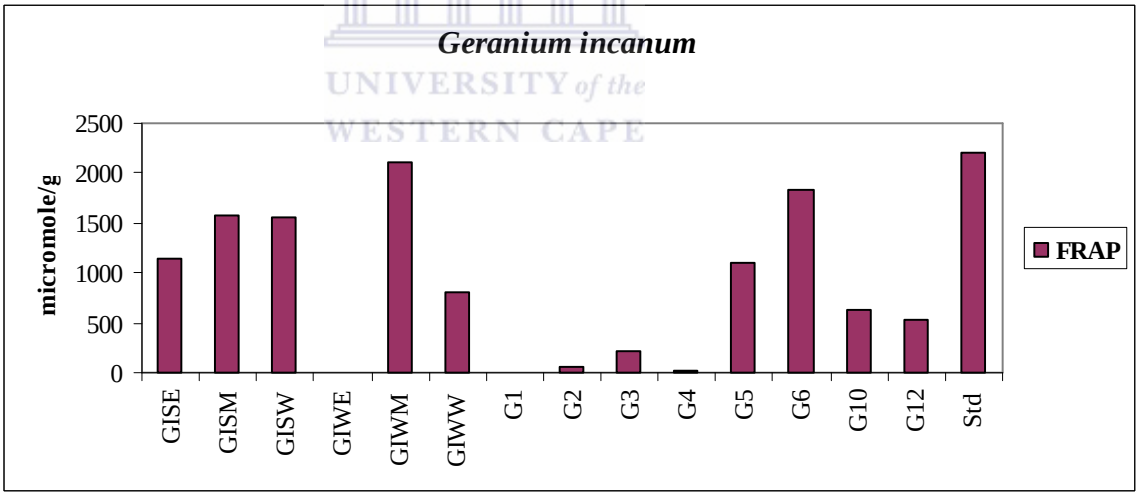
Figure 33D – Bar chart showing the amount of anthocyanins present in the various extracts of *G. incanum.*

The **ORAC** (Figure 33E), **FRAP** (Figure 33F) and **TEAC** (Figure 33C) followed the same pattern, where the antioxidant capacity or power were fairly distributed except for compounds **G1**, **G2** and **G4** with very low ORAC values as shown in Figure 33E. The same pattern is found in the FRAP and TEAC evaluations as shown in Figures 33F and 33C respectively. There is a reasonable indication that the methanolic extract of the winter collection would be a strong antioxidant as shown by the high value in Figure 33F.



Extracts and isolates of *G. incanum*

Figure 33E – Bar chart showing the ORAC values (The antioxidant capacity) of some extracts and isolates present in *G. incanum.*



Extracts and isolates of *G. incanum*

Figure 33F – Bar chart showing the FRAP values (Ferric reducing antioxidant power) of some extracts and isolates present in *G. incanum.*

CHAPTER 7

RESULTS AND DISCUSSION

7.1 *GETHYLLIS* SPECIES

The whole plants of *G. multifolia, G. villosa* and *G. gregoriana* were harvested in September 2007 from the Western Province in South Africa. These were dried, milled and extracted as illustrated in section 3.9.1 – 3.9.3 where only water, methanol and 70% ethanol were used in the extraction. The 70% ethanol was used just to mimic the method used in preparing the Koekemakranka brandy, a popular alcohol infusion drink used in traditional medicine to treat colic, flatulence and indigestion (Smith, C.A., 1966; Cillie, A.M., 1992; Rood, B., 1994; Watt and Breyer-Brandwijk 1962 and Van Wyk and Gericke, 2000).

Gethyllis gregoriana afforded a methanol (26.79 g, 8.37%) and water extract (18.92 g, 5.91%), ***Gethyllis multifolia*** afforded a methanol (17.98 g, 8.36%) and water extract (21.11g, 9.82%) and ***Gethyllis villosa*** yielded a methanol (10.65 g, 10.14%) and water extracts (7.43g, 7.08%).

