Phytochemical Analysis and Selected Biological Activity of *Phyllanthus parvulus* Sond. *var garipensis*

by

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BSc Chemistry, BSc (Honours) Chemistry

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KwaDlangezwa

2009

Declaration

I declare that this project was carried out by Clerentia Mbali Mdlolo, in the Department of Chemistry, under my supervision.

....30/3/09.

Signature Date

Dr O.A. Oyedeji

Supervisor

Dedication

I dedicate this work to all the people
who have assisted in making this research project a success

and

My family for their understanding, support and undying love for me.
Their faith in me and their prayers for my success, caused me to carry on
and not look back, even when things were not going too well.

Acknowledgements

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- My supervisor, Dr O A Oyedeji; Doc it is your support, love, guidance and encouragement that made me continue when I felt like stepping back and for that, I thank you so much;
- Prof. G A Kolawole for first introducing the plant to us.
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- The academic and technical staff of the Department of Chemistry, Botany, Biochemistry and Microbiology for their various contributions towards the success of this project;
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- The National Research Foundation and the University of Zululand Research Funding for the financial assistance without which this study would not have been possible; and most importantly
- I thank my God Almighty for His mercy and love for me; through it all His loving hand was always there, giving me all I needed to make this study possible – answering all my prayers and granting all my wishes. It is true Lord, your ear is always listening and you fulfill our requests.

Abstract

From the huge family of *Euphobiceae* stems a large *Phyllanthus* genus. The *Phyllanthus* genus has about 800 species world wide of which about 22 species are native to Southern Africa. A number of the *Phyllanthus* species have been reported to have history in herbal medicine systems, and studies have been done to support their use. Stemming from the South African species *is Phyllanthus parvulus* Sond. *var garipensis*, a shrub that is known botanically but, its medicinal potentials are unknown. This project focuses on the chemical and biological properties of *Phyllanthus parvulus* Sond. *var garipensis*.

The phytochemical study reveals that the plant possesses some secondary metabolites namely: alkaloids, saponins, flavonoids, tannins and antraquinones. A compound lanosterol-amide linked glycoside was isolated from the ethyl acetate fraction.

The hexane, chloroform, ethyl acetate, methanol, ethanol and water extract were tested for antibacterial and antioxidant activities. All the plant extracts showed activity with chloroform extract exhibiting high activity against the reference strains used. It exhibited high sensitivity greater than the standard compound ampicillin. The n-hexane and water extract had the least activities, while in the antioxidant studies the methanol and ethanol extract were the most active.

The toxicity of the extract was tested using the brine shrimp lethal test and was found not to be toxic after 12hours since less than 70% of the brime shrimps were still alive.

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Chapter One

1. Introduction

1.1. Medicinal Plants

Plants are a source of fuel, building materials, craftwork material, dyes, food supplements and medicine for people, all over the world. Plants used for medicine contain a wide range of substances that can be used to treat chronic illness as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants are still of great importance (Diallo *et al.*, 1999).

The medicinal value of plants lies in some chemical substances or group of compounds that produce a definite physiological action in the human body. These chemical substances are called secondary metabolites. The most important of these bioactive groups of plants are alkaloids, terpenoids, steroids, flavonoids, tannins and phenolic compounds (Edeoga *et al.*, 2005).

Western medicine is well-known and in use, but at the same time it has created problems due to some side-effects such as carcinogenicity caused by synthetic drugs. This has enhanced the interest in search for natural products with medicinal property e.g. naturally occurring antioxidants and antibiotics for use in foods and medicine. Therefore, phytotherapy has been considered an alternative to alleviate side-effects associated with synthetic drugs (Sanchez-Lamar *et al.*, 1999). Information on the chemical constituents does not only aid in discovering new therapeutic drugs, but such information can alsohelp in disclosing new sources of

economic materials such as tannins, oils, gums, that are precursors for the synthesis of complex chemical substances (Fansworth, 1996).

According to World Health Organization (WHO), traditional medicine is estimated to be used by up to 80% of the population of most developing countries. These plant-based medicines are used for primary health care needs (Bulletin WHO, 2002). Traditional medicine of late has been viewed by the pharmaceutical industry as a source of "qualified leads" in the identification of bioactive agents for use in the production of synthetic modern drugs. Between 25-50% of modern drugs are derived from plants. Although plants are unique in their activities but it has also been found that a particular plant may be used by different tribes or countries for different ailments, this shows that plants possess a very wide range of healing powers which are attributed to their chemical composition. Despite the wealth of human experience and folklore concerning the medicinal uses of plants, proper scientific investigation has only been applied to a small fraction of the world's plants. This is a cause of grave concern as plant species continue to disappear. The demand for medicinal plants is increasing in both developing and developed countries. A response to this situation is urgently needed to prevent the disappearance of plant species and the ethno-pharmacological knowledge that accompanies them. (de Silva, 1997).

For thousands of years, Africans have relied on medicinal plants and knowledge thereof, to treat ailments (van Wyk *et al.*, 1997). The use of medicinal plants predates the introduction of antibiotics and other modern drugs into the African continent. Africans have been able to cure a lot of diseases by using concoctions made from different plants and, these have been passed from generation to generation. Indigenous medicinal plants are used by more than 60% of

South Africans in their health care needs or cultural practices South Africa has over 10% of the world's flora and is rich in the knowledge of their use; approximately 3,000 plant species are used by an estimated 200,000 indigenous traditional healers (http:// herbalafrica.co.za/Index.htm). Common indigenous medicinal plants used include *Agathosma betulina, A. crenulata,* Aloe *ferox, Artemisia afra, Balanite maughamii, Bersama tysoniana, Boophane disticha, Bowiea volubilis, Cassine papillosa, Clivia miniata, Cryptocarya latifolia, Gunnera perpensa* and *Siphonochilus aethiopicus,* to mention a few (Coetzee *et al.*, 1999).

The use of medicinal plant by a large number of South African has led to the sales of plants part in open market of which Durban Muthi Market is one. In a case study, conducted by Myles Mander in 1998, revealed that more than 700 indigenous and 20 alien plant species are sold for medicinal purposes in the KwaZulu-Natal Province alone. In the study he also found out that the black population in Durban has indicated that they rely on both health care systems, giving a 60:40 percentage split in favour of Western health care systems to traditional medicine. The ratio is relatively close, indicating that a large number of people still depend on traditional medicine. This is despite the fact that indigenous medicine is more expensive than subsidized Western health care provided by the Government. It is estimated that about six million indigenous medicine consumers are in KwaZulu-Natal and 27 million in South Africa. Households are spending between 4-6% of their annual income on indigenous medicine and services. A massive demand is thus generated in terms of the number and mass of plants consumed. In KwaZulu-Natal more than 4000 tonnes of plant material is traded annually at a value of R60 million (a third of the value of the annual maize harvest for the province). Nationally, 20 000 tonnes may be traded annually, valued at R270 million. Urban consumers

anticipate that their consumption of indigenous medicine will either remain at current levels or increase. This implies that indigenous medicine is a basic consumer good and often essential for the welfare of Black households (Mander, 1998).

1.1.1 *Phyllanthus* species

The plant genus, *Phyllanthus* (Euphorbiaceae), is widely distributed in most tropical and subtropical countries. It is a very large genus of herbs, shrubs or trees, monoecious or sometimes dioecious consisting of approximately 550 to 800 species. It is subdivided into 10 or 11 subgenera: *Botryanthus, Cicca, Co-nani, Emblica, Ericocus, Gomphidium, Isocladus, Kirganelia, Phyllanthodendron, Phyllanthus* and *Xylophylla* (Unander *et al.*, 1995, Calixto *et al.*, 1998). About 22 species are native to Southern Africa (Archer, 2000).

The Southern Africa species are common through Zimbabwe, Malawi, Zambia, Mozambique, South-West Africa and Angola. Several trees are in the Kruger National Park e.g. *Phyllanthus amapondensis* (red pepper) (Pretoria, 1988). *Phyllanthus cedrifoluis* is confined to the forests of the Transkei and the Ngoye forest in Zululand.

Phyllanthus parvulus Sond. *var garipensis* (Fig. 1) is, however, indigenous to South Africa, though it is well-known botanically and regarded as weed. It is a biennial shrub that grows to about 0.9 m; it has a greenish-brown stem and glabrous leaves. This plant can be found in low altitude hot dry scrubland and Themeda triandra grassland with scattered trees, also on fixed coastal sand dunes sea-level to 900 m. Within South Africa, *P. parvulus* Sond. *var garipensis*

can be found in the Limpopo, Cape and KwaZulu-Natal Provinces. It is very abundant within the University of Zululand campus.

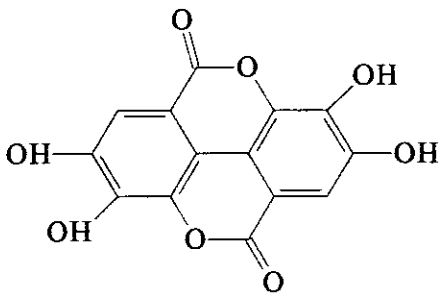
Fig 1: *Phyllanthus parvulus* Sond. *var garipensis* (***E. Mey. Ex Drege)*** in its natural habitat (left), a closer view (right).

1.2 Literature Review

1.2.1. Phytochemistry

The *Phyllanthus* is a genus of species with high medicinal value in which bioactive compounds have been isolated and used in treating some bacterial or viral infections. Unander in his book referred to the *Phyllanthus* species as a source of new antiviral compounds and showed the versatility of this genus (Unander *et al.*, 1990). A wide range of plant species belonging to the genus *Phyllanthus* has been phytochemically investigated. Among the widely studied species are *P. niruri, P. urinaria, P. emblica, P. flexuosus, P. amarus* and *P. sellowianus.* Several compounds such as alkaloids, tannins, flavonoids, lignans, phenols and terpenes have been

isolated and identified in various species of *Phyllanthus* and have shown antinociceptive action in mice and other therapeutic activities (Rojas *et al.*, 2005). Some of the compounds reported include: Ellagic acid, geraniin, phyllemblin and phyllanthoside (Fig. 2).



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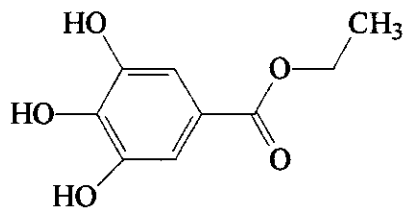

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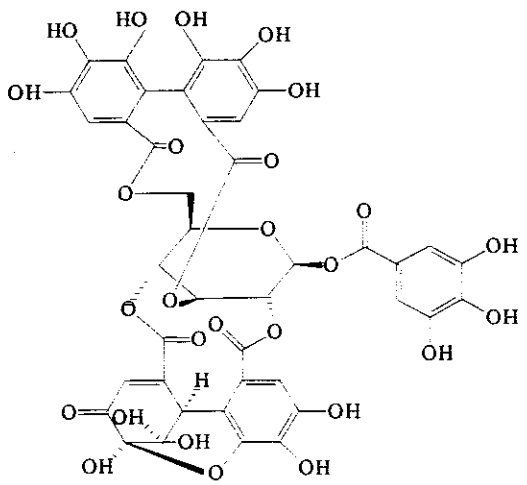

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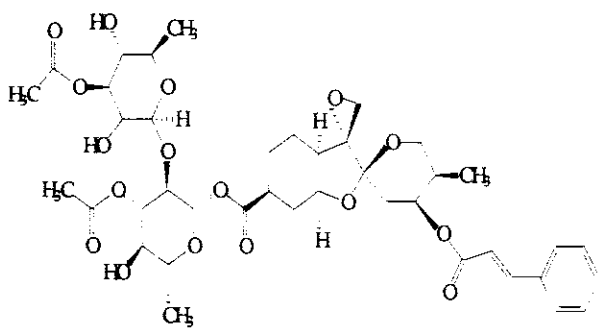

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Fig 2: Ellagic acid (1), Geraniin (2), Phyllemblin (3) and Phyllanthoside (4)

A dichloromethane extract of the leaves of *Phyllanthus polyphyllus* var. *siamensis* afforded two new monoacetylated triterpene arabinosides. The isolates and synthesized derivatives

from the isolates were evaluated biologically and, the presence of an arabinosyl moiety among the hederagenin glycosides evaluated was found to be important for the modulation of cytotoxic activity against cancer cells (Youkwan *et al.*, 2005).

Two tannins namely pinocembrin-7-O-[4", 6"-(S)-hexahydroxydiphenoyl]-D-glucose and pinocembrin-7-O-[3"-O-galloyl-4", 6"-(S)-hexahydroxydiphenoyl]-D-glucose were isolated from *Phyllanthus tenellus* (Huang *et al.*, 1998). A lignan neonirtetralin was isolated from *P. niruri* Linn (Wei *et al.*, 2002). From the preliminary investigation and literature survey, no record was found on the biological or phytochemistry of *P. parvulus* Sond. *var garipensis*.

1.2.2 Ethnopharmacology

The use of *Phyllanthus* plant in folklore medicine can be dated back to the 18th century. Researches interest on *Phyllanthus* genus is largely attributed due to the wide distribution of the genus in many tropical and subtropical countries, large number of species availability, diversity of secondary metabolites and their broad therapeutic use in folk medicine

Studies have supported the use of *Phyllanthus* genus in traditional medicine which in turn has revealed the potential and beneficial therapeutic actions in the management of certain illness and diseases such as inflammatory reactions, intestinal problems, hepatitis B, kidney and urinary problems (Calixto *et al.*, 1998). In India, there has been a great interest in the *Phyllanthus* genus regarding their therapeutic potential for the management of a number of diseases (Kumaran *et al.*, 2007). Infusion of leaves, stem and roots of *Phyllanthus* species are commonly used in folk medicine in Brazil, for treating intestinal infections, diabetes, hepatitis

B virus and alignment of the kidney and urinary bladder. Antiviral effects of the plant extract against the hepatitis B virus and possibly against the reverse transcriptase of retroviruses have also been reported (Thygarajan *et al.*, 1998; Venkateswaran *et al.*, 1987 a&b; Shead *et al.*, 1992).

Most *Phyllanthus* species have been reported to have pharmacological properties, *e.g. P. niruri* has been reported to exhibit *in vitro* antibacterial action against *staphylococcus, micrococcus* and *pastuurella* bacteria as well as *in vivo* and *in vitro* antimalarial properties (Veeramuthu *et al.*, 2006). Extracts of *P. emblica* showed good inhibitory effects against *Staphylococcus aureus, S. typhosa and Candida albicans* (Khanna *et al.*, 1973). The lyophilized aqueous extract (LWE) from the leaves of *P. discoideus* was found to show antibacterial activity. The alkaloid fraction obtained from LWE also inhibited the growth of *Escherichia coli, Enterococcus faecium, Pseudomonas aeruginosa, Staphylococcus aureus* and *Mycobacterium smegmatis* (Mensah *et al.*, 1998).

