STUDIES ON THE CHEMICAL CONSTITUENTS OF THE LEAVES AND SEEDS OF *HYPTIS SPICIGERA LAM.*

BY

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JUNE, 2011

DECLARATION

I declare that the work in this thesis entitled, "**STUDIES ON THE CHEMICAL CONSTITUENTS OF THE LEAVES AND SEEDS OF *HYPTIS SPICIGERA LAM.*"** has been performed by me in the Department of Chemistry, Ahmadu Bello University, Zaria, under the supervision of Professor J.O. Amupitan and Professor A.O Oyewale. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

_____ _____

LADAN, Zakari **Date**

CERTIFICATION

This thesis entitled "**STUDIES ON THE CHEMICAL CONSTITUENTS OF THE LEAVES AND SEEDS OF *HYPTIS SPICIGERA LAM.*"** By LADAN, Zakari meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God for His mercies, and faithfulness and to my darling wife Mrs Esther Omowumi Ladan, my beloved sons: Benjamin Kuzayet Ladan , David Kazah-Yet Ladan and Othniel Kazah-Chat Ladan.

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ABSTRACT

The extracts of the leaves of *H.spicigera* were screened for the presence of secondary metabolites: alkaloids, glycosides, flavonoids, carbohydrates, tannins, sterols, terpenoids and resins. The results of the phytochemical screening showed all the extracts with the exception of hexane extract to contain the secondary metabolites analysed in high and moderate amounts. Antimicrobial activities of the crude extracts of *H.spicigera* against a broad spectrum of micro organisms namely: *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Corynebacterium ulcerans, Salmonella typii, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Neisseria gonorrheae* and *Candida albicans* were carried out. All the extracts showed bactericidal activity against the entire antibiotic resistant micro organisms tested with some degree of variations against standard drug, penicillin. The methanolic extract of *H.spicigera* exhibited significant bactericidal activity at low concentration of 2.5mg/ml while *S.aureus, N.gonnorhea* and *C.albicans* were resistant at MBC of 5.0mg/ml. The insecticidal properties of *H.spicigera* leaf extracts (hexane, ethyl acetate and methanol) tested against *Callosobruchus maculatus* on cowpea was carried out. Azadirachtin was used as standard check along with the extracts tested. Ethyl acetate extract showed the highest percent mortality of 98% each at 72hrs, while 6% and 10% were recorded for the emergence of the *C.maculatus.* Ethyl acetate extract and azadirachtin standard check showed ovi-position deterrent at 4.16% and 2.78% respectively. Hexane extract showed the least percent (2%) seed damage while the control gave the highest percent (88%) of seed damage at 16 weeks. *Hyptis spicigera* seed oil was characterized using GC-MS and UV-VIS spectrometry for its fatty acid profile, tocopherol and physicochemical properties. The oil content was 21% while unsaturated fatty acids were linoleic acid (71.85 %) and palmitic acid (16.06%) as predominant fatty acids. Tocopherol content was 186.15mg/ml while Vitamin A was absent. The study showed potentials of *Hyptis spicigera* seed oil to have high oxidative stability which could be suitable for food and beverage as well as other industrial applications while the tocopherol content could improve human health. Purification of the ethyl acetate fraction by High Performance Liquid Chromatography (HPLC) yielded 3-buten-2-enol on the basis of GC-MS. The best separation using the analytical High Performance Liquid Chromatography (HPLC) was