The extracts were subjected to phytochemical screening as shown in section 3.5.0. Results indicated the presence of tannins, flavonoids, phenolics, saponins, anthraquinones, glycosides as well as essential oils in all the extracts while alkaloids were absent (Table 71) which is interestingly contrary to the observed trend in the Amaryllidaceae family acclaimed to contain alkaloids (Elgorashi and Van Staden, 2004).

Table 71:- Phytochemical screening result of the *Gethyllis* species

Test materials	Tann	Phen	Glyc	Sapo	Flav	Alka	Anth	Esse
GGM	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	P+ve	P+ve
GGW	P+ve	P+ve	P+ve	N-ve	P+ve	N-ve	N-ve	P+ve
GMM	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	P+ve	P+ve
GMW	P+ve	P+ve	P+ve	N-ve	P+ve	N-ve	N-ve	P+ve
GVM	P+ve	P+ve	P+ve	P+ve	P+ve	N-ve	P+ve	P+ve
GVW	P+ve	P+ve	P+ve	N-ve	P+ve	N-ve	N-ve	P+ve

GGM = *G. gregoriana* MeOH extract; **GGW** = *G. gregoriana* H_2O extract; **GMM** = *G. multifolia* MeOH extract;**GMW** = *G. multifolia* H_2O extract;**GVM** = *G. villosa* MeOH extract;**GVW** = *G. villosa* H_2O extract

7.2 BIOLOGICAL EVALUATION

7.2.1 Brine shrimp lethality bioassay of Gethyllis species

The brine shrimp lethality bioassay was conducted on all the extracts as shown in section 3.10.2.0. Results indicated that all the *Gethyllis* extracts were active except the water extract of *G. villosa* (Tables 72, 73 and 74). The highest value in this evaluation was recorded for *G. gregoriana* (0.223; Table 74) which confirmed the highly toxic nature of the Amarydaceae family (Muller-Doblies, 1986; Elgorashi and Van Staden, 2004). It should be noted that *G. gregoriana* was very active at all the concentration levels tested which is a clear indication that it may be a good antimicrobial and antiviral agent (Mc Laughlin et al., 1991; Kaur et al., 2009). It is further noteworthy that to the best of our knowledge, this is the first time *G. gregoriana* will be reported on for any bioassay and that cytotoxicity will be evaluated for the 3 plants in this report using the brine shrimp bioassay.

Table 72: Average number of dead nauplii after 24hours for *Gethyllis* species

Test materials	Vials at 1000 µg/ml			Vials at 100 µg/ml			Vials at 10 µg/ml			Vials for control expt.
	1	2	3	1	2	3	1	2	3	
GGM	0	0	0	0	0	0	0	1	0	10
GGW	1	1	1	3	4	4	7	6	7	10
GMM	0	0	0	0	1	1	3	3	4	10
GMW	2	2	2	4	3	4	6	7	8	10
GVM	0	1	0	1	1	0	3	4	4	10
GVW	9	8	9	10	9	10	10	10	10	10

Table 73: **Average number of survived nauplii after 24hours for *Gethyllis* species**

Test materials	Vials at 1000 µg/ml		Vials at 100 µg/ml		Vials at 10 µg/ml		Vials for control expt.
	Ave. surv.	% surv.	Ave. surv.	% surv.	Ave. surv.	% surv.	% surv.
GGM	0	0	0	0	0.33	3.33	100
GGW	1	10	3.67	36.67	6.67	66.67	100
GMM	0	0	0.67	6.67	3.33	33.33	100
GMW	2	20	3.67	36.67	7	70	100
GVM	0.33	3.33	0.67	6.67	3.67	36.67	100
GVW	8.67	86.67	9.67	96.67	10	100	100

Table 74: LC_{50} and average number of dead nauplii after 24hours for *Gethyllis* species

Test materials	Vials at 1000 µg/ml	Vials at 100 µg/ml	Vials at 10 µg/ml	**LC_{50}**	General remarks
GGM	10	10	10	**0.23**	**Very active**
GGW	9	6	3	120.66	Active
GMM	10	9	7	**6.20**	**Very active**
GMW	8	6	3	139.55	Active
GVM	10	9	6	**4.23**	**Very active**
GVW	1	0	0	1956.39	Inactive

7.2.2 Antimicrobial evaluation

The antimicrobial evaluation was carried out using the same diffusion method described for *Cissampelos capensis*. The assay was set up as described in section 3.10.3.1.