Extracts of *P. emblica* L. have been reported to possess several pharmacological actions, *e.g.* analgesic, anti-inflammatory, antioxidant, chemoprotective, hypolipidaemic and anti-HIV-1 (Human immunodeficiency virus-1) (Asha *et al.*, 2007). Additional studies on *Phyllanthus* callus and roots extracts of different species, have indicated the presence of phyllembin, a tannin (hydrolysable tannin) with antimicrobial activity and also inhibits DNA polymerase, HIV reverse transcriptase and angiotensin-converting enzyme involved in diabetic complications (Unander *et al.*, 1995; Ogata *et al.*, 1992). Geraniin and derivatives of geraniin have been reported to be highly active in the inhibition of HIV reverse transcriptase (Ueno *et*

al., 1998). Hexane extracts of *P. maderaspatensis* revealed significant hepatoprotective effect on carbon tetrachloride and thioacetamide induced liver damage in rats (Santos *et al.*, 1994). Pharmacological studies carried out with callus extracts of *P. niruri*, *P. tenellus* and *P. urinaria* have shown antinociceptive properties and the main compounds identified in the extracts were flavonoids, tannins and phenols (Pettit, 1990).

Glycosides isolated from *Phyllanthus acuminatus* have been reported to exert antitumor activity on murine P-388 lymphocytic leukemia and B-16 melanoma cell lines (Powis and Moore, 1985; Lee *et al.*, 1996). *P. amarus* inhibited hepatitis B virus polymerase activity, decreased episomal hepatitis B virus DNA content and suppressed virus release into culture medium (Venkatewaran *et al.*, 1987). An aqueous extract of *P. niruri* inhibited DNAp of HBV and binded to the surface antigen of hepatitis B *in vitro*. The extract also inhibited woodchucks hepatitis virus (WHV) DNAp and binds to the surface antigen *in vitro* (Da Wei *et al.*, 2002).

1.2.2.1 General traditional uses of the *Phyllanthus* species:

According to literature and interviews conducted, the *Phyllanthus* species are commonly used to expel kidney stones, support kidneys, increase urination, relieve pain, protect and detoxify the liver, reduce spasms and inflammation, kill viruses and bacteria, aid digestion, reduce fever and blood sugar, lower blood pressure and cholesterol, treat malaria, prevent mutation, as a mild laxative and as a worm expellant.

1.3 Research Problem

Three *Phyllanthus* species were found growing wildly in the University of Zululand Campus and these are commonly used by traditional healers in the area for the treatment of diabetics and kidney problem. Traditional healers admitted that the three plants are different but that they are used commonly and interchangeably for the same or almost the same aliment There is also the rumor that a particular species of the South African *Phyllanthus* is toxic, hence its unpopularity amidst most Healers despite its acclaimed used by some countries. Due to their close resemblance, the three species were taken for identification at the University of Zululand Herbarium, Natal Herbarium in Durban and National Herbarium in Pretoria. The three species were first identified as synonyms of *P. burchellii.* Preliminary screening indicates that the three plants were different thus our curiosity in identifying the different species. Two species were later identified as *P. parvulus sond var garipensis* and *P. burchellii,* while the third species is yet to be identified. While the identification process was in progress at the National Herbarium in Pretoria, phytochemical and antimicrobial screening was carried out on the first two species identified at UniZulu Herbarium and Durban Herbarium. The results of these screenings confirmed that the two plants were not the same even though they are often reported as synonyms (Mdlolo *et al.*, 2008). This indicated that the plants were not synonyms.

The use of these plants by traditional healers interchanging was worrisome coupled with the slim literature reports of the South African species which was limited to morphology and botanical survey with no chemical information on the constituents and my preliminary finding; arouse a research interest in me. Furthermore, *Phyllanthus* species are used in

countries like China, India, America, Brazil Germany, and Nigeria for use for the treatment numerous illnesses such as cancer, diabetes, cholesterol and hepatitis B; some of which are life-threatening diseases in South Africa. These countries are into packaging and selling of *Phyllanthus* plants at pharmacies and yet the South African species remain unknown. A study of the chemical composition and biological activities is therefore eminent to ascertain the ethnomedicinal and pharmaceutical potentials of the South African species for economic and international recognition. This research project focuses on one species, which is *Phyllanthus parvulus* Sond. *var garipensis.*

1.4 Research Aims

This project was aimed at identifying and analyzing the phytochemicals present in *P. parvulus* Sond. *var garipensis*, and evaluation of some biological properties of the plant with the following specific objectives:

i. To carry out qualitative and quantitative phytochemical analysis of the secondary metabolites present in *P. parvulus* Sond. *var garipensis.*
ii. Isolate some compounds in *P. parvulus* Sond. *var garipensis*
iii. Identify and elucidate the isolated compounds using Mass Spectrometry (MS), Fourier transformer Infrared (FTIR), Ultraviolet Spectrometry (UV), Nuclear Magnetic Resonance (NMR) and X-ray (if need be).
iv. Conduct the antimicrobial and antioxidant assays of the plant for biological evaluation.
v. Determine the toxicity of the plant using Brine Shrimp Lethal test.

Chapter Two

2. Experimental

2.1 Plant Collection

Mature whole plant sample was collected at the University of Zululand, KwaDlangezwa Campus and was identified, by Dr. Stefan Siebert, as *Phyllanthus parvulus* Sond. *var garipensis* and authenticated at Natal Herbarium and Pretoria National Herbarium. The voucher specimen (number 197) was deposited at the University of Zululand Herbarium. The plant material was dried at room temperature and used for all the extracts prepared.

2.2 **Phytochemical Screening** (Edeoga *et al.*, 2005; Harbone, 1973)

2.2.1 Qualitative

Plant filtrate was prepared by boiling 20 g of the fresh plant in distilled water. The solution was filtered through a vacuum pump. The filtrate was used for the phytochemical screening of flavonoids, tannins, saponins, alkaloids, reducing sugars, anthraquinones and anthocyanosides.

Alkaloids

1 ml of the plant filtrate was mixed with 2 ml of Dragendoff's reagent; a turbid orange colour indicated the presence of alkaloids. The confirmation test was done using Mayer's reagent; a yellow precipitate indicated the presence of the alkaloids.

Tannins

1 ml of the filtrate was mixed with 2 ml of $FeCl_3$; a dark green colour indicated a positive test for the tannins.

Saponins

1 ml of the plant filtrate was diluted with 2 ml of distilled water; the mixture was vigorously shaken and left to stand for 10 min during which time, the development of foam on the surface of the mixture lasting for more than 10 min, indicates the presence of saponins.

Anthraquinones

1 ml of the plant filtrate was shaken with 10 ml of benzene; the mixture was filtered and 5 ml of 10% (v/v) ammonia was added, then shaken and observed. A pinkish solution indicates a positive test.

Anthocyanosides

1 ml of the plant filtrate was mixed with 5 ml of dilute HCl; a pale pink colour indicates the positive test.

Flavonoids

1 ml of plant filtrate was mixed with 2 ml of 10% lead acetate; a brownish precipitate indicated a positive test for the phenolic flavonoids. While for flavonoids, 1 ml of the plant filtrate was mixed with 2 ml of dilute NaOH; a golden yellow colour indicated the presence of flavonoids.

Reducing Sugars

1 ml of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown colour with Fehling B and a green colour with Fehling A indicate the presence of reducing sugars.

2.2.2 Quantitative Analyses

Saponins

About 20 g each of dried plant samples were ground and, put into a conical flask after which 100 ml of 20% aqueous ethanol was added. The mixture was heated using a hot water bath, at about 55 °C, for 4 h with continuous stirring, after which the mixture was filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and then shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated three times. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a waterbath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material.

Flavonoids

About 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol, at room temperature. The whole solution was filtered through Whatman filter paper No 42. The filtrate was later transferred into a crucible and evaporated into dryness over a water bath; the dry content was weighed to a constant weight.

Tannins

About 500 mg of the plant sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h on a mechanical shaker. This was then filtered into a 50 ml volumetric flask and made up to the marked level. Then, 5 ml of the filtrate was transferred into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min. The tannins content was calculated using a standard curve of gallic acid.

Alkaloids

5 g of the plant sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, the reaction mixture was covered and allowed to stand for 4 h. This was later filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered; the residue being the alkaloid, which was dried and weighed to a constant mass.

2.3 Extract Preparation

Two extraction methods were used for the plant sample; this was done to assess which method would give larger amounts of the extracts and purer compounds during isolation. All the solvents used in the extraction processes were purchased from Merck Chemical (Pty) Ltd Laboratory Supplies Division (Durban) and Capital Lab Supplies CC (New Germany).

2.3.1 Method 1

About 515.32 g of the fresh plant was collected within the University of Zululand Campus between the months of February and March 2007 and was air-dried at room temperature. Fig. 3 is a schematic diagram of the extraction procedure for the preparation of different fractions.

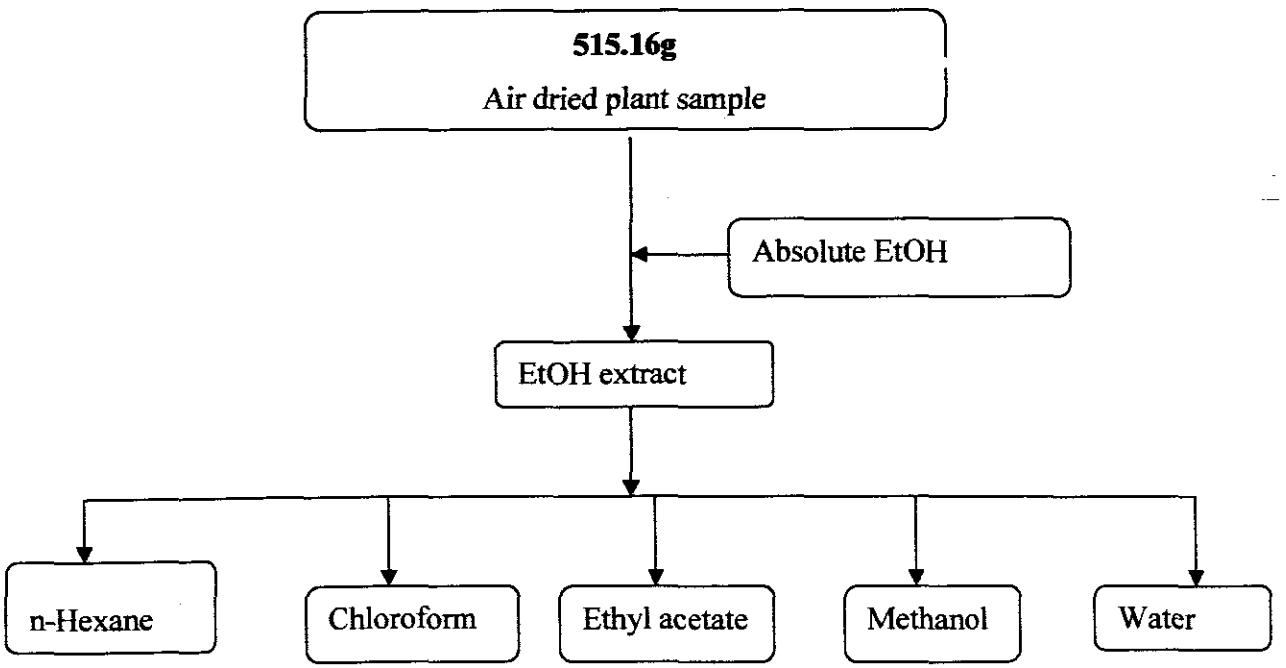

Fig. 3: Route 1 Extraction Method

The air-dried plant sample was soaked in absolute ethanol for 48 h, with continuous shaking in an electric shaker. After 48 h the extract was subsequently filtered and concentrated *in vacuo* at 40 °C. About 49.16 g of the crude ethanol extract was obtained. This crude extract was then partitioned successively into five fractions using n-hexane, chloroform, ethyl acetate, methanol and water (Fig 3). The partitioning was achieved by first dissolving the ethanol extract in water after which n-hexane was added to the solution; the mixture was shaken vigorously and poured into a separatory funnel, shaken and then allowed to rest; two

layers were observed, the lower layer (organic layer) was transferred into a 500 ml conical flask; this was done repeatedly until the organic layer (n-hexane) was clear. The same procedure was performed subsequently for ethyl acetate, chloroform and methanol.

2.3.2 Method 2

About 509.76 g plant sample was collected at the University of Zululand Campus during the month of November 2007. The plant sample was air-dried at room temperature. Fig 4 shows a schematic diagram of the method used.

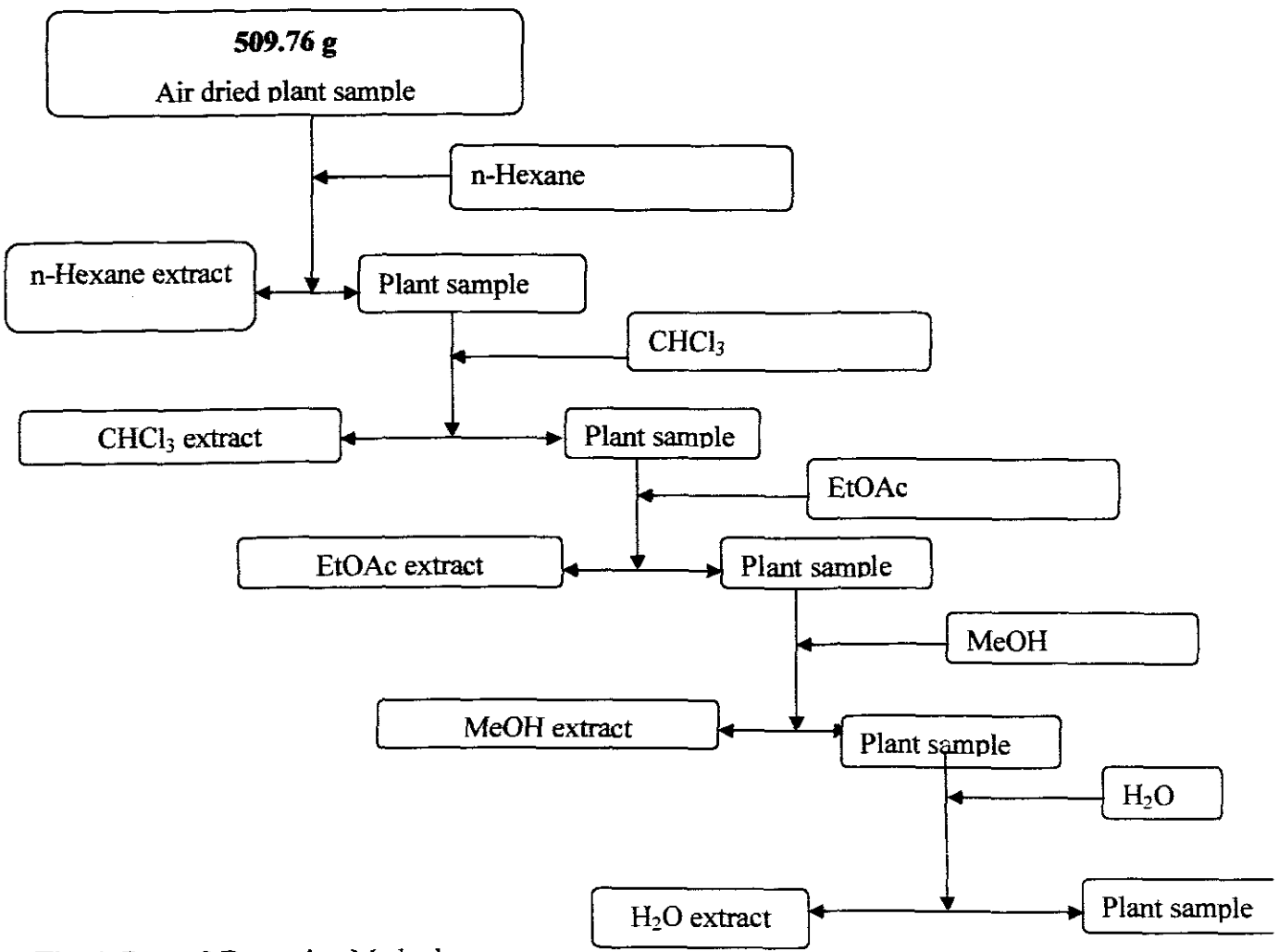

Fig. 4: Route 2 Extraction Method

The air-dried plant sample was extracted with n-hexane three times until the solvent became clear. The extract was filtered and concentrated *in vacuo* at 40 °C. The same procedure was applied consecutively to chloroform, ethyl acetate, methanol and water.