achieved at 0.8ml/min. The hydrodistillation of the fresh leaves of *H.spicigera* gave a colourless volatile oil with yield of 0.65%.The volatile oil gave forty compounds on the basis of GC-MS with low composition of cineole (4.11%) and caryophyllene (2.61%) while α-pinene (12.16%) β-pinene (9.47%) and α-phellandrene (10.19%) were predominant compounds. Crude ethyl acetate extract of *H.spicigera* leaves was fractionated through solvent separation followed by thin layer and column chromatography. Two relatively pure constituents were obtained and characterized through spectral studies. The structure elucidation of the constituents was established as ursolic acid and its derivative (3β-hydroxy-urs-12-en-28-oic acid) and 3β-hydroxy-20-isopropyl-urs-12-an-28-oic using spectroscopic techniques including 1D and 2D NMR such as ^{1}HNMR, ^{13}CNMR, DEPT, HMQC, HMBC, COSY, NOESY as well as MS, and IR. Toxicity studies on the dichloromethane and methanolic extracts of *Hyptis spicigera* were carried out on mice intraperitoneally. The LD_{50} was calculated using the method of Karber (LD_{50}, 2534mg/kg) which showed the plant to be moderately toxic based on the WHO toxicity rating. The biological activity of one of the new compounds (ursolic acid) was determined against a trypanosome (*T.brucei brucei*).The compound was evaluated in vitro for activity against *Trypanosoma brucei brucei (Tbb)* and was found to possess anti-trypanosoma activity in vitro in a dose dependent pattern at 0.4µg/ml in 3 minutes.

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Abbreviations

$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
C_5D_5N	Deuterated pyridine
$(CD_3)_2CO$	Deuterated acetone
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
COSY	Correlation Spectroscopy
d	Doublet (NMR)
DEPT	Distortionless enhancement by polarisation transfer
DMSO	Dimethyl sulfoxide
DMSO-D_6	Deuterated Dimethyl sulphoxide
EI	Electronic impact
EtOAc	Ethyl acetate
EtOH	Ethanol
FT-IR	Fourier transform Infrared
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High Performance Liquid Chromatography
m	Multiplet (NMR)
Me	Methyl
MeOH	Methanol

Min	Minute
M pt.	Melting point
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PE	Petrol ether
ppm	Parts per million
PTLC	Preparative thin layer chromatography
R_f	Retention factor
s	Singlet (NMR)
t	Triplet (NMR)
TLC	Thin Layer Chromatography

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Natural products can either be of pre-biotic origin or originate from microbes, plants, or animal sources (Nakanishi, 1999). As chemicals, natural products include such classes of compounds as terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acids, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and so forth. Natural products do not just occur by accident or products of convenience of nature, but more than likely are a natural expression of the increase in complexity of organisms (Jarvis, 2000). Interest in natural sources to provide treatments for pain palliatives, or curatives for a variety of maladies or recreational use reaches back to the earliest points of history. Nature has provided many things for humankind over the years, including the tools for the first attempts at therapeutic intervention (Nakanishi, 1999). The *Nei Ching* is one of the earliest health science anthologies ever produced and dates back to the thirtieth century B.C. (Nakanishi, 1999). Some of the first records on the use of natural products in medicine were written in cuneiform in Mesopotamia on clay tablets and date to approximately 2600 B.C. (Cragg and Newman, 2001; Nakanishi, 1999). Indeed, many of these agents continue to exist in one form or the other to this day and are used for the treatment of inflammation, influenza, cough, and parasitic infestation among others. Chinese herb guides document the use of herbaceous plants as far back in time as 2000 B.C (Holt and Chandra, 2002).

For a variety of different reasons, the interest in natural products continues to this very day (Barron and Vanscoy, 1993; Bhattaram *et al.,* 2002; Chan, 1995; Holt and

Chandra, 2002; Koh and Woo, 2000; Marriott, 2001). The first commercial pure natural product introduced for therapeutic use is generally considered to be the narcotic morphine, marketed by Merck in 1826 (Newman *et al.*, 2000). The first semi-synthetic pure drug based on a natural product, aspirin, was introduced by Bayer in 1899.

The World Health Organization estimates that approximately 80 percent of the world's population relies primarily on traditional medicines as sources for their primary health care (Farnsworth *et al*, 1985). Over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 different plants. Approximately 75 percent of these substances were discovered as a direct result of chemical studies focused on the isolation of active substances from plants used in traditional medicine (Cragg and Newman, 2001).The number of medicinal herbs used in China in 1979 has been estimated to be about 5267 (Nakanishi,1999).More current statistics based on prescription data from 1993 in the United States show that over 50 percent of the most prescribed drugs had a natural product either as the drug or as the starting point in the synthesis or design of the actual end chemical substance (Newman *et al*,2000). Thirty-nine percent of the 520 new drugs approved during the period 1983 through 1994 were either natural products or their derivatives (Harvey, 2000). Of the 20 top-selling drugs on the market in the year 2000 that are not proteins, 7 of these were either derived from natural products or developed from leads generated from natural products. This select group of drugs generates over 20 billion U.S. dollars of revenue on an annual basis (Grabley and Sattler, 2003; Harvey, 2000). Drug development over the years has relied only on a small number of molecular prototypes to produce new