Table 75: Antimicrobial profile of *Gethyllis* species (The inhibition zones)

	Psa	Prv	Esc	Bas	Sta	Bal	Caa	Cae	Asn
GGM	41	34	29	32	30	33	27	29	23
GGW	16	19	19	22	19	14	19	15	17
GMM	18	21	26	19	18	18	10	11	11
GMW	10	13	11	12	10	11	0	0	14
GVM	15	13	19	22	28	30	15	17	17
GVW	0	0	0	0	0	0	0	0	0
Amx	61	44	49	42	53	47	0	0	0
Flu	0	0	0	0	0	0	46	53	39

The results showed that only *G. villosa* was inactive for all 9 organisms tested (Table 75). The maximum zone (41 mm) of antibacterial effect was observed in the methanolic extract of *G. gregoriana* against the Gram-negative organism *Pseudomonas aeruginosa*. The results obtained for the antimicrobial evaluation showed fair similarities with that of the cytotoxicity test.

7.3. CHEMICAL EVALUATION (ANTIOXIDANT ASSAY)

Total antioxidant evaluations were also carried out on all the extracts of *Gethyllis* species and which included the 70% ethanolic extracts. This was carried out in order to mimic the way the traditional healers usually administer the extract of Gethyllis (Muller-Doblies, 1986; Elgorashi and Van Staden, 2004).

7.3.1 Polyphenolic content

The polyphenolic content was also determined as shown in 3.11.2. The results showed that the amount of polyphenols present in the extracts were varied as shown in Figure 34A in which *G. gregoriana* has both the highest amount in the methanolic and ethanolic extracts while the lowest was recorded for *G. villosa*. This result further confirms the fact that methanol is a better solvent for the extraction of the polyphenolic content in Gethyllis as it was observed in most of the plant extracts in this project.

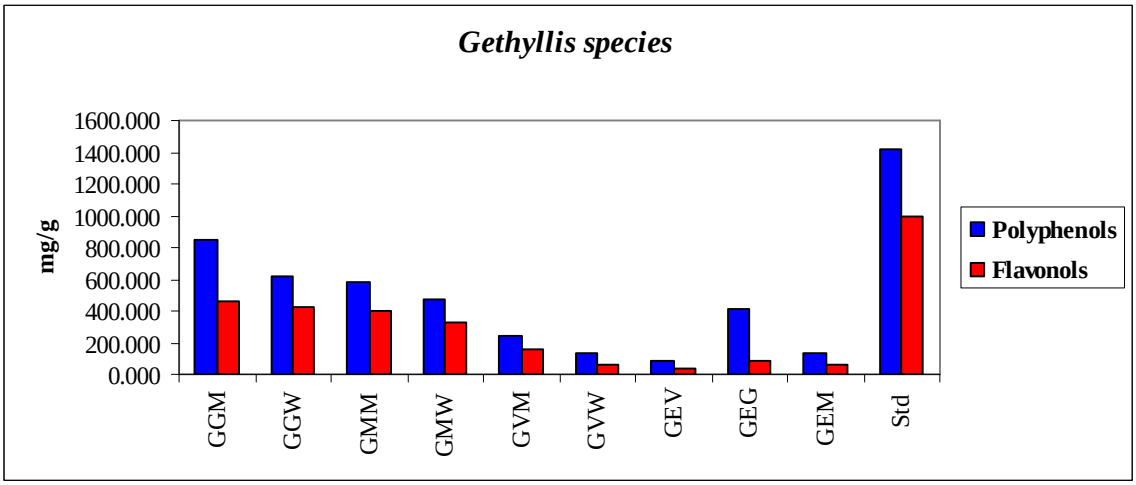


Figure 34A – Bar chart illustrating and comparing the amount of polyphenols and flavonols present in the various extracts of *Gethyllis* species.