2.4. Isolation

2.4.1 Materials

All the chemicals used were purchased from Merck Chemical (Pty) Ltd Laboratory Supplies Division (Durban) and Capital Lab Supplies CC (New Germany).

i. Silica gel 60 0.040-0.063 mm (230-400 mesh ASTM)

ii. Silica gel 60 0.063-0.200 mm (70-230 mesh ASTM)

iii. Silica gel 60 0.2-0.5 mm (30-70 mesh ASTM)

iv. Purified sand

v. TLC aluminium sheets 20 x20 cm Silica gel 60 F_{254}

vi. Columns of different sizes

vii. FT-IR

viii. UV-Visible

ix. NMR

2.4.2. Isolation Techniques

In this study, three isolation methods or techniques were routinely used, namely:

- Thin Layer Chromatography (TLC)
- Vacuum Liquid Chromatography (VLC)
- Column chromatography (CC)

2.4.2.1. Thin Layer Chromatography (TLC)

TLC is primarily used as an inexpensive method for separation, qualitative identification or for the semi quantitative visual analysis of samples. TLC chromatographic technique gives a clue as to how many components are in a mixture or an extract. It is also used to determine the purity of isolated compound. Commercially prepared TLC aluminum sheets of 20 x 20cm Silica gel 60 F_{254} was used. The plate was cut to size of 5 x 5cm and the extract was spotted at the bottom the TLC plate (about 0.5cm from the base). The plate was then placed in a developing tank containing chosen solvent system. The initial solvent system used was 100% hexane. The polarity of solvent system was increased by adding ethyl acetate at various ratios of 95:5, 90:10, 85;15, 80:20 until 50:50 ratio of heaxane:ethyl acetate respectively was achieved. The spots were developed using UV light, iodine vapor and anasaldehyde. This method afforded 12 different spots.

2.4.2.2. Vacuum Liquid Chromatography (VLC)

This method is used to group compounds present in an extract according to their similar functional group. The column was prepared in a sintered glass funnel using TLC grade packing silica gel. Uniform packing is achieved by initially tapping the funnel and followed by application of a vacuum below the funnel. The sample was then applied uniformly at the top of the support. Step-gradient elution was achieved by using solvent with varying polarity. Each eluting gradient was collected allowed to dry and then used for the column chromatography.

2.4.2.3. Column chromatography (CC)

This is preparative application of chromatography which is used to isolate pure chemical compounds from a mixture of compounds, on a scale from micrograms up to kilograms depending on the size of column used. A glass tube with a diameter 50 mm and a height of 50 cm with a tap at the bottom was used for the column chromatography technique. A plug of cotton wool was placed at the bottom of the column very close to the tap so as to prevent the stationary phase from blocking the column.

The adsorbent which is the silica gel (70-230 mesh size) was weighed using a ratio of 30 gm of the adsorbent to 1gm of the crude. The column was filled with a little quantity of hexane. Slurry of weighted adsorbent was prepared using 100% hexane and then carefully poured into the column. Air bubbles were prevented from forming. A solution of the extract was then pipetted on top of the stationary phase. This layer was topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the flow of the eluting solvents. The eluting solvent initially was 100% hexane and the polarity was gradually increase at a 95:5 hexane:ethyl acetate ratio until a 50;50 ratio was used. About 25mls of eluting mixture was collect until there appears to be no more solute in the column. The fractions collected were monitored by TLC and similar fractions were combined together. Solvent was then removed from the bulked fraction, allowed to dry and weighed. TLC analysis was carried out on the semi dry bulked fraction using various polar solvent to ensure the purity of the fraction.

2.4.3. Isolation of compound

A small amount of the ethyl acetate extract from method 1, was dissolved in 5 ml of the Ethyl acetate solvent. TLC of this extract was carried out; using different solvent systems of 100% n-hexane, 9:1, 8:2, 7:3, 5:5 and 1:9 n-hexane:ethyl acetate, a maximum number of 13 compounds appeared on the TLC plate after spraying with anasaldehyde and placing the plate in the oven for about 10 min. 6 g of the ethyl acetate extract was subjected to Vacuum Liquid Chromatography (VLC). Silica gel 60 0.063-0.200 (70-230 mesh ASTM) and silica gel 60 0.04-0.063 (230-400 mesh ASTM) were used, about a hundred fractions of 250 ml were collected and the TLC of each was done. Those with same or similar TLC solute pattern were bulked and concentrated *in vacuo*. Fractions 12-16 (96:4) n-hexane: ethyl acetate showed seven-spots with one prominent blue spot. The solvent was allowed to dry completely and brownish yellow crystals with a mass of 25 mg were formed. The crystals were further subjected to open column chromatography using silica gel 60 (0.040-0.063mm), 95:5 n-hexane and ethyl acetate as the mobile phase. 24 fractions of 30 ml were collected. Fractions 12-20 collected from the column chromatography showed a single blue spot with some impurities on the TLC plate, after spraying with anasaldehyde. This spot had a retention factor of 0.29. The fractions were bulked and concentrated *in vacuo* and light yellow crystals formed when the solvent was completely evaporated. The crystals were further purified by dissolving them in hexane after which the mixture was placed in ice for recrystalisation. The solvent was decanted and the same procedure was repeated until the crystals were white. When dissolved in hexane and spotted on the TLC plate, a single blue spot was observed after spraying and heating. The white crystals, weighing about 5 mg, were obtained. Melting point

measurements were done and the compound was taken for further analyses with FT-IR, UV-Visible and NMR.

2.5 Biological Studies

The plant extracts from method two were used for antimicrobial screening and antioxidant activity.

2.5.1 Antimicrobial Activities

Resistance of some microorganisms to treatment is a growing problem; some of this being due to overuse of antibiotics by humans and some due to the use of antibiotics as growth promoters in foods for animals (Johnson *et al.*, 2006). Resistance to β-lactam antibiotics has become more serious in recent decades as strain producing extended-spectrum β-lactamases render many, if not all of the penicillins and aphalosporins, ineffective as therapy. Again "nosocomial" or "hospital- acquired infection" is alarming in the 21st century as antibiotic resistance spreads. Of late, the focus has gone back to the use of plants that have been used by different groups of people, to treat some of the infections that are caused by bacterial strains. The *phyllanthus* species have been reported to posses some antiviral and antibacterial properties; therefore it was eminent to evaluate the antimicrobial properties of *P. parvulus* Sond. *var garipensis*, since there is no information on this plant.

In this project two methods were used to evaluate the antimicrobial activities of the plant extracts:

i. The agar disc diffusion and

ii. Minimum inhibitory concentration (MIC)

Reference isolates of *Escherichia coli* ATCC 8739, *Pseudomonas aerugenosa* ATCC 19582, *Enterobacter cloacae* ATCC 13047, *Bacillus cereus* ATCC 10702, *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 29212, *Streptococcus faecalis* ATCC 29212, *Serratia marcescens* ATCC 9986, *Shigella flexineri* KZN, *Salmonela* spp. KZN, *Micrococcus kristinae, Micrococcus leteus* and *Acinecto calcococeuticus anatratus* CSIR were obtained from the University of Fort Hare, Department of Microbiology.

2.5.1.1 Agar Disc Diffusion Method

20 mg/ml of each extract was prepared by dissolving 0.2 g of the plant extract in 10 ml of the solvent used for extraction. 1 mg/ml of ampicillin was used as the positive control while the solvent was used as the negative control for each extract. This experiment was done in triplicate. Muller Hinton agar was prepared by dissolving 38 g of the agar in 1000 ml of distilled water and autoclaved at 121 °C for 15 min. After 15 min., the agar was allowed to cool for a while and 15ml of the agar was dispensed into the plates. The agar was allowed to solidify and 100 µl of the microorganism was inoculated on the agar plate. A paper disc that had been soaked in the plant extract was placed at the center of the plate. The plates were incubated for 24 h at 37 °C and the zone of inhibition measured; the zone of inhibition of the negative controls was subtracted from the zone of the plant extracts so as to find the true zone of inhibition of the extract (Chan *et al.*, 1993, Afolayon *et al.*, 1997).

2.5.1.2 Minimum Inhibitory Concentration (MIC)

The 96 well microtitre plates were used using Ellof method (Ellof, 1998). Distilled water, nutrient broth and pipette tips were autoclaved and the cultures of bacteria were prepared and

left for 24 h to grow. Dilute concentrations (50:50) of bacterial strains were used. A stock solution of each plant extracts was prepared by dissolving 0.4 g of the extract in 10 ml of the solvent; 1 mg/ml of ampicillin was used as a positive control. 100 ml of sterile water was added to the wells, then a stock solution was added and was mixed well; then 100 µl of the mixture was removed from A and transferred to B (serial dilutions) and from B downwards up to H and then 100 µl was removed from H and discarded. 100 µl of bacteria culture was then added to all the wells. The microplates were covered and incubated overnight at 37 °C. The following day iodonitrotetrazolium chloride (INT) solution (0.2 mg/ml) was prepared and 40 µl of the prepared solution was added to all the wells; this was incubated for 30 min; the red-coloured plates indicated the reduction of INT by the mitochondrial dehydrogenase which is an evidence of living bacteria. This was done in triplicate.

2.5.2 Antioxidant Activities

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules which can produce free radicals to initiate chain reactions that damage cells (Sies, 1997). An antioxidant terminates the chain reaction by removing the free radical intermediate and inhibits other oxidation reactions by being oxidized itself. Some antioxidants are produced in the body, while others must be sequestered from the diet or supplemented. Most citrus and dried fruits, cruciferous vegetables, garlic, onions, carrots, tomatoes, sweet potatoes, sesame and olive oil are rich sources of antioxidants. There are thousands of naturally-occurring and synthetic antioxidants. These antioxidants belong to different classes of compounds, such as caratenoids, polyphenolics, polyamides, garlic acid derivatives, tannins and catechins. Phenolic phytochemicals can be categorized into three:

i. Non-flavonoid polyphenols

ii. Flavonoids

iii. Phenolic acids

The phenolic compounds have aromatic rings containing one or more hydroxyl groups, while polyphenols contain multiple phenol rings within their structures. All classes of phenols possess antioxidant activities, depending on the number of hydroxyl groups present on the benzene ring (Packer *et al.*, 2002). Recently, research interest has increased in finding antioxidants that are naturally-occurring for use in food stuffs and medicinal materials, to replace the synthetic ones which are restricted due to their side effects. Furthermore, natural antioxidants have the capacity to improve food quality and stability; they can also be used as neutraceuticals to terminate free radical chain reactions in biological systems and may thus provide improved health benefits for the consumers. A number of experimental models have been developed for the determination of antioxidant activities. They are classified into two major categories namely (Vaya *et al.*, 2001):

i. Measuring the potential of the sample to donate an electron or a hydrogen atom to a specific reactive species or to any electron acceptor.

ii. Measuring the ability to remove any source of oxidative initiation e.g. inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

The *Phyllanthus* species have been reported to possess antidiabetic, anticancer, anti-inflammatory activities where free radicals are sources of such diseases. As far as our literature survey could ascertain, no information was available on the antioxidant activities of *Phyllanthus parvulus* Sond. *var garipensis*. The antioxidant activity of *Phyllanthus parvulus*

Sond. *var garipensis* was evaluated, using different antioxidant assays such as free radical scavenging, reducing power and metal ion chelating activities. Furthermore, the total phenolic content, flavonoid and proanthocynadin content were also determined and their correlations, with the antioxidant activities, were ascertained.

2.5.2.1 Preparation of Stock Solution

Stock solution for each extract was prepared by dissolving 0.02 g of the plant extract in 20 ml of methanol to make a concentration of 1 mg/ml. Six concentrations of 5, 10, 25, 50, 125 and 250 µg/ml were prepared from this stock solution to give the primary standards. Ascorbic acid and butylated hydroxytoluene (BHT) were used as standard antioxidants.

2.5.2.2 DPPH Free Radical Scavenging Assay (Kumaran et al., 2007)

About 0.3 mM of 1, 1-diphenyl-2-picrylhydrazy (DPPH) in MeOH was prepared; this solution was kept in the dark. For all the different concentrations, 2 ml of the extract was mixed with 1 ml of DPPH; for the blank 1 ml of ethanol was used instead of DPPH and for the control, ethanol was used instead of the extract. Triplicates solutions were prepared. The reaction mixtures were kept in the dark for 30 min and the absorbance read at 517*nm*. The equation below was used to calculate the percentage scavenging activities (AA) of each extract.

$$AA\% = 100 - [(Abs_{(sample)} - Abs_{(blank)} / Abs_{(control)}) \times 100] \quad \ldots\ldots 1$$

Where $Abs_{(sample)}$ is the absorbance of the sample, $Abs_{(blank)}$ is the absorbance of the blank and $Abs_{(control)}$ is the absorbance of the control.

2.5.2.3 **Reducing Power Method** (Kumaran et al., 2007)

1 ml of plant sample was mixed with 2.5 ml phosphate buffer (0.2 M) pH 6.6 and 2.5 ml (1%) ($K_3(Fe(CN)_6)$; the mixture was incubated at 50 °C for 20 min, after which 2.5 ml (10%) trichloroacetic acid was added. The whole mixture was then centrifuged (650 x *g* at room temperature) for 10 min, 2.5 ml upper layer was removed and 2.5 ml H_2O and 0.5 ml (0.1%) $FeCl_3$ were added. Triplicates solutions were prepared. This was allowed to stand for 30 min.; the absorbance was measured at 700 nm.

2.5.2.4 **Metal Ion Chelating Activity** (Kumaran et al., 2007)

To 0.5 ml of extracts, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml of ferrozine (5 mM) was added. The mixture was shaken vigorously and allowed to stand at room temperature for 10 min. Triplicates solutions were prepared. The absorbance of Fe^{2+}-Ferrozine was measured at 562 nm. The chelating activity of the extracts for Fe^{2+} was calculated as:

$$\text{Chelating rate (\%)} = (A_0\text{-}A_1)/\ A_0 \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots 2$$

where A_0 is the absorbance of the blank (without the extract) and A_1 is the absorbance in the presence of the extract.