medicines (Harvey, 2000). Indeed, only approximately 250 discrete chemical structure prototypes have been used up to 1995, but most of these chemical platforms have been derived from natural sources. While recombinant proteins and peptides are gaining market share, low molecular- weight compounds still remain the predominant pharmacologic choice for therapeutic intervention(Grabley and Sattler,2003).A small sampling of the many available examples of the commercialization of modern drugs from natural products along with their year of introduction, indication, and company are: Orlistat, 1999, obesity, Roche; Miglitol, 1996, antidiabetic (Type II), Bayer; Topotecan, 1996, antineoplastic, SmithKline Beecham; Docetaxel, 1995, antineoplastic, Rhône-Poulenc Rorer; Tacrolimus, 1993, immunosuppressant, Fujisawa; Paclitaxel, 1993, antineoplastic, Bristol-Myers Squibb. The overwhelming concern today in the pharmaceutical industry is to improve the ability to find new drugs and to accelerate the speed with which new drugs are discovered and developed. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. Analysis of the human genome will provide access to a myriad number of potential targets that will need to be evaluated (Grabley and Sattler, 2003). The process of high-throughput screening enables the testing of increased numbers of targets and samples to the extent that approximately 100,000 assay points per day are able to be generated. However, the ability to accelerate the identification of pertinent lead compounds will only be achieved with the implementation of new ideas to generate varieties of structurally diverse test samples (Grabley and Sattler, 2003; Harvey, 2000). Experience has persistently and repeatedly demonstrated that nature has evolved over thousands of years a diverse chemical library of compounds

that are not accessible by commonly recognized and frequently used synthetic approaches. Natural products have revealed the ways to new therapeutic approaches, contributed to the understanding of numerous biochemical pathways and have established their worth as valuable tools in biological chemistry and molecular and cellular biology. Just a few examples of some natural products that are currently being evaluated as potential drugs are (natural product, source, target, indication, status): manoalide, marine sponge, phospholipage- A2 Ca^{2+}-release, anti- inflammatory, clinical trials; dolastatin 10, sea hare,microtubules, antineoplastic, nonclinical; staurosporine, streptomyces, protein kinase C, antineoplastic, clinical trials; epothilone, myxobacterium, microtubules, antineoplastic, research; calanolide A, B, tree, DNA polymerase action on reverse transcriptase, acquired immunodeficiency syndrome (AIDS), clinical trials; huperzine A, moss, cholinesterase, Alzheimer's disease, clinical trials(Grabley and Sattler,2003). The costs of drug discovery and drug development continue to increase at astronomical rates, yet despite these expenditures, there is a decrease in the number of new medicines introduced into the world market. Despite the successes that have been achieved over the years with natural products, the interest in natural products as a platform for drug discovery has waxed and waned in popularity with various pharmaceutical companies. Natural products today are most likely going to continue to exist and grow to become even more valuable as sources of new drug leads. This is because the degree of chemical diversity found in natural products is broader than that from any other source, and the degree of novelty of molecular structure found in natural products is greater than that determined from any other source (Cragg *et al.,* 1997). The introduction of active agents derived from natural sources into the anticancer weaponry has already significantly