GEV = *Gethyllis villosa* ethanolic extract
GEG = *Gethyllis gregoriana* ethanolic extract
GEM = *Gethyllis multifolia* ethanolic extract

7.3.2 Flavonoid content

A closely related profile was observed for the presence of flavonoids in the three plants except for flavanol which is higher in *G. multifolia* than in *G. gregoriana* (Figures 34A, 34B and 34C). The results further showed the presence of flavanone in higher concentration when compared to the standard (Figure 34C).

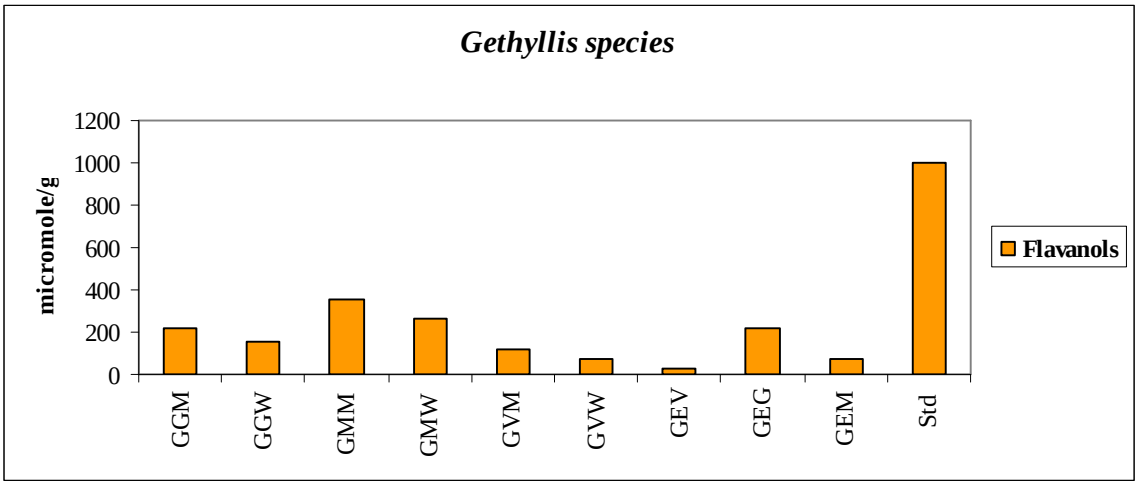


Figure 34B – Bar chart showing the amount of flavanols present in the various extracts of *Gethyllis* species.