2.5.2.5 **Flavonoid Content** (Kumaran et al., 2007)

1 ml (1 mg/ml) plant sample was mixed with 4.3 ml 80% EtOH, 0.1 ml Al $(NO_3)_3$ and 0.1 ml potassium acetate. Triplicates solutions were prepared. The mixture was allowed to stand for

40 min and the absorbance read at 415 nm. The flavonoid content (x), where y was the absorbance of the solution, was calculated using the equation:

$$y = 0.6648x + 0.01586 \quad \text{.....3}$$

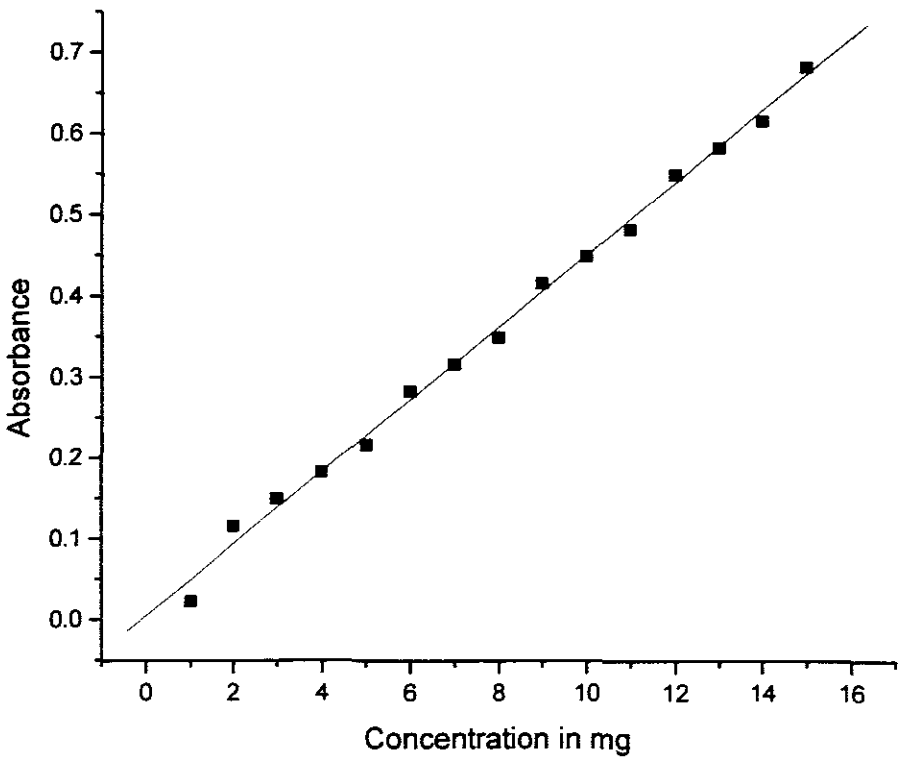

Figure 5: Quetin standard curve

2.5.2.6 Total Phenolic Content (Kumaran et al., 2007)

100 µl (1 mg/ml) of the plant sample was mixed with 1.5 ml Folin-Ciotalteu reagent (FCR); this was allowed to stand for 2 min and then 1.2 ml of (20%) Na_2CO_3 was added. This reaction mixture was left for 30 min after which the absorbance was read at 765 nm. The total phenolic content (x) was calculated from the regression equation of calibration curve (Gallic acid) where y is the absorbance:

$$y = 10.564x + 0.103 \quad \text{.....4}$$

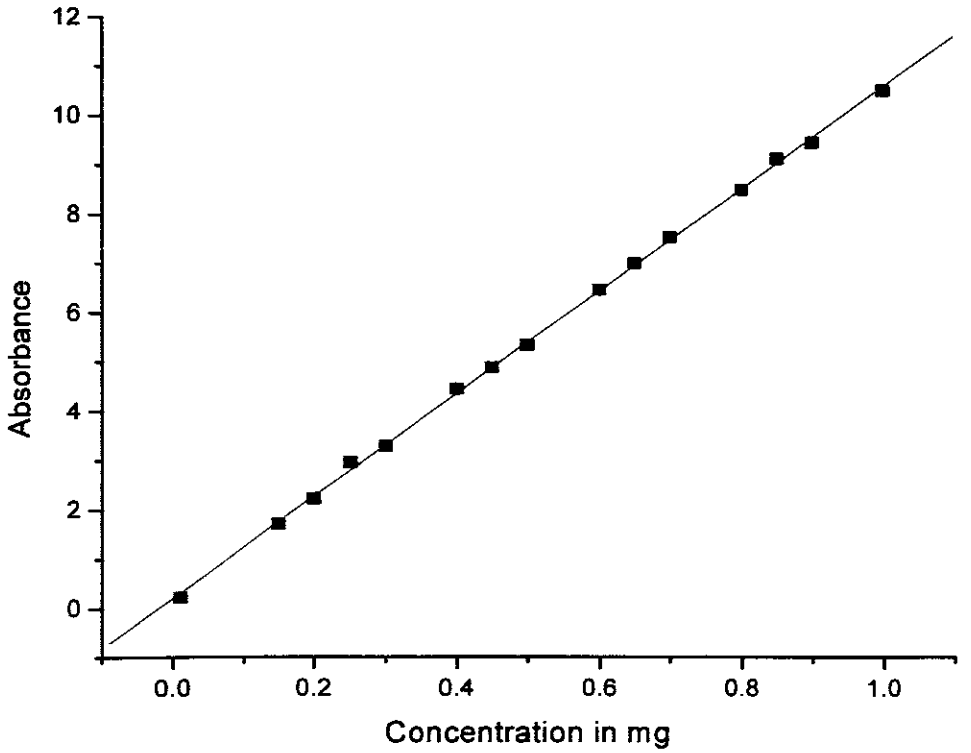

Figure 6: Gallic acid standard curve

2.5.2.7 Proanthocyanidin (Kumaran et al., 2007)

0.5 ml (1 mg/ml) of plant sample was mixed with 3 ml of 4% vanillin-MeOH and 1.5 ml of dilute HCl; this was left for 15 min after which the absorbance was read at 500 nm. The proanthocyanidin content (x) was calculated using the equation:

$$y = 0.5825x \quad \text{.....5}$$

where y is the absorbance.

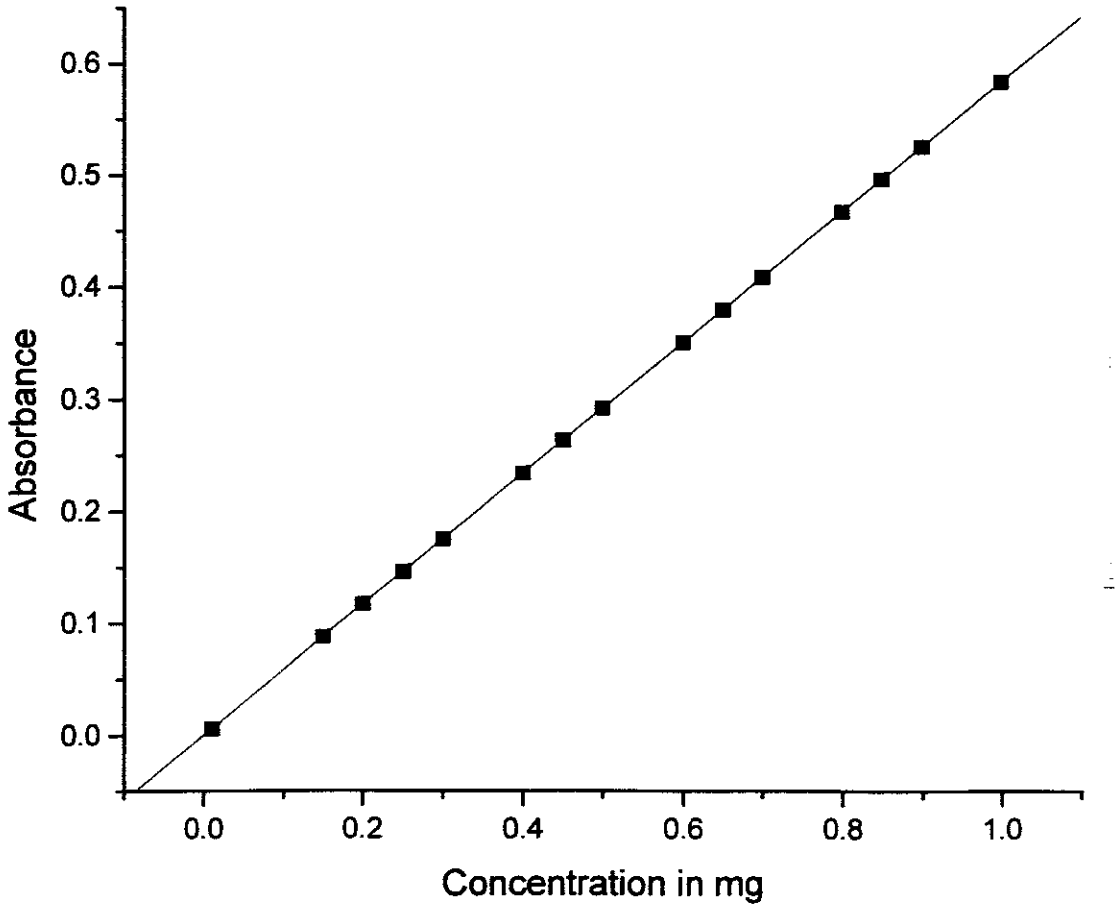

Figure 7: Catechin standard curve

2.6 Toxicity

2.6.1 Brine Shrimp Lethal Test

The assay was carried out according to the principle and protocol described by Ojala *et al.*, (1999) and Waller *et al.*, (1996) with slight modification. Brime shrimp eggs (*Artemia salina*) were hatched in artificial sea water (40g of coarse non-iodized salt per a 1L of distilled water) and poured into 500 cm by 2 meter long glass column. Aeration was supplied through an opening at the top of the column via a rubber tube that touches the bottom of the column. Three heaped teaspoons of the eggs was added to the column and the wall of the column was covered with aluminium foil to create a dark environment. The temperature of the hatching

water was between 27 to 30 ^{O}C using 100W incandescent light bulb over the hatching column. The test was done using n-hexane, chloroform, ethyl acetate, methanol, ethanol and water extracts. Concentrations of 5, 10, 25, 50, 125 and 250 µg/ml were prepared for each extract. The experiments were done in duplicate for each concentration at room temperature. The number of shrimps was determined by placing two drops of water with shrimps under microscope; two drops had an average of 15 shrimp larvae. About 10 ml of degassed water was placed in a Petri dish and 100 µl of plant extract in methanol was added; the mixture was lightly shaken to ensure a homogeneous solution. The 15 shrimp larvae were placed in the mixture. For the control, methanol was used instead of the plant extract. The number of survivors was counted after 3, 12, 18 and 24 h.

Chapter Three

3. Results and Discussion

3.1 Phytochemical Screening

Qualitative screening confirmed the present of five of the secondary metabolites in both plant samples of *P. parvulus* Sond. *var garipensis* (Table 1). The present of these five compounds support the use of the plant in folklore medications. Alkaloids are known to contain a lot of pharmacological properties. They are mostly used as antidepressant (morphine), stimulants (caffine), anaesthetic (cocaine), antitumor (vinblastine) antimalaria (quinine), antibacterial (berberine) and amoebicide (emetine). (Bruneton, 1995; Cowan, 1999; Heinrich *et al.*, 2004; Gurib-Fakim, 2006). Flavonoids are known to have anti-inflammatory, anti-allergic, antiviral, antispasmodic and diuretic effect (Bruneton, 1995; van-Wyk *et al.*, 1997; Cowan, 1999) while Saponins are known to be immune booster. Extracts of plants, rich in saponins, are said to demonstrate antiflammatory, homolytic, allelopathic, cholesterol lowering and anticancer properties (Sauvaire *et al.*, 1996; Sowmya *et al.*, 1999; Mandeaau *et al.*, 2005). Tannins are good antimicrobial agent which precipitate protein thereby providing waterproof layer on the skin when used externally or protect the underlying layers of the skin and limit the loss of fluid. They are also known to be good antiviral agents (Bruneton, 1995; Cowan, 1999).

Table1: Qualitative Analysis of *P. parvulus* Sond. *var garipensis*

Secondary Metabolites	*P. parvulus* Sond. *var garipensis*
Alkaloids	+
Saponins	+
Tannins	+
Flavonoids	+
Reducing compounds	_
Antraquinones	+
Anthocyanosides	_

+ =Presence of constituents and intensity; - = absence of constituent

There was no much difference in the quantitative analysis of the five secondary metabolites present in the plant samples of *P. parvulus* Sond. *var garipensis* collected at various times. Quantitatively, saponins predominant the chemical profile followed by flavonoids (Table 2). Since saponins and flavonoids are known to be anti-inflammatory and antiviral, it therefore means that the South African *P. parvulus* Sond. *var garipensis* can used to treat such related disease.

Table 2: Quantitative (Percentages) of Secondary Metabolites in *P. parvulus* Sond. *var garipensis*

Plant	Alkaloid	Saponins	Flavonoids	Tannins
P. pavulus sond var garipensis	0.8	14.2	4.2	2.1

3.2 Extraction

The results shown by the two extraction methods used revealed that there are no significant differences in the extract masses, although the aqueous extract of the first method gave a greater yield as compared to the second method. Though, the plant samples were collected at different seasons. The chloroform extract was accidentally poured into a plastic container and the chloroform solvent dissolved the container overnight thus the chloroform extract was lost (Table 3).

Table 3: Masses of extracts for the two extraction methods, the chloroform extract spilled.

Solvent	**Method one** **Weight of the extract(g)**	**Method two** **Weight of the extract(g)**
n-Hexane	5.15	5.61
Ethyl acetate	6.07	3.62
Chloroform	X	5.15
Methanol	3.73	4.30
Water	10.59	5.45

3.3 Isolation and Elucidation of lanosterol- glycoside compound

The ethyl acetate fraction from method one was subjected to VLC and CC separation technique to afford a clean isolate with molecular weight 633 g/mol, ($C_{37}H_{61}O_7N$) and melting point of 131.6-135.7 °C. The molecular weight obtained along with the ^{1}H- and ^{13}C-NMR (Fig 9 and 10) suggests a saponin compound (Fig 8) that is likely linked to a glucose compound or compounds. Further analysis of the ^{1}H, ^{13}C and ^{13}C DEPT NMR (Fig 11)

indicates the presence of 12 CH_2. The presence of 3 olefinic protons between 5.4-4.95 ppm (Table 4) suggest that 2 of the olefinic protons (δ 5.4 and 5.0 ppm) are close to the amide group CH = CH – CO - NH -. Table 4 displays the information deduced from the 1D and 2D-NMR spectra which helped in elucidating the structure of the isolated compound. This substructure fitted well into the IR information obtained. The IR spectra data of the isolated compound further confirms the presence of C=O, N-C, N-H and O-H functional groups at 1625, 2933, 2860 and 3442 cm^{-1} respectively (Fig 12). This is further supported by the HMBC, HMCQ and NOSEY spectra (Fig 13-15). The compound was suggested to contain lanosterol carbon skeleton with an amide linkage side chain between a methylene glycoside and the lanosterol (Fig 16 and 17). This is an unusual saponin type sterol which has been reportedly isolated from marine animals (Vincken *et al.*, 2007, Oyaiza, 1986). Similar steroids with an amide linkage and various side chains have been isolated and reported by Mandeau *et al.* (2005).