changed the future of many individuals afflicted with cancer of many different types. Continued research into natural sources will continue to deliver newer and more promising chemicals and chemical classes of anticancer agents with novel mechanisms of action that will improve survival rates to even higher degrees. Human immunodeficiency virus infection is a devastating, globally widespread disease that consumes significant health-care dollars in the due course of management of patients (Jarvis, 2000). Most of the currently useful anti-HIV agents are nucleosides and are limited in use due to severe toxicity and emerging drug resistance. Natural products, with their broad chemical structural diversity, provide an excellent opportunity to deliver significant therapeutic advances in the treatment of HIV (Yang *et al*., 2001). Many natural products with novel structures have been identified as having anti-HIV activities (Javis, 2000; Yang *et al*., 2001). Betulinic acid, a triterpenoid isolated from *Syzigium claviflorum*, has been found to contain anti- HIV activity in lymphocytes. The quassinoside glycoside isolated from *Allanthus altissima* has been found to inhibit HIV replication. Artemisinin, isolated from *Artemisia anuua*, is a sesquiterpene lactone that is of special interest because of its novel structure, potent antimalarial activity, and activity against *Pneumocystis carinii*. A novel phorbol ester isolated from *Excoecaria agallocba* has been reported to be a potent inhibitor of HIV-1 reverse transcriptase. Indeed, most of the natural product chemicals that are attracting interest in this area of research are secondary metabolites such as terpenes, phenolics, peptides, alkaloids, and carbohydrates and are also inhibitors of HIV reverse transcriptase. Other target opportunities in the life cycle of the human immunodeficiency virus available for exploitation are: (1) attachment of virus to cell surface, (2) penetration and fusion of the virus with the cell membrane, (3) reverse

transcription via reverse transcriptase, (4) integration into the host genome, (5) synthesis of viral proteins including zinc fingers, and (6) processing of viral polypeptide with HIV protease and assembly of viral proteins and DNA into a viral particle, maturation, and extrusion of the mature virus (Yang *et al*., 2001). Infectious viral diseases remain a worldwide problem. Viruses have been resistant to therapy or treatment longer than most other forms of life because their nature is to depend on the cells that they infect for their multiplication and survival (El-Sayed, 2000). Such a characteristic has made the development of effective antiviral chemotherapeutic agents difficult. Today there are few effective antivirals available for use. In order to confidently wage the war against viruses, research efforts are now turning to the molecular diversity available from natural products. For the period 1983 to 1994, seven out of 10 synthetic agents approved by the Food and Drug Administration (FDA) for use as antivirals were based on a natural product. Natural products are indeed viable sources and resources for drug discovery and development (Artuso, 1997). Indeed, without natural products, medicine would be lacking in therapeutic tools in several important clinical areas such as neurodegenerative disease, cardiovascular disease, solid tumors treatment, and immune-inflammatory disease (Banerji, 2000;Harvey, 2000; Nisbet and Moore, 1997).Furthermore, the continual emergence of new natural product chemical structure skeletons, with interesting biological activities along with the potential for chemical modification and synthesis bode well for the utility of natural products. Finally, the uses of natural products need to be by no means restricted to pharmaceuticals but can also be expanded to agrochemicals. For example, the use of pyrethrins obtained from *Chrysanthemum* spp. as insecticides has been very popular over the years and persists today. Research continues into the use of

natural products as pesticides. While the success stories have not been as numerous or spectacular for herbicides as they have been for drugs and pesticides, there have been victories along the way and the future holds strong potential for this field also (Duke *et al*., 2000; Majetich,1993).

In recent times, focus on plant research has increased all over the world and a large body of evidence has accumulated to show immense potential of medicinal plants used in various medical, pharmaceutical, cosmetic, agrochemical applications. Plants have been the subject of human curiosity and use for thousands of years (Ram *et al.*, 2004).These plants have played important roles in many countries of the world for centuries by providing food, shelter, clothing, agrochemicals, flavours and fragrances and more importantly, medicines (Gurib-Fakim, 2006).Traditional people have relied on medicinal plants to combat various ailments and many others, and this information has been passed down from generation to generation (vonMaydell,1996). A large proportion of these ailments occur due to the presence of microorganisms such as bacteria, fungi and viruses that infect the body system. In some countries particularly the African continent, medicinal plants are sold in open market places or prescribed by traditional herbal healers in their homes (Fyhrquist *et al*., 2002) to help improve human and animal health. Plants have indeed formed the basis of sophisticated traditional medicine systems glob will continue to provide mankind with new remedies for all forms of ailments (Gurib-Fakim, 2006). Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute no less than 25% to the total (Gurib-Fakim,2006).Natural products, particularly medicinal plants, will remain an important source of new drugs, new leads and new chemical entities (NCEs)(Newman *et*