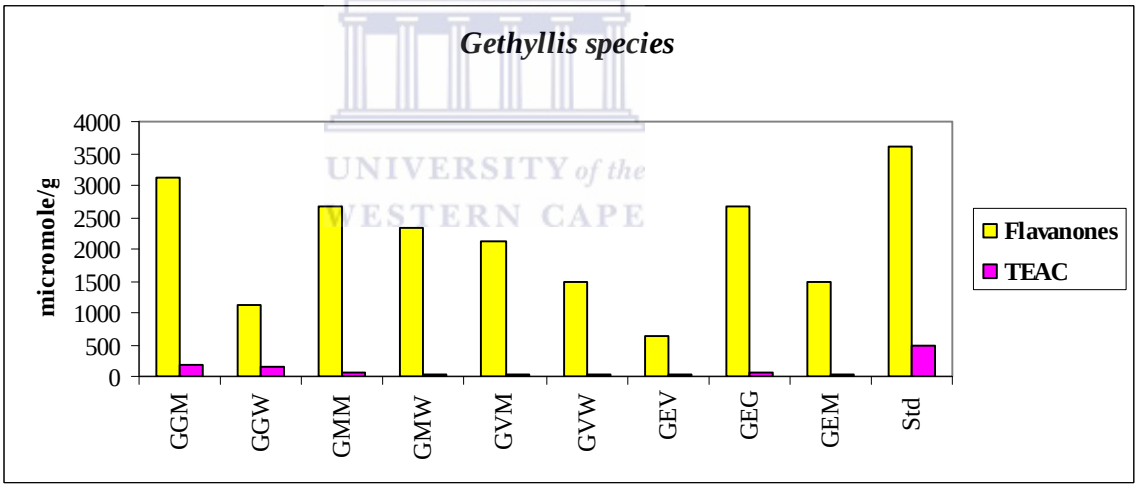


Figure 34C – Bar chart illustrating and comparing the amount of flavanones and the Trolox equivalent antioxidant capacity present in the various extracts of *Gethyllis* species.

7.3.4 Anthocyanin content

Results for anthocyanin content (Figure 34D) showed that only *G. multifolia* has an appreciable amount of anthocyanins in the methanol, water and ethanol extracts while it is completely absent in the water and methanol extracts of *G. gregoriana* but is present in fair concentration in its ethanolic extract as shown in Figure 34D.

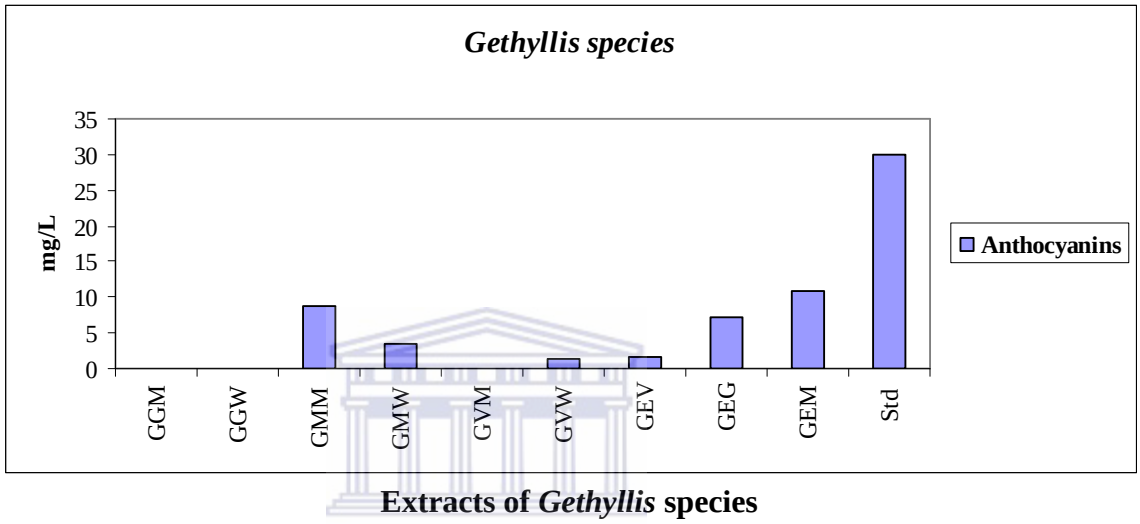


Figure 34D – Bar chart showing the amount of anthocyanin present in the various extracts of *Gethyllis* species.

The results for **ORAC** (Figure 34E), **FRAP** (Figure 34F) and **TEAC** (Figure 34C) follow a similar profile, where *G. gregoriana* still has the higest capacity except for some minor deviation in some cases as shown by FRAP where the *G. gregoriana* water and methanol extracts were nearly the same and the *G. multifolia* water extract was low as shown in Figure 34F.