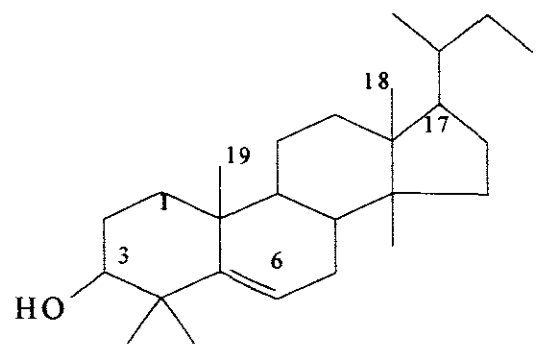

Figure 8: Lanosterol skeletal structure

Table 4: NMR Spectroscopic Data

^{1}H-^{13}C	^{1}H-^{1}H	No. of Protons	^{1}H Position	$^{13}C(CH_3$ and CH)	^{13}C (CH_2)
0.6-12.04	3.55-1.59	1	3.4-3.6	11.98	18.97
0.75-12.28	3.59-3.55	1	4.9-5.1	12.05	21.21
3.57-77.80	3.59-1.50	1	5.2-5.3	19.40	23.06
5.34-121.8	5.39-1.95	1	5.35-5.40	19.03	24.07
2.29-44.50	2.26-3.55	2	2.28-2.39	19.82	28.25
0.98-55.28	1.59-5.00	2	1.90-2.27	21.08	31.91
1.99-39.90	2.80-5.39	2	1.79-1.90	29.1	31.92
1.18-39.90	1.59-1.90	49	0.39-1.79	31.92	33.94
1.79-37.5	0.82-1.75			71.82	37.25
1.50-30.28	5.39-2.00			121.77	39.68
	2.26-2.20			129.27	39.78
	1.16-1.80			42.22	42.31
				56.06	
				56.77	
				56.86	