al.,2000;Newman *et al*.,2003;Butler,2004). It has been indicated that in 2001 and 2002 approximately one-quarter of the best selling drugs worldwide were natural products, or were derived from natural products (Butler, 2004). Bioactive natural products have enormous economic importance as specialty chemicals. They can be used as drugs, lead compounds, biological or pharmaceutical tools, feed stock products, excipients and nutraceuticals (Pieters and Vlietinck, 2005). More than 80% of the population in developing countries depends on plants for their medical needs (Farnsworth, 1988; Balick *et al*., 1994). Nearly 88% of the global population turns to plant-derived medicine as their first line of protection for maintaining health and combating diseases (Samy *et al*., 1999). Due to this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994). With the increasing acceptance of herbal medicines as alternative form of health care delivery, the screening of medicinal plants for bioactive compounds is imperative (Masoko *et al*., 2005; Cowan, 1999). It has been estimated that one-quarter to one-half of all pharmaceuticals dispensed in the United States are from higher plant origins. The use of plant extracts as well as other alternative forms of medical treatments is currently enjoying great popularity. It is estimated that there are over 250,000 higher plant species on this planet. Of these figure, only about 6% have been screened for biological activity and a reported 15% have been evaluated phytochemically (Ayensu and DeFilipps, 1978; Verpoorte, 2000).With increase now in the popularity of medicinal plant research, these figures may have risen. An advantage of natural bioactive molecule is that they have a milder side effect on the body in comparison to chemically synthesized drugs (Badisa *et al.*, 2003).

With considerable interest in the screening of plant and other natural product extracts in modern drug discovery programs structurally novel chemotypes with potent and selective biological activity may be obtained (Kinghorn and Balandrin, 1993; Gullo, 1994; Colegate and Molyneux, 1993; Cragg *et al.,* 1997). A consideration of biological activity in addition to the isolation and structure elucidation stages in a phytochemical investigation may add a great deal to the overall scientific significance of the work. Phytochemists may gain considerable information by using panels of simple bioassays and/or more specialized *in vitro* bioassays to follow each step of a purification procedure (Colegate and Molyneux, 1993).

Plant secondary metabolites also show promise for the cancer chemoprevention, which has been defined as "the use of non-cytotoxic nutrients or pharmacological agents to enhance physiological mechanisms that protect the organism against mutant clones of malignant cells." There has been considerable prior work on the cancer chemopreventive effects of extracts and purified constituents of certain culinary herbs, fruits, spices, teas, and vegetables, which have shown the ability to inhibit the development of cancer in laboratory animal models (Ho *et al*.,1994).Clinical trials as cancer chemopreventive agents under the auspices of the United States National Cancer Institute are planned for plant products such as curcumin, ellagic acid, and phenethyl isothiocyanate (Kelloff *et al.,* 2000).

Figures show that about 25% of all prescriptions sold in the United States are from natural products, while another 25% are for structural modifications of a natural product (Farnsworth, 1977). Furthermore, Farnsworth (1977) claims that 119 characterized drugs are still obtained commercially from higher plants and that 74% were

found from ethnobotanical information. Of the several hundred thousand plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically. When one considers that a single plant may contain up to thousands of constituents, the possibilities of making new discoveries become evident. The crucial factor for the ultimate success of an investigation into bioactive plant constituents is thus the selection of plant material.

In view of the large number of plant species potentially available for study, it is essential to have efficient systems available for the rapid chemical and biological screening of the plant extracts selected for investigation. Phytochemical studies on medicinal plants have served the dual purpose of bringing up new therapeutic agents and providing useful leads for chemotherapeutic studies directed towards the synthesis of drugs modeled on the chemical structure of natural physiological products. The studies in the correlation of chemical structure and physiological activity through functional variation in active constituents of the plant materials in this case, can be promoted further.