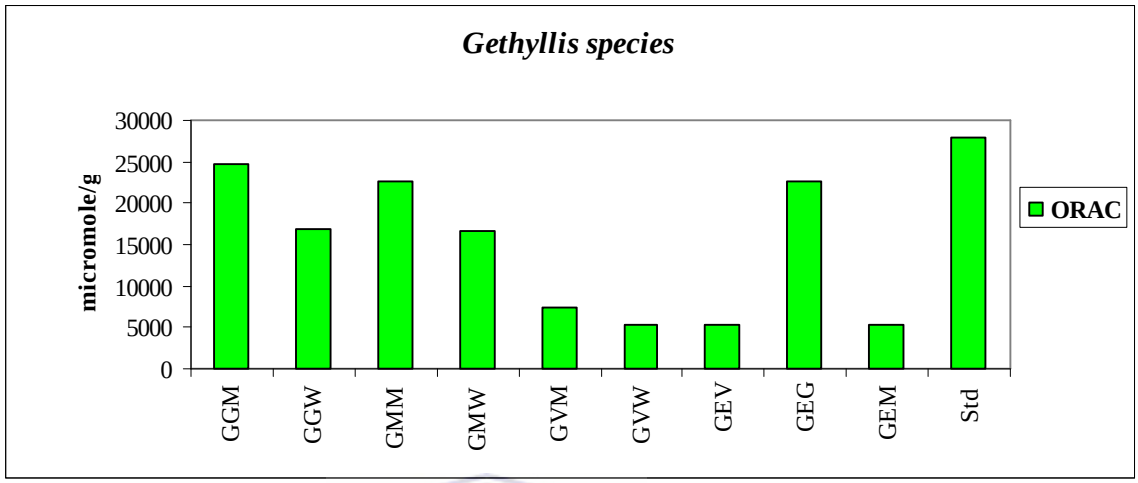


Figure 34E – Bar chart showing the ORAC values (The antioxidant capacity) of some extracts present in *Gethyllis* species.

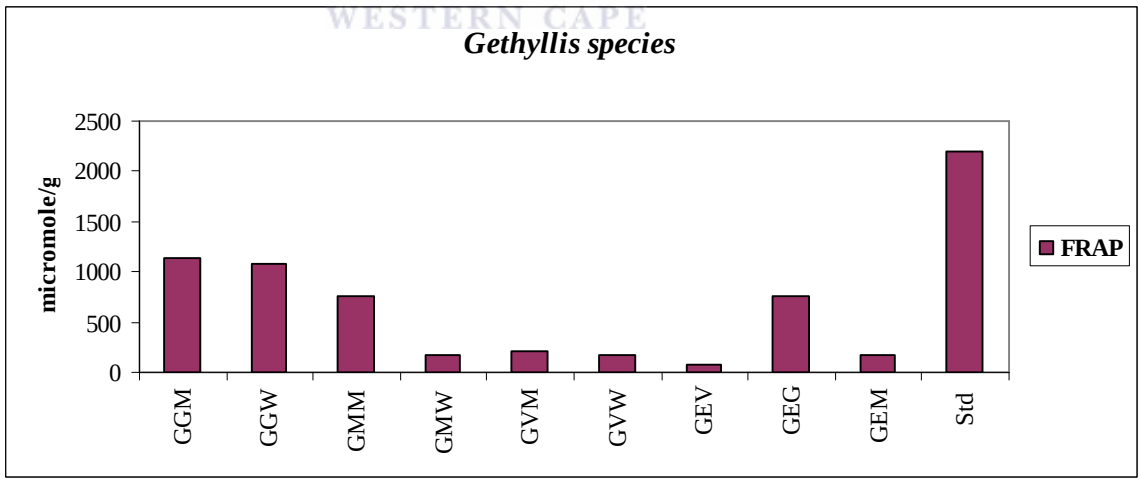


Figure 34F – Bar chart showing the FRAP values (Ferric reducing antioxidant power) of some extracts present in *Gethyllis* species.