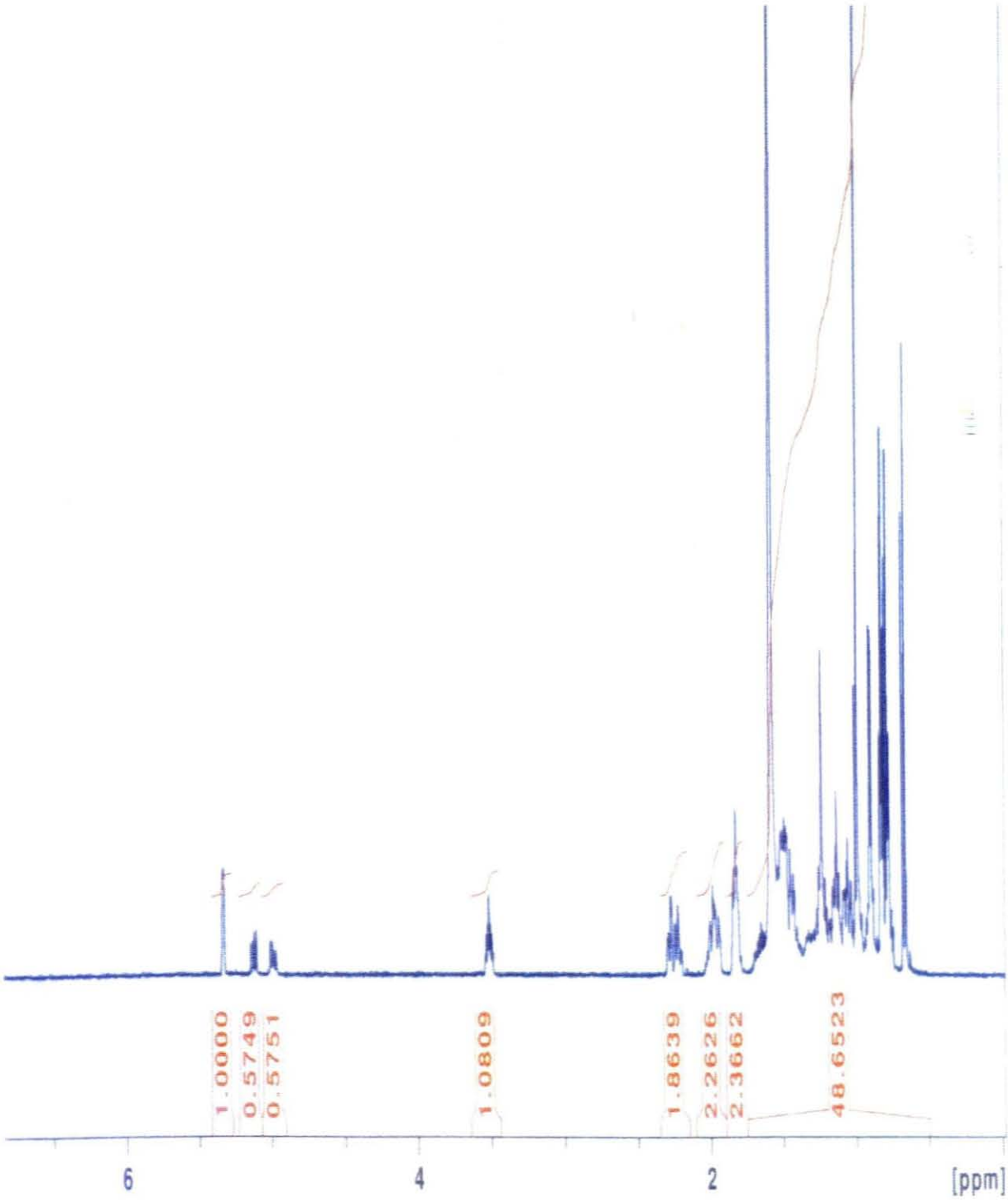

Figure 9: ^{1}H-NMR of Isolated Compound

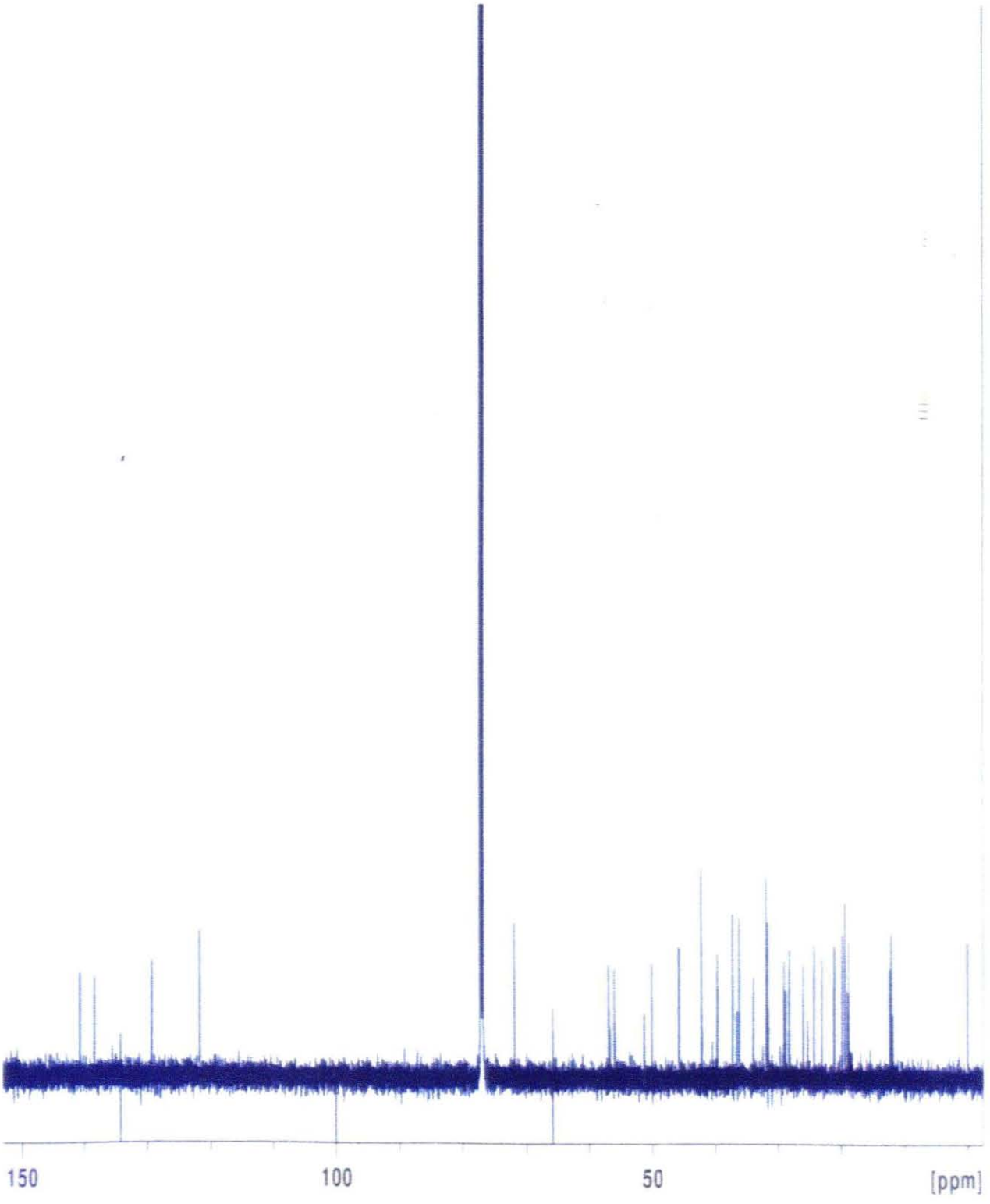

Figure 10: ^{13}C-NMR of Isolated Compound

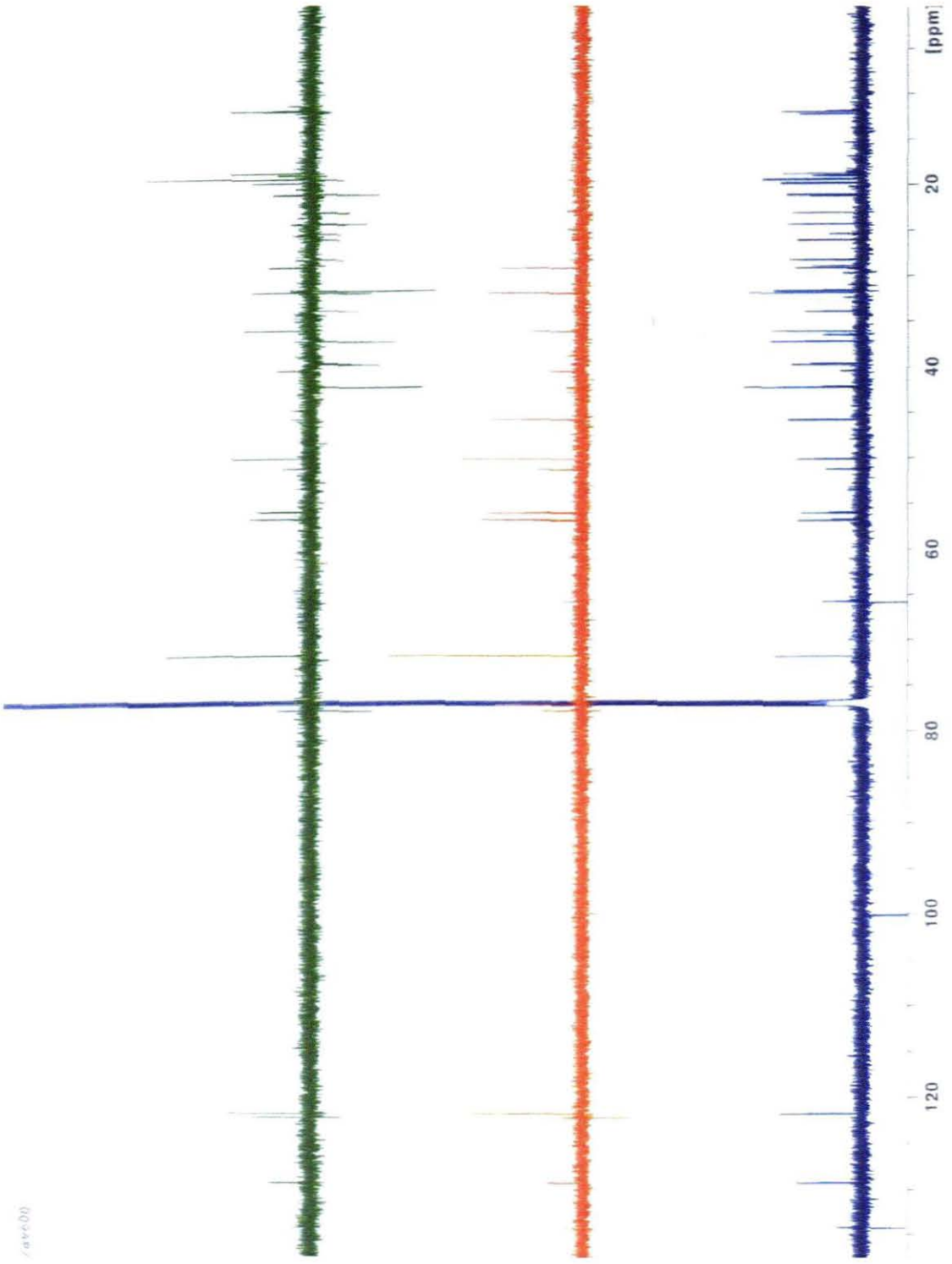

Figure 11: ^{13}C- DEPT NMR of Isolated Compound

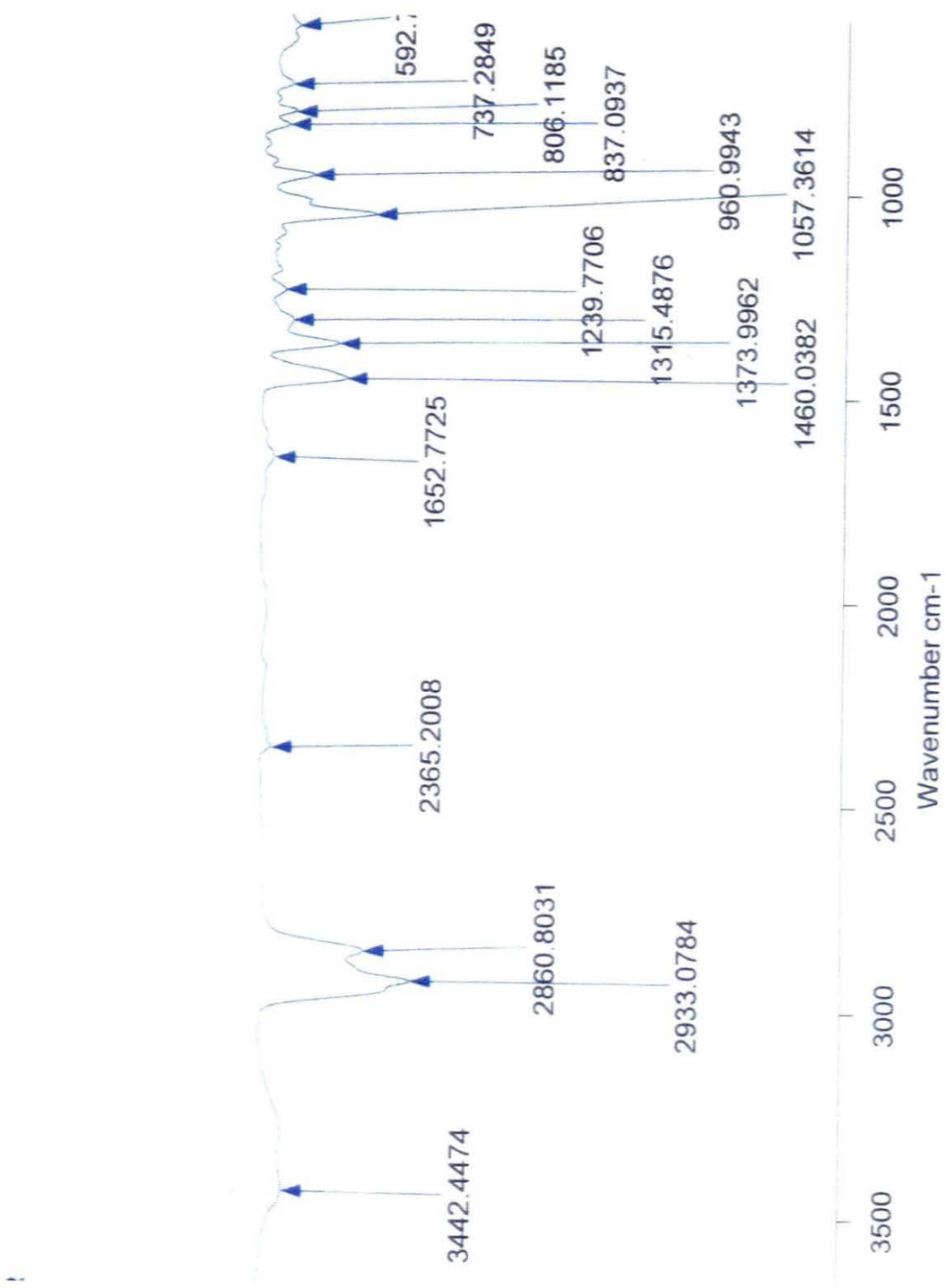

Figure 12: IR Spectrum of Isolated Compound

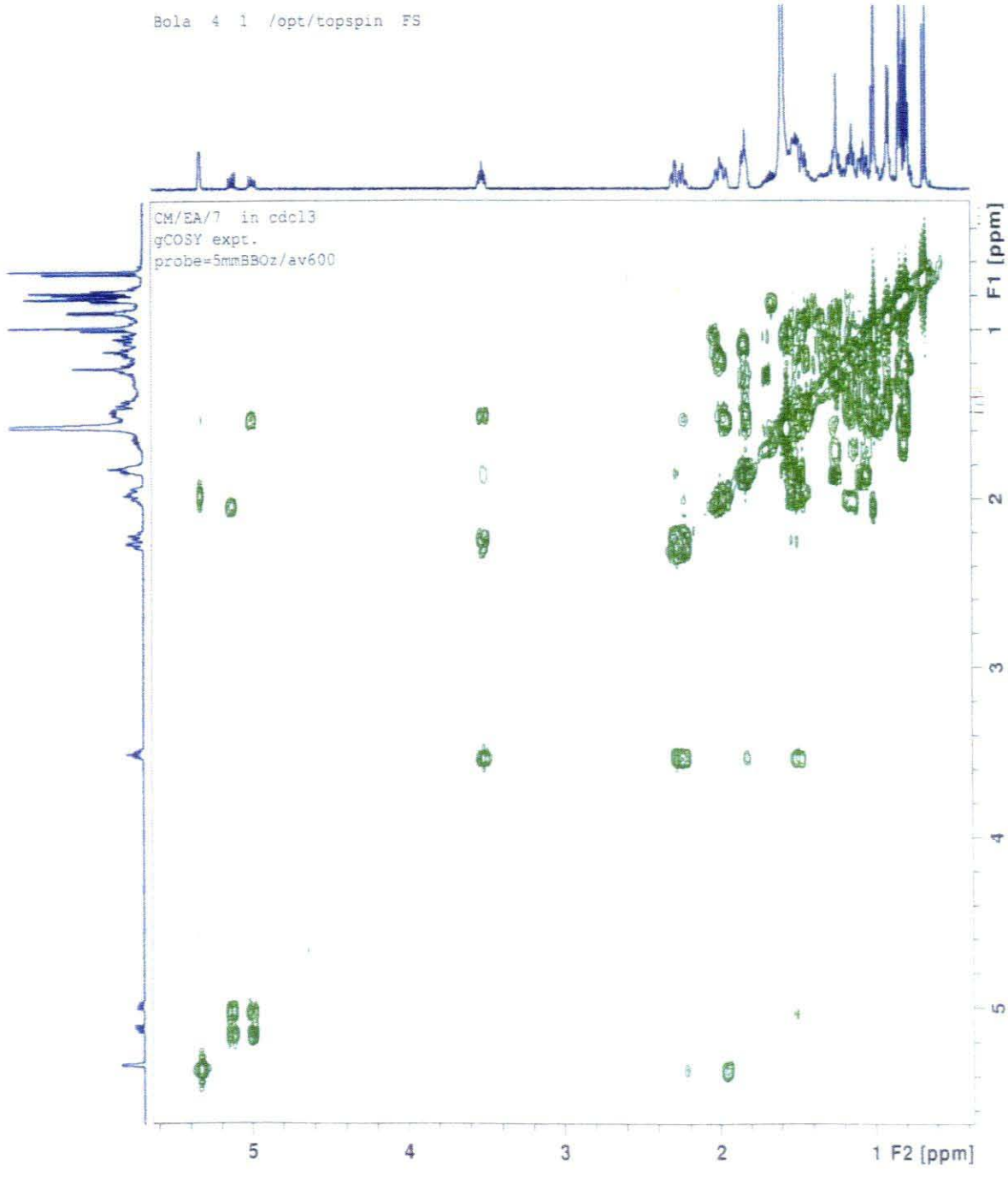

Figure13: ^{1}H-^{1}H COSY NMR

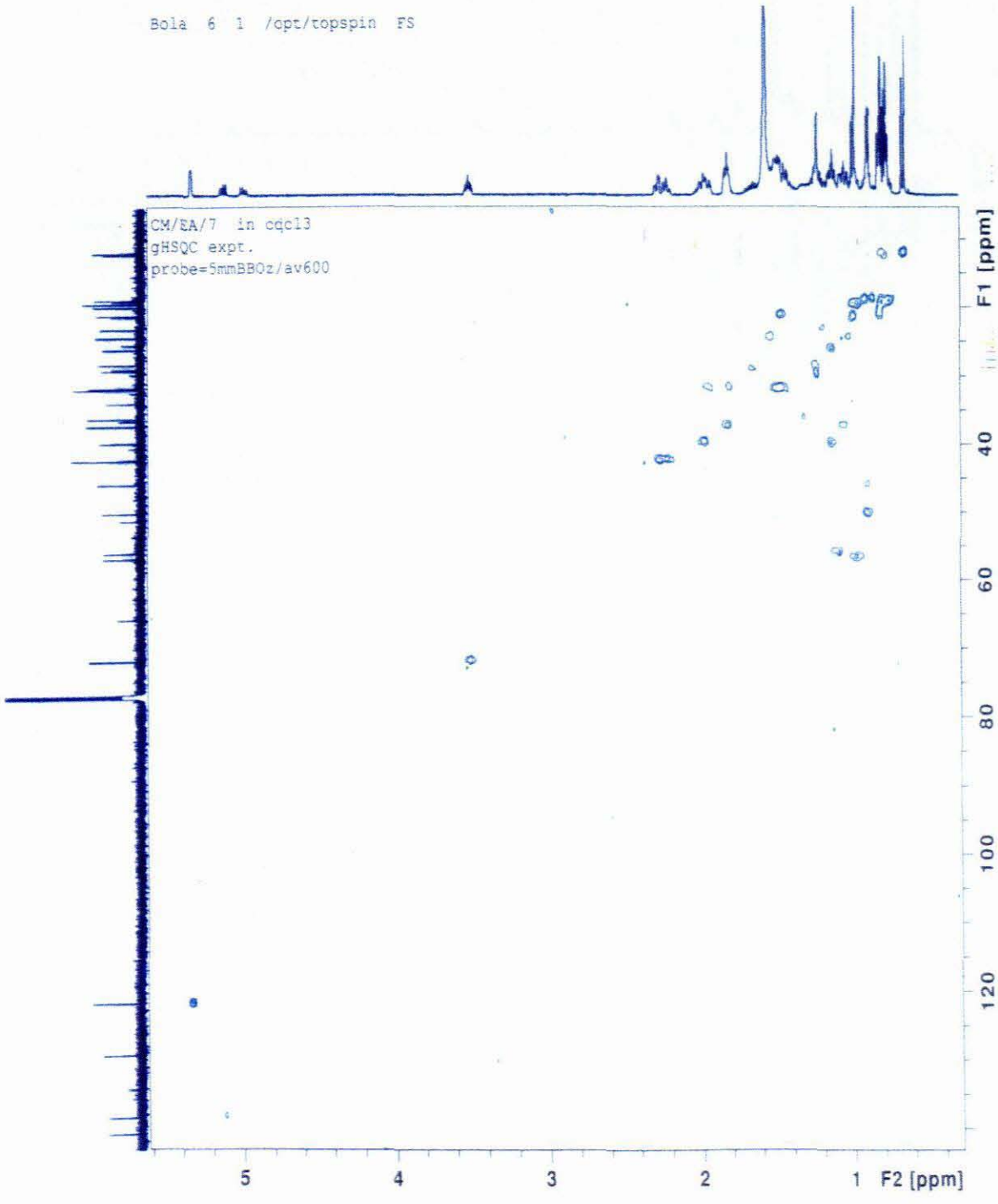

Figure 14: ^{1}H- ^{13}C COSY (HSQC) NMR of Isolated Compound

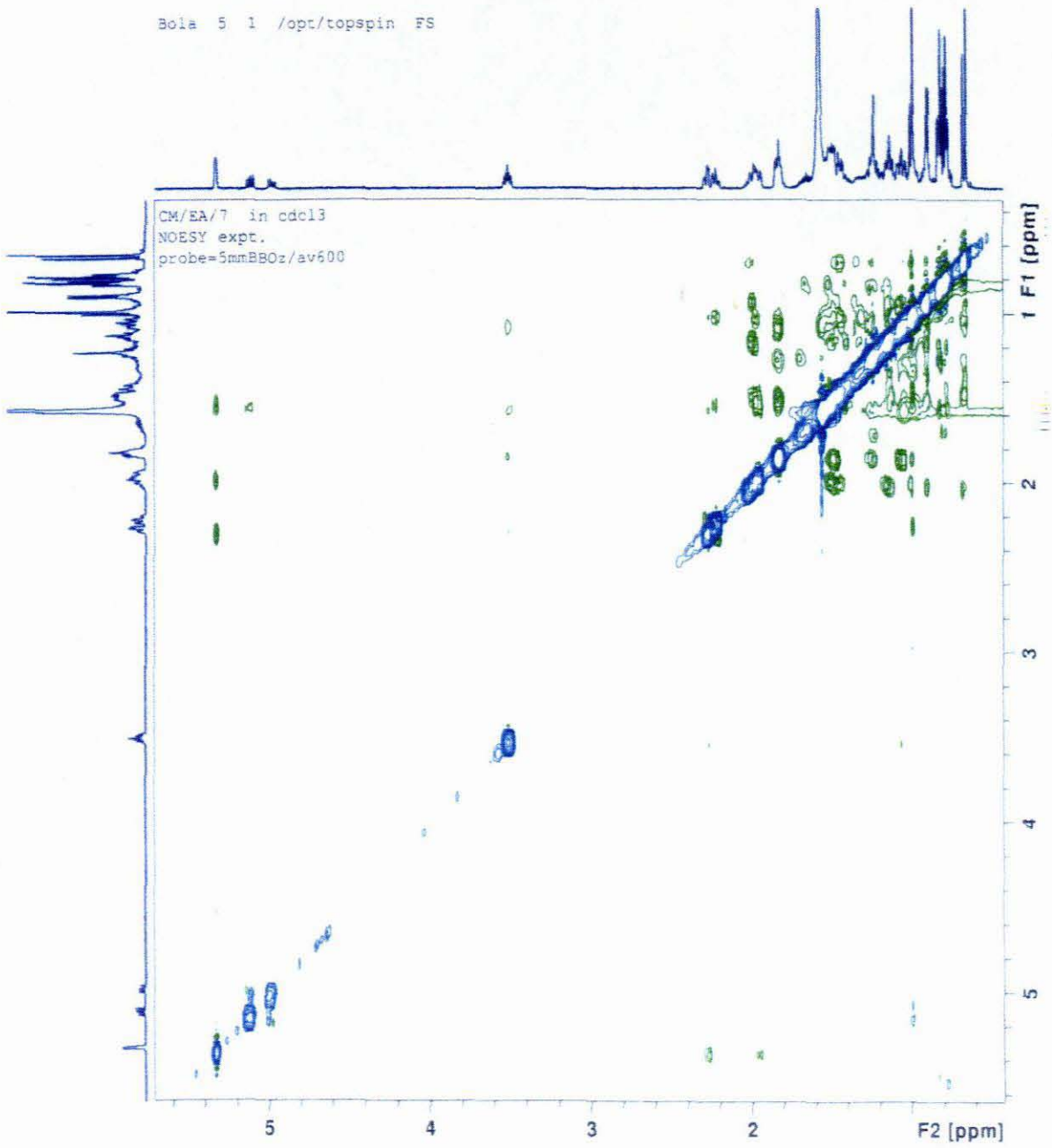

Figure 15: NOSEY NMR of the Isolated Compound

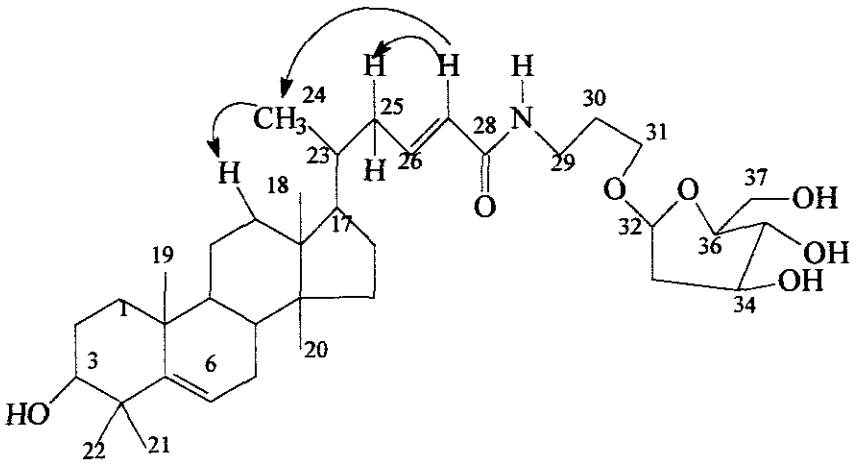

Figure 16: NOSEY representation of the Lanosterol-amide glycoside compound

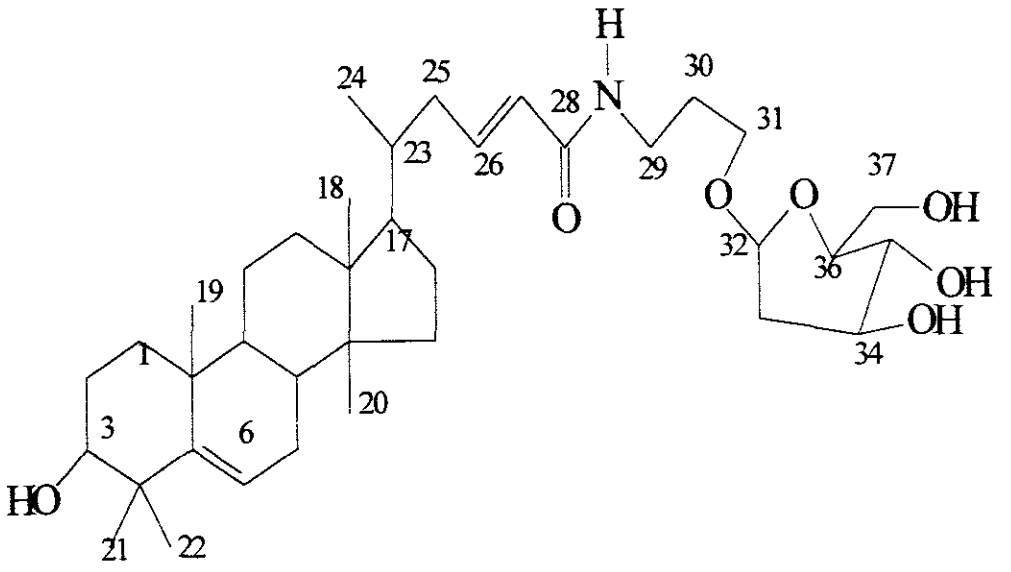

Figure 17: Lanosterol-amide linked glycoside

3.4 Antibacterial Assays

Solvent extracts from method two was used for the biological assay. The chloroform extract shows the highest inhibitory activity against 9 microorganisms with inhibition zones ranging between 15 and 25.5 mm. This was followed by the methanol extract which inhibited the growth of 8 microorganisms, with inhibition zones ranging between 8.5 and 15 mm. The ethyl acetate showed moderate inhibitory effect on 8 microorganisms while n-hexane extract inhibited only 5 organisms. The n-hexane extract was not active against *E. coli,* when comparing the activities of the other solvent extracts on *E. coli.* The least activity was recorded for the water extract showing positive inhibitory effect on only 3 microorganisms i.e. *E. coli, Enterobacter cloacae* and *Micrococcus leteus.*