Today's pharmaceutical companies rely heavily upon the exploration of botanicals for providing "new leads". Some traditional examples of natural product leads that have worked their way into common pharmaceutical use (Table 1) are shown below.

Table 1.0: Traditional plants with their uses

Name of Compound	Botanical Source	Uses
d-tubocuraine	Chondodendron	muscle relaxant
Quinine	Cinchona	Anti-malarial
Pilocarpine	Pilocarpus jaborandi	Glaucoma
Amphotericin	Streptomyces nodosus	Anti-fungal
Cephalosporin	Cephalosporium	Antibiotic
Reserpine	Rauwolfia serpentine	Tranquilizer
Cocaine	Erythroxylon coca	Local anesthetic
Morphine	Papaver somniferum	Analgesic
Mitomycin	Streptomyces caespitosus	Anti-cancer
Mescaline	Lophophora williamsii	Hallucinogen

The exploration of plant extracts for effective therapeutic molecules is certainly ongoing. In fact, industrial drug discovery process has historically relied heavily upon the application of chemical methods as a means of screening plant/animal species for new drugs. It is the job of the chemist to utilize certain techniques to isolate compounds that may eventually lead to the discovery of the discrete components of a molecule (pharmacophore) that are necessary for a molecule to elicit a biological action. In turn, the pharmacophore can then be refined into a new molecule that can ultimately be synthesized in the laboratory. The goal is to synthesize new molecules (that may, in some cases, look radically different from the original compound) that retain the intrinsic activity of the natural compound or even enhance its potency and efficacy. In the last few decades there has been an increase in the demand for herbal drugs in various branches of medicinal care and this has made it imperative for scientist to study the therapeutic claims of reputed traditional medical practitioners. The pharmacopoeias of many countries have recorded large number of drugs of plant origin. Interestingly, many African plants are used in traditional medicine as antimicrobial agents and for treatment of other ailments, but only a small proportion of such plants, plant parts and uses are documented. Also very few of these uses have been subjected to scientific investigation and this necessitates the screening of more plants species, since only a minute percentage of plants on earth have been explored in research studies (Badisa *et al*., 2003).

Today plant based products, essential oils, plant extracts, natural resins and their preparations have a wide range of applications mainly in perfume and cosmetic industry, in food technology, in aroma industry and in pharmaceutical industry. This large spectrum of uses stimulated studies on natural products. The methods used in the analysis

of plants that started at the end of the 19th century, only allowed investigations on crystalline constituents isolated from these extracts. Subsequent developments on vacuum distillation techniques provided the possibility to determine the volatile components of this extracts.Along with the developments in extraction techniques, the development of chromatographic techniques primarily with planar chromatography (thin layer chromatography (TLC)) and other novel analytical methods were introduced to the benefit of scientists. Gas chromatography (GC) in the 1950's had opened a new dimension in the analysis of volatile compounds. In the meantime high performance liquid chromatography (HPLC) was introduced for the fractionation and isolation of more polar and non-volatile compounds.

The combination of gas chromatography and mass spectrometry (GC-MS) allows the rapid identification of not only volatile components but also plant extracts, by comparing their mass spectra with available libraries which build up with reference substances recorded under the same experimental parameters. The same principle has been applied in the last decade for liquid chromatography and mass spectrometry (LC-MS) for non-volatile plant constituents. Moreover, the invention of chiral stationary phases for gas chromatography, mostly based on cyclodextrins, has facilitated the identification of the enantiomeric composition of the isolated substances, especially in essential oils. Simultaneously, the advances in spectroscopic methods such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have increased the speed of the identification and structure elucidation of natural products.

The present work aims at studying the chemical constituents of *Hyptis spicigera* plant found in Northern Nigeria for better utility in industries such as pharmaceuticals,

agrochemicals, cosmetics, oleochemicals and as lead for the synthesis of biologically active compounds.