CHAPTER 8
SUMMARY AND CONCLUDING REMARKS

In screening several plant species from an inventory of common medicinal plants from both South and West Africa for diverse medicinal purposes, 6 plants were selected because of their interesting and useful ethnomedicinal values. These plants are *Piliostigma reticulatum, Cissampelos capensis, Geranium incanum, Gethyllis gregoriana, Gethyllis multifolia and Gethyllis villosa.* This study successfully attempt to relate specific constituents present in these plants with their widespread ethnomedicinal uses. The extractions were carried out using hexane, dichloromethane, ethyl acetate, methanol and water. All the extracts were phytochemically screened for identification of secondary plant metabolites. The results revealed a variety of profiles indicating plants with or without alkaloids, tannins, flavonoids, glycosides, saponins, anthocyanins and essential oils. It was generally evident that anthocyanins was virtually absent in most of the extracts of the plants except *Gethyllis* while it was only *C. capensis* that contained alkaloid. Nearly all the plants contain flavonoids, saponins and polyphenols. Fractionation was carried out using standard chromatographic techniques. A total of thirthy seven (37) compounds were isolated from three of the plants namely: *Piliostigma reticulatum,* while only extractions were carried out on the *Gethyllis* species. Total of 18 isolates were characterised and their structures were unambiguously established by spectroscopic methods including infrared and ultraviolet spectroscopy, high resolution mass spectrometry and one- and two-dimensional nuclear magnetic resonance experiment. **(P1)** *6,8-di-C-methylquercetin- 3,7,*3'-*trimethyl ether*, (**P2)** *Piliostigmol.* (**P3)** *6, 8-di-C-methylkaempferol- 3,7-dimethyl ether* , (**P4)** *6,8-di-C-methylquercetin -3,*3'*-dimethyl ether*, (**P5)** *6,8,*3'*-tri-C-methylquercetin- 3,7-dimethyl ether*, (**P7)** *6-C-methylquercetin -3,7,3'-trimethyl ether*, (**P8)** *6-C-methylquercetin- 3-methyl ether*, (**P9)** *6,8-di-C-methylkaempferol- 3-methyl ether* and (**P13)** *Quercetin* were isolated from *P. reticulatum* while **C1)** *5,6-dehydro-4,5-dihydroxy-1,3,6-trimethoxy-17-methylmorphinan-7-one,* (**C2)** *1,2-methylenedioxy-3-hydroxy-9,10-imethoxyaporphine,* (**C3)** *5,6-didehydro-4-hydroxy-3,6-dimethoxy-17-methylmorphinan-7-one,* (**C5)** *3,7,8 3'-tetramethoxy- 6 - C-methyl- 5,4'-*

dihydroxyflavone (*6 -C-methylquercetin 3, 3',7, 8 –tetramethyl ether*), (**C6)** *5,7,8 -trihydroxy-2', 5'-dimethoxy-3',4' methylenedioxyisoflavanone* and (**C11)** *3 -methoxy-6 -C-methyl-3',4',5,7,8 -pentahydroxyflavone* (*6 -C- methylquercetin -3-methyl ether*) were from *C. capensis G. incanum* offered **(G5)** *Quercetin,* **(G10)***16α-hydroxy-(-) kauran-18-oic acid,* and **(G12)** *6,8-di-C-methylquercetin 3, 7-dimethyl ether*. Ten (10) of the isolated compounds were novel, including the first ever C-methyl-2-phenoxychromonol, Piliostigmol(2-O-(p-hydroxyphenyl)-3,5-dihydroxy-6-C-methyl-7-methoxychromone), the diterpene16α-hydroxy-(-)-kauran-18-oic acid, and five C-methylflavonols, isoflavanone, aporphine and a dehydromorphinandienone. The different extracts fractions, and isolates were tested for cytotoxicity using brine shrimp lethality test and antimicrobial capability using Nine microbes with three Gram –ve and +ve bacteria and three fungi which are Gram-negative bacteria were *Pseudomonas aeruginosa* (NCTC 10332), *Proteus vulgaris* (NCTC 4175) and *Escherichia coli Sero type 1* (NCTC 09001), while the Gram-positive were *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (NCTC 13134) and *Bacillus licheniformis* (NCTC 01097). The fungal species used were *Candida albicans* (ATCC 90028), *Candida eropiralis* (ATCC 750) and *Aspergillus niger* ATCC 10578. *Gethyllis gregoriana* showed the highest cytotoxicity level of LC_{50} = 0.23 where as it was observed that all the hexane fractions were not active while most activities resides in the methanolic extracts. The total tertiary alkaloid (TTA) showed the highest activity against the *Bacillus substillis* a Gram +ve bacteria at 45mm while the same trend in the cytotoxicity were equally observed in the antimicrobial where all the hexane fractions showed no activity against any of the nine pathogens. The *Piliostigma reticulatum* was also found to exhibit antimicrobial activity against some bacteria and fungi such as *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Bacillus subtilis* (NCTC 8236), *Proteus vulgaris* (NCTC 4175), *Aspergillus niger* (ATCC 10578) and *Candida albicans* (ATCC 10231). Piliostigmol, showed the highest activity against E. coli (MIC = 2.57 μg/ml, 0.006 μmol), which was found out to be three times more active than the Amoxicillin standard used. The total antioxidant assay also reveal a huge amount of information into the nature and properties of each extracts and isolates in which