Bacillus cereus, Shigella flexineri, Staphylococcus aureus, Salmonella sp. and *Acinecto calcococeuticus anitratus* showed some resistance behaviour to few extracts. In general, the results showed that the plant extracts possess broad antibacterial activities because some of their activities are even higher than the standard antibiotic (Ampicillin) used in this study. Ampicillin which was the positive control used in this experiment was only active against six bacteria *with M. kristinae* having an inhibitory zone of 28mm. (**Table 5**).

The minimum inhibitory concentration (MIC) results showed that the ethyl acetate had the lowest MIC value of 0.313 mg/ml against *Staphylococcus aureus*; this indicates that small concentration of ethyl acetate extract is needed to inhibit the growth of *S. aureus*. Thus this extract can be used in treating *S. aureus* infections. The chloroform methanolic and water extract had moderate MIC value against most bacteria while the MIC value for n-hexane and water extracts were high (5 mg/ml) for most of the bacteria inhibited. The standard

antibacterial Ampicillin had the MIC value of 0.3125 mg/ml against *Salmonella ssp* and 0.156 mg/ml against *E. coli.*

Table 5: Antibacterial activity of different extracts using disc diffusion method (inhibition zones, mm).

Microorganisms	**n-Hexane**	**$CHCl_3$**	**EtOAc**	**MeOH**	**H_2O**	**Ampicillin**
Escherichia coli ATCC 8739	-	21.5±1.76	13±1.41	8.5±0.71	9.5±0.71	16±1.41
Pseudomonas eurugenosa ATCC 19582	-	16±2.83	12±2.83	11.5±0.71	-	-
Enterobacter cloacae ATCC 13047	9.5±0.71	-	-	09±0.71	10±0.50	-
Bacillus cereus ATCC 10702	-	-	15±4.24	-	-	08±1.41
Klebsiella pneumoniae ATCC 10031	06±0.50	20±4.24	07±0.50	10±0.50	-	-
Staphylococcus aureus ATCC 29212	-	15±4.24	10±1.41	-	-	-
Streptococcus faecalis ATCC 29212	9.5±3.54	-	7.5±0.71	12±2.12	-	-
Serratia marcescens ATCC 9986	12.5±2.12	25.5±3.54	12.5±0.71	-	-	-
Shigella flexineri KZN	-	20±2.83	-	-	-	15±1.41
Salmonella ssp. KZN	-	22±1.41	-	09±0.50	-	20.2±0.28
Micrococcus kristinae	-	20±1.41	-	15.5±2.12	-	28±2.83
Acinecto calcococeuticus anitratus CSIR	12.5±2.12	20±1.41	-	-	-	-
Micrococcus leteus	-	19±1.41	-	10.5±0.71	08±0.50	22±1.41

Each value is represented as mean±SD (n = 3)

Table 6: Minimal inhibitory concentration (MIC) of plant extracts with antibacterial activity

(Effective concentration of extract against the microorganism in mg/ ml)

Microorganisms	n-Hexane	$CHCl_3$	EtOAc	MeOH	H_2O	Ampicilin
E-coli ATCC8739	-	5.000	2.500	2.500	2.500	0.156
Psuedomonas eurogenosa ATCC 19582	-	1.250	2.500	5.000	-	-
Enterobacter cloacae ATCC 13047	5.000	-	-	2.500	5.000	-
Bacillus cereus ATCC 10702	-	-	5.000	-	-	1.250
Klebsiella pneumoniae ATCC 10031	5.000	2.500	2.500	2.500	-	-
Staphylococcus aureus ATCC 29212	-	2.500	0.313	-	-	-
Streptococcus faecalis ATCC 29212	5.000	-	5.000	2.500	-	-
Serratia marcescens ATCC 9986	5.000	2.500	5.0	-	-	-
Shigella flexineri KZN	-	2.500	-	-	-	1.250
Salmonella ssp. KZN	-	5.000	-	2.5	-	0.313
Micrococcus kristinae	-	2.500	-	1.250	-	1.250
Acinecto calcococeuticus anitratus CSIR	1.250	2.500	-	-	-	-
Micrococcus leteus	-	5.000	-	2.500	2.500	1.250

3.5 Antioxidant Assays

3.5.1. DPPH Free Radical Scavenging Method

Table 7 shows the percentage scavenging activities represented as mean ± SD (n = 3) of the extracts and the standard antioxidants.

In all the extracts, there was an increase in absorbance with increasing concentration, which was also supported by the color intensity of the samples used during the experiment; the more intense the color, the higher the absorbance. Although, when one looks at Figure 18, the graphs of H_2O, $CHCl_3$ and EtOAc showed increasing absorbance but after 5 μg/ml, there seems to be a constant trend in the absorbance pattern or absorptivities. If one looks at the MeOH, EtOH and the two standards, they have absorbances that are directly proportional to the concentration; that is, as the concentration of the extracts increases, the absorbances also increase. There could be a number of reasons for this behavior. One could assume that the extract with this constant behavior does contain compounds that possess antioxidant activities but in very small quantities, whereas those that showed high antioxidant behaviors, have high concentration.

In the DPPH free radical scavenging method, all the extracts show broad scavenging activities with MeOH and water extracts having the highest activity followed by EtOH, EtOAc, $CHCl_3$ and n-hexane. The n-hexane extract shows a constant scavenging activity, even at high concentrations. It should be noted that for a compound to be considered an antioxidant, it must be able to donate an electron; such an electron usually come from the hydroxyl group or an electron-rich compound. Since n-hexane a non-polar solvent, it could be assumed that there are no electron-donating species present in the hexane extract; this accounts for the results obtained.

Table 7: Percentages for the DPPH method scavenging activities of the plant extracts

Plant Extract	**5 µg/ml**	**10 µg/ml**	**25 µg/ml**	**50 µg/ml**	**125 µg/ml**	**250 µg/ml**
n-hexane	23.86±0.030	24.46±0.014	24.82±0.032	24.89±0.0045	24.89±0.0045	27.00±0.023
$CHCl_3$	8.00 ±0.013	9.86 ±0.009	18.09±0.004	22.76±0.25	22.76±0.25	36.92±0.040
EtOAc	27.68±0.0093	39.28±0.001	40.01±0.0078	46.92±0.0032	46.92±0.0032	65.20±0.0050
MeOH	83.53±0.028	84.40±0.0075	85.57±0.0036	86.66±0.0051	86.66±0.0051	87.27±0.0039
EtOH	48.30±0.018	56.80±0.0027	72.93±0.021	88.24±0.0021	88.24±0.0021	89.72±0.0016
H_2O	44.62±0.025	49.38±0.032	67.08±0.0075	91.44±0.018	91.44±0.018	95.61±0.019
Asc. Acid	43.06±0.079	64.44±0.10	97.70±0.0017	98.15±0.00071	98.15±0.00071	97.64±0.00071
BHT	79.97±0.081	85.71±0.18	83.63±0.061	93.89±0.0073	93.89±0.0073	97.64±0.001

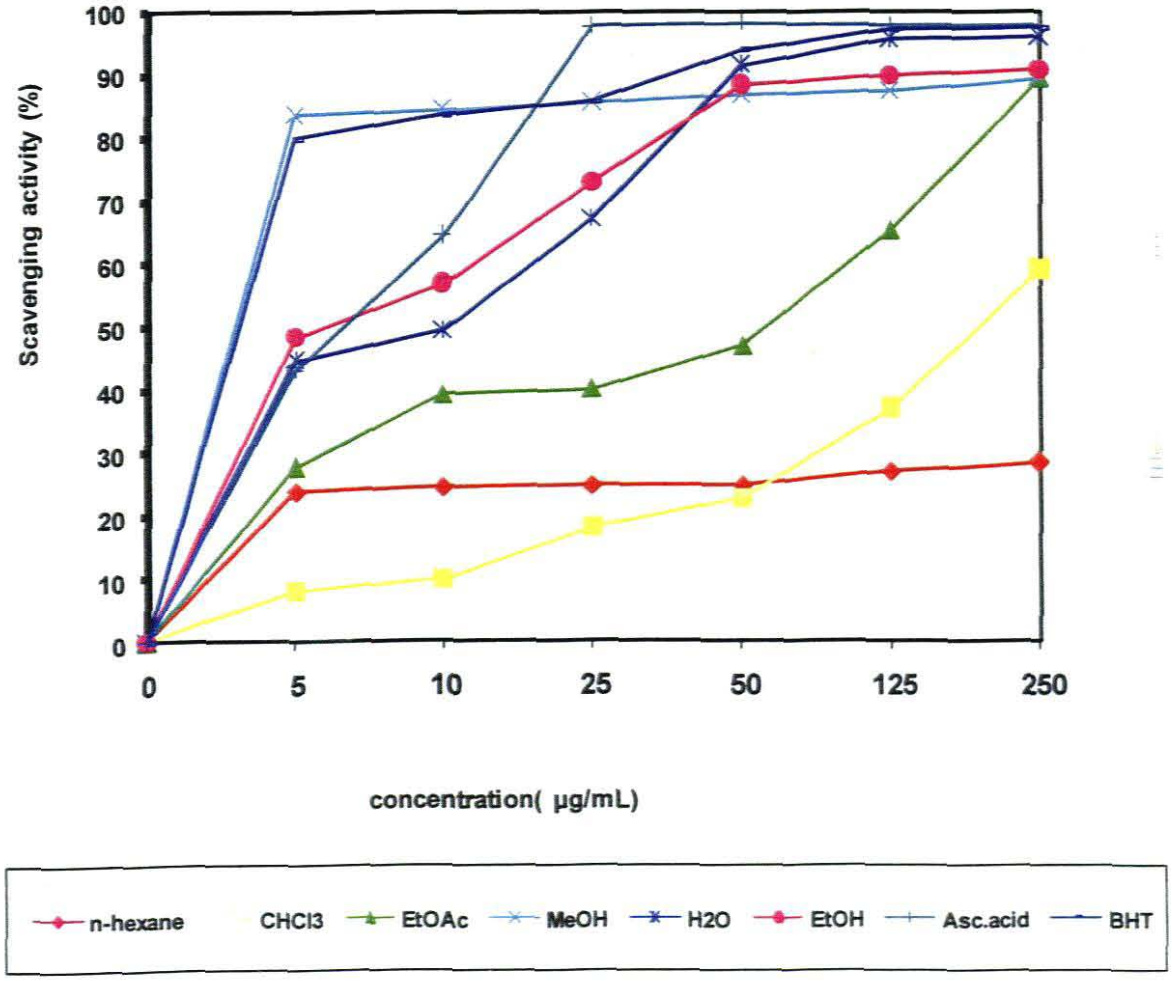

Figure 18: Scavenging activity of the extracts against 1, 1-diphenyl-2-picrylhydrazy (DPPH) radical with ascorbic acid and BHT as standard antioxidants.

3.5.2 Reducing Power Method

Table 8 shows the reducing power of the extracts represented as mean ± SD (n = 3). The extracts showed increasing reducing power with increasing concentration **(Fig. 19)**. From graphical representation of values in Table 8, the methanol extract showed the highest activity, which is even greater than the standard antioxidants; this is closely followed by the ethanol extract. The other extracts showed activities which are less than BHT and ascorbic

acid standard antioxidant. One could infer that the other extracts do have the antioxidant properties but maybe the concentration of the compounds responsible for such activities, are in trace amounts.

Table 8: Reducing power of the extracts of *P. parvulus* Sond. *var garipensis*

Plant Extract	5µg/ml	10 µg/ml	25 µg/ml	50 µg/ml	125 µg/ml	250 µg/ml
n-hexane	0.364±0.012	0.367±0.014	0.377±0.0077	0.375±0.0026	0.403±0.0045	0.424±0.0035
$CHCl_3$	0.398±0.0096	0.401±0.019	0.396±0.005	0.407±0.015	0.428±0.0074	0.462±0.0035
EtOAc	0.557±0.026	0.563±0.003	0.570±0.0021	0.569±0.0017	0.596±0.0021	0.640±0.0045
MeOH	0.586±0.011	0.640±0.065	0.854±0.12	1.196±0.025	2.362±0.12	3.938±0.11
EtOH	0.299±0.024	0.284±0.0071	0.368±0.0071	0.450±0.011	0.647±0.0098	0.945±0.042
H_2O	0.198±0.0016	0.203±0.061	0.224±0.0026	0.256±0.0036	0.289±0.004	0.321±0.002
Ascorbic acid	0.614±0.026	0.642±0.029	0.660±0.0035	0.859±0.039	1.472±0.080	2.299±0.070
BHT	0.414±0.0073	0.434±0.0041	0.528±0.0036	0.723±0.035	1.128±0.041	1.683±0.058

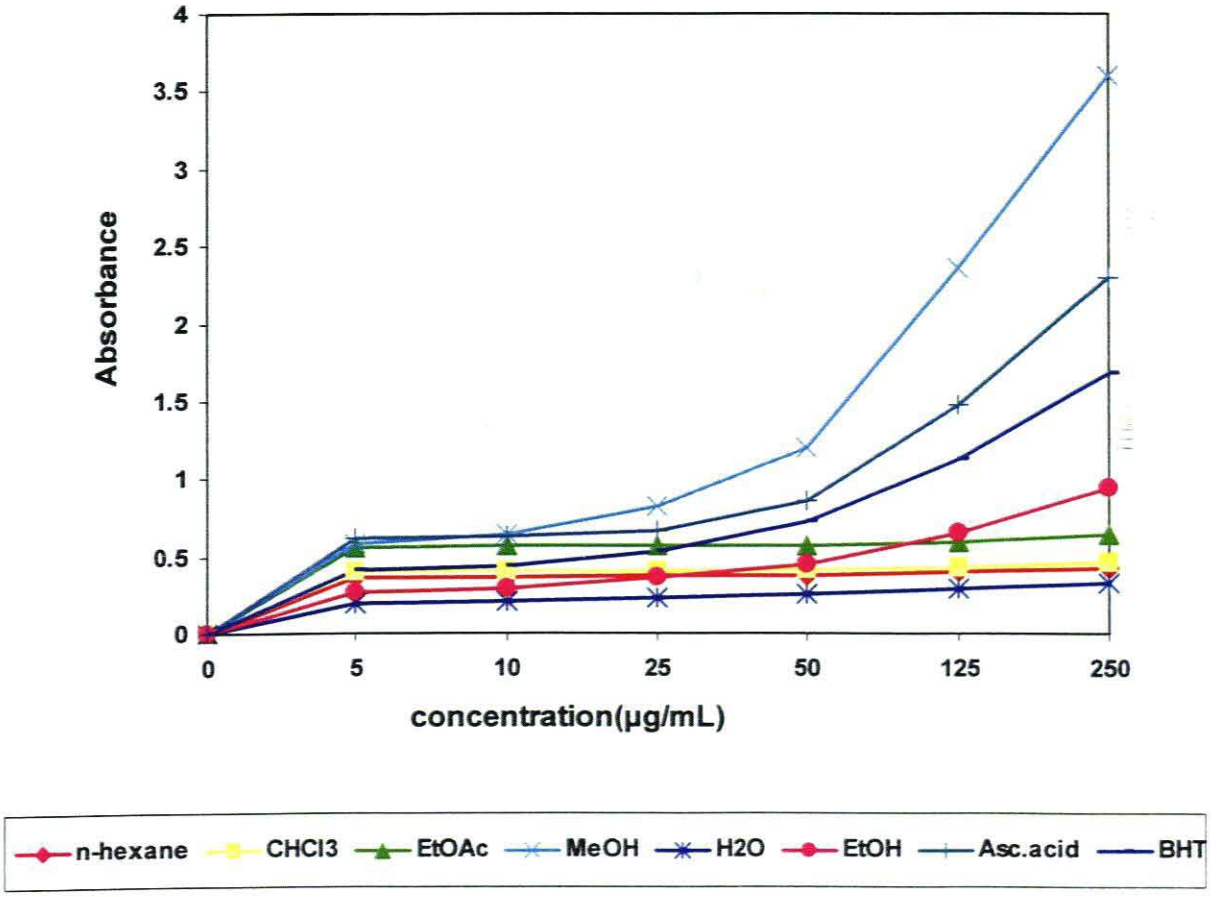

Figure 19: Reducing power of the extracts and the standard antioxidants (BHT and ascorbic acid)

3.5.3 Metal Ion Chelating Activity

The chelating effect was shown by the reducing absorbance of the extract samples with increasing concentration of which, when the chelating capacity **(Table 9)** is calculated using equation 2, portrays an increasing capacity **(Fig. 20)**. All the extracts show increasing capacity with increasing concentration although; none of them possess capacity higher than the standard antioxidant (BHT). The n-hexane extract showed the highest capacity increase at concentrations above 10 µg/ml.

Table 9: Metal Scavenging Capacity represented as mean ± SD (n=3)

Plant Extract	**5 µg/ml**	**10 µg/ml**	**25 µg/ml**	**50 µg/ml**	**125 µg/ml**	**250 µg/ml**
n-hexane	6.75±0.0017	15.36±0.0035	28.96±0.033	32.23±0.034	38.88±0.029	43.71±0.043
$CHCl_3$	22.71±0.097	25.91±0.020	26.31±0.049	30.48±0.019	33.59±0.020	35.71±0.0061
EtOAc	23.91±0.061	25.08±0.024	26.39±0.014	28.35±0.043	35.31±0.031	38.27±0.040
MeOH	13.52±0.046	16.89±0.030	18.01±0.018	19.94±0.023	20.78±0.0052	21.54±0.016
EtOH	17.28±0.017	19.96±0.0056	20.67±0.022	21.31±0.012	22.07±0.025	22.75±0.033
H_2O	12.52±0.056	16.24±0.035	17.51±0.0067	18.38±0.031	21.07±0.10	24.15±0.018
BHT	26.55±0.039	30.60±0.017	32.57±0.048	35.47±0.054	45.54±0.021	47.25±0.053

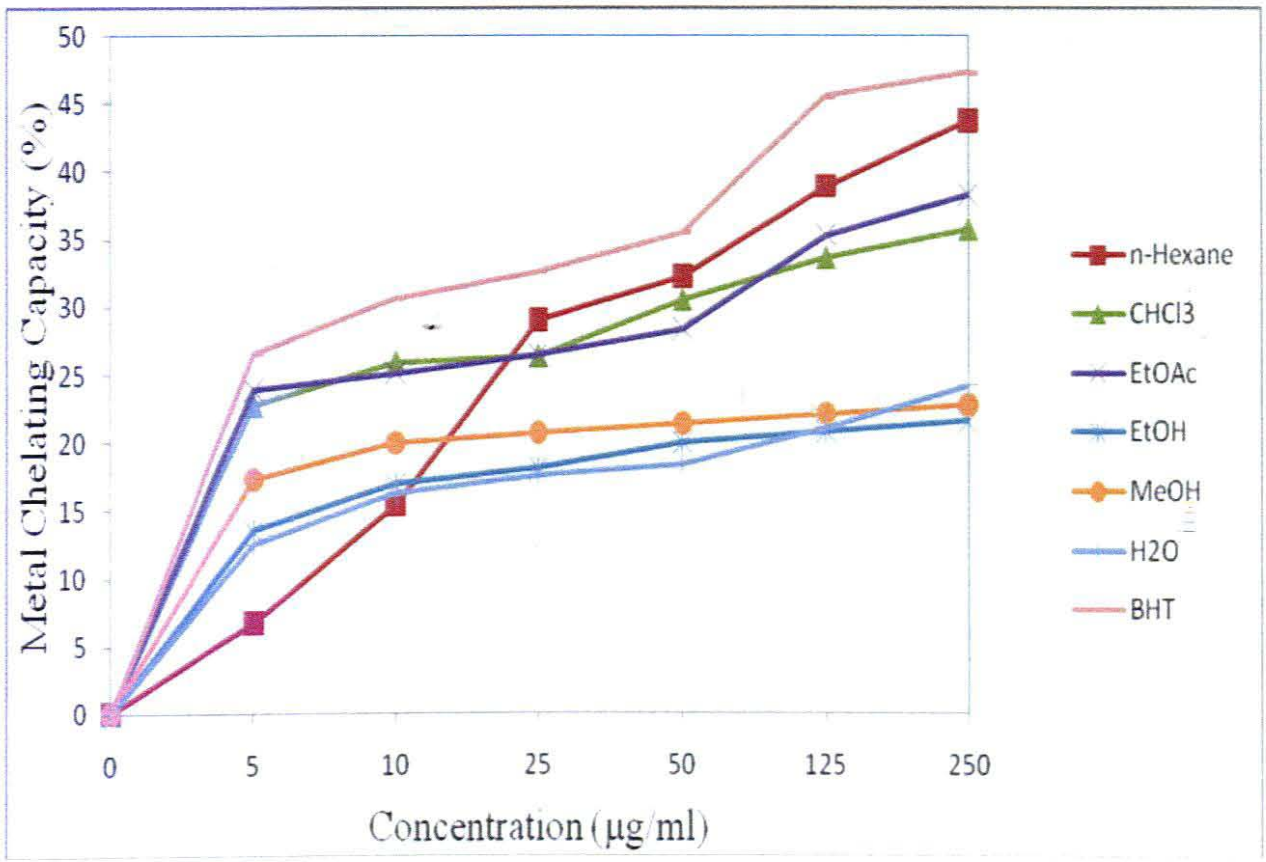

Figure 20: Metal chelating effects of n-hexane, chloroform, ethyl acetate, methanol, ethanol extracts and BHT

3.5.4 Total Phenol, Flavonoid and Proanthocyanidin Contents

Studies into the flavonoid, total phenol and the proanthocyanidin contents indicates that an extract with a high amount of one or all of these compounds must have good antioxidant activities because these compounds are known to be antioxidants themselves. This is not true for the chloroform and the n-hexane extracts, when the results presented in **Table 10** are compared to the DPPH and reducing power results; their contents are very high and they have the least activities. This type of results leaves one wondering whether there are other components to be found in the non-polar compounds that could also be responsible, since it is

believed that compounds with the hydroxyl groups play a major role in the antioxidant activities. When one considers the MeOH, EtOH and the EtOAc extracts, there is a moderate correlation between flavonoid, total phenol and the proanthocyanidin contents and their antioxidant activities.

Table 10: Calculated values for the total phenol, flavonoid and proanthocyanidin contents of solvent extracts of *P. parvulus* Sond. *var garipensis* represented as mean ± SD (n=3).

Extract	**Flavonoid (µg/ml)**	**Total phenol (µg/ml)**	**Proanthocyanidin (µg/ml)**
n-Hexane	2.18±0.0060	0.0225±0.005	0.736±0.0062
$CHCl_3$	4.465±0.0046	0.0328±0.020	0.896±0.016
EtOAc	2.950±0.0078	0.0288±0.017	0.812±0.010
MeOH	2.473±0.0099	0.0487±0.025	0.575±0.0041
EtOH	2.275±0.023	0.118±0.098	0.508±0.0012
H_2O	1.789±0.0045	0.0318±0.012	0.476±0.0012

3.6 Toxicity

Table 11 and **12** shows the number of mortalities and the percentage mortalities taken at different times. The shrimps were considered dead if there was no movement after several seconds of observation. The number of the mortality was taken after 3 h from the onset of the experiment. This was done to verify that the shrimps were still alive before they were left over night. Readings taken after 12 h showed that the extracts were not toxic since the lethality concentration was not detected up to 250µg/ml. Records showed that after 24 h all the shrimps were dead. Literature normally ignores mortality rated after 24 h and regards the substances to be of less or non-toxic effect; that is, the potency of an extract is important within the first 24 h.

Table 13 shows the lethal concentration at 50% of the EtOAc, EtOH and H_2O extracts after 18 hours. This indicates that at 13.305 µg/ml (EtOAc), 112.97µg/ml (EtOH) and 117.12µg/ml (H_2O) the extracts are lethal to the shrimps. However the n-hexane, $CHCl_3$ and MeOH extracts showed no lethality at 50% concentrations.

Table 11: Number of mortalities

Extract (µg/ml)	Time (hours)	n-Hexane	$CHCl_3$	EtOAc	MeOH	EtOH	H_2O	Control
5	3	0	0	0	0	0	0	0
	12	01	0	02	02	0	01	0
	18	05	03	06	07	06	05	02
	24	15	15	15	15	15	15	07
10	3	0	0	0	0	0	0	0
	12	01	01	04	03	0	01	01
	18	06	07	08	07	05	06	02
	24	15	15	15	15	15	15	06
25	3	0	0	0	0	0	0	0
	12	01	03	04	03	02	01	01
	18	05	07	06	06	04	04	04
	24	15	15	15	15	15	15	05
50	3	0	0	0	0	0	0	0
	12	01	03	05	03	02	01	01
	18	05	06	07	05	05	04	05
	24	15	15	15	15	15	15	05
125	3	0	0	0	0	0	0	0
	12	04	04	05	04	04	03	02
	18	07	06	08	07	08	06	05
	24	15	15	15	15	15	15	05
250	3	0	0	0	0	0	0	0
	12	05	06	05	04	05	03	04
	18	07	09	07	07	06	06	05
	24	15	15	15	15	15	15	05

Table 12: Percentage mortality

Extract (µg/ml)	Time (hours)	n-Hexane	$CHCl_3$	EtOAc	MeOH	EtOH	H_2O	Control
5	3	0	0	0	0	0	0	0
	12	6.7	0	13.3	13.3	0	6.7	0
	18	33.3	20	40	46.7	40	33.3	13.3
	24	100	100	100	100	100	100	46.7
10	3	0	0	0	0	0	0	0
	12	7	6.7	26.7	20	0	6.7	6.7
	18	40	46.7	53.3	46.7	33.3	40	13.3
	24	100	100	100	100	100	100	40
25	3	0	0	0	0	0	0	0
	12	7	20	26.7	20	13.3	6.7	6.7
	18	33.3	46.7	40	40	26.7	26.7	26.7
	24	100	100	100	100	100	100	33.3
50	3	0	0	0	0	0	0	0
	12	7	20	33.3	20	13.3	6.7	6.7
	18	33.3	40	46.7	33.3	33.3	26.7	33.3
	24	100	100	100	100	100	100	33.3
125	3	0	0	0	0	0	0	0
	12	26.7	26.7	33.3	26.7	26.7	20	6.7
	18	46.7	40	53.3	46.7	53.3	53.3	33.3
	24	100	100	100	100	100	100	33.3
250	3	0	0	0	0	0	0	0
	12	33.3	40	33.3	26.7	33.3	20	26.7
	18	46.7	46.7	46.7	46.7	40	53.3	33.3
	24	100	100	100	100	100	100	33.3

Table 13: LC_{50} of three extracts after 18 hours

Extract (after 18 hours)	LC_{50} (µg/ml)
EtOAc	13.3050
EtOH	112.9661
H_2O	117.11864

3.7 Conclusion

The present of saponins in large percentages confirms its used by traditional healers in treating infected wounds and ulcers. Flavonoids are known to be antiinflammtory, antiallergic, antiviral, antibacterial, antiviral and antihormorrhagic agents (Formica and Regelson, 1995; Slowing *et al.*, 1996). The high percentage flavonoid content of *P. parvulus* Sond. *var garipensis* indicates that this plant can be used as antiinflammatory and antiviral like those from Nigeria, India and Brazil. The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity (Carballo et al., 2002). The n-hexane, $CHCl_3$ and MeOH were not lethal to the shrimps since the LC_{50} concentrations were not detected, but after 18h the EtOAc, EtOH and H_2O were lethal against shrimps. The challenge is to do the *in vivo* cytotoxicity test as to confirm the toxicity of the plant.

Looking at the results obtained it can be stated that there isn't a huge difference between the two extraction methods, because they both gave almost similar yields which is the most important thing when working with plants.

The isolated compound, lanosterol-amide linked glycoside, has been reportedly isolated from the marine animals. However, this is the first time this compound was isolated from the *Phyllanthus* species.

When comparing the plant with the other species of the same genus, the plant can be said to possess antimicrobial and antioxidant activities, as the other species from the same genus. The challenge now is to try and do the *in vivo* investigations so as to ascertain the potency and

also compare with those from other countries. This is a good indicator for the South African species which is regarded as a weed to possess these biological potentials.

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