the distribution of polyphenols, flavonoids and anthocyanins present in the plant extracts were reavealed. The ORAC, FRAP and TEAC showed intensively the antioxidant capacity of the extracts and isolates in which it was evident that the methanolic extracts and isolates obtained from them are good antioxidant agents. The results obtained for this study give a useful profile for development of a good antimicrobial and antioxidant agents for the future from the results obtained. The observed antimicrobial and antioxidant activities of the isolated compounds were correlated with their structures, and the structural requirements for activity in both test systems were defined. Finally, the traditional use of the extracts in infections and inflammatory conditions was rationalized based on the content of the isolated compounds, and it has been proposed that the total crude extract, with its content of so many bioactive compounds, could be formulated for use in skin infections, microbial, viral and inflammatory conditions.

This study has therefore contributed to the body of knowledge relating to the phytochemistry of some African medicinal plants in particular and the chemistry and biological activities of natural products in general. This research has successfully created a huge data bank concerning the nature and chemistry of the six plants especially the three plants that isolation and characterization were carried out on. The presence of large amount of flavonoids in *Piliostigma reticulatum*, alkaloids in *Cissampelos capensis* and tannins in *Geranium incanum* and the revelation of possessing both antimicrobial and antioxidant activities from the results generated has been used to rationalized the traditional uses of the plant in infections and inflammatory conditions. This rationalization is further supported by the high activity of these C-methylflavonols, especially Piliostigmol and 6-C-methylquercetin-3-methyl ether against the common wound pathogen *Staphylococcus aureus*, alkaloid from Cissampelos in treatment of cancer and related tumor cells as well as the finding that many of these compounds were potent as antioxidants as well as antimicrobial.

Since many of the isolated compounds are novel, and the biological activities of all the flavonoids have not been completely and extensively studied, there is a need to evaluate futher these compounds isolated from all the plants in other biological test system. Thus, the effect of piliostigmol on biliary excretion should

be determined, as the first 2-phenoxychromone isolated (capillarisin) was found to be a potent biliary excretion stimulant. The compounds should especially be evaluated in those conditions for which the plant is widely used, including cough, diarrhea and dysentery as recorded for *Geranium incanum* by Amabeoku 2009 and *Cissampelos* species (Amresh et al., 2004; 2007a; 2007b; 2007c).

It is also of interest to investigate the non-occurrence of an alkaloid in the Gethyllis species which are known to contain alkaloid from the family they came from (Elgorashi and Van staden, 2004; Viladomat et al., 1997; Van Wyk et al., 2008). It should be noted that there were so many extracts, fractions, subfractions and even isolates that has not been worked on which would be of interest to work on in the nearest future.

Finally, it can be expected that many of the crude extract, especially the methanolic extracts with its content of so many bioactive compounds will be more effective in treatment of various infections and inflammations than any individual constituent. The suggestion has therefore been made to formulate the various crude extracts into a suitable preparation for use in both microbial and viral infections especially in bacterial skin infections as indicated for *P. reticulatum*. Such a preparation will, if effective, provide a cheaper and less toxic alternative to the currently available drugs which are known to be expensive.

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