1.2 Aim

The study aimed at achieving the following:

i. Phytochemical screening of the various extracts of leaves of *Hyptis spicigera* for use as a bio-control agents in agriculture.

ii. Antimicrobial study of the extracts of the leaves of *Hyptis spicigera* on some selected pathogens.

iii. Evaluation of the insecticidal properties of the various extracts on grain weevils using cowpea.

iv. Isolation and purification of compounds from the extracts of *Hyptis spicigera* plant.

v. To elucidate structures of the isolated compounds using Nuclear Magnetic Resonance Spectroscopy (NMR), Mass Spectrometry (MS) and other spectroscopic techniques.

vi. To identify active ingredient/s.

vii. To further conduct biological evaluation of the pure fractions.

The above aims are individually of special problem-solving relevance. Moreover, they are mutually complimentary in helping to provide the much needed information on the scientific data of the botanical extracts of the leaf part of *Hyptis spicigera* which may find use as pharmaceuticals, agrochemicals, and cosmetics among others.

1.3 Statement of the Problem

Plant resources have provided the basic needs of life such as food, fibre, fuel and shelter and will continue to provide these needs and much more on a renewable basis. Plants have also been a valuable source of flavours, fragrances, colourants, and phytochemicals for industries and pharmaceuticals. The rising incidence of health related problems in both developing and developed countries has prompted research in the development of drugs from leads identified from traditional medicinal uses as an alternative approach to manage new deadly diseases and those that have become resistant to available drugs. There is also a resurgence of interest on plant- based medicines due to the undue side effects of modern synthetic therapeutic agents and their inability to cure diseases, many of them being for the treatment of symptoms. Herbal healthcare products could also help in uplifting the quality of life of the ageing populations.

The World Health Organisation has initiated global efforts to urge governments to take steps to upgrade the traditional medical systems and treatments of their respective countries through validation of their quality, safety and efficacy. Guidelines have been developed by WHO on standardisation, quality control and analysis of herbal medicinal products. These initiatives have sparked off considerable interest in the international health-related scientific community to re-evaluate traditional therapies based predominantly on the use of medicinal plants. Presently, research on the untapped plant resources and leads from traditional uses that would hopefully identify useful bioactive compounds/extracts with therapeutic relevance is being extensively carried out by scientists in developed and developing countries. Additionally the role of plant- based products as dietary/ health supplements, nutraceuticals, cosmecuticals and personal care

products are being investigated and many countries have recognized the need for such products. It is clear from the high number of ongoing and completed studies in this research area that plant- derived pharmaceuticals and healthcare products can meaningfully contribute towards the management of the biochemical and physiological functions of a wide range of disorders. However in many cases, the issue of mechanism of action and pharmacodynamics is a major concern and it is necessary to conduct research extensively focused on molecular level activities and clinical trials that will contribute to our understanding of the efficacy and safety of many extracts with therapeutic potential.

1.4 Significance of the study

The significance of the study lies in the need to authenticate claims by traditional herbal healers and farmers on the efficacy of the plant in treating ailments and as pest repellents in protecting grains from damage. This will also lead to the generation of scientific data on the biological properties of the plant which is in abundance in the Northern part of Nigeria. The scientific data generated will help in the development of soft natural bio-control agents that can replace chemical insecticides imported into the country. The use of bio-control agents will reduce pesticide load in the environment and conserve foreign exchange from the importation of synthetic pesticides.

1.5 Justification for the study

Medicinal plants constitute an important source of bioactive molecules (Sharma, 2002).Plants synthesize secondary metabolites as defense mechanisms to protect them

against microbial infections. The presence of these compounds provide an invaluable resource that has been used to find new drug molecules (Gurib-Fakim,2006).Compounds such as muscarine, physostigmine, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al*.,1996).It is estimated that 60% of anti-tumor and anti-infectious drugs already on the market or under clinical trials are of natural origin (Yue-Zhong,1998).Many of these chemical compounds cannot be easily synthesized but can be obtained from wild or cultured plants (Rates,2001).Recently, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank *et al*.,1982;Vulto and Smet,1988;Mentz and Schenkel,1989).The interest in drugs of plant origin is due to several reasons, for example, conventional medicine can be ineffective or may result in side effects, can be very costly and unaffordable especially by the poor population in the African continent and lot of other problems. A large percentage of the world population does not have access to to conventional pharmaceutical products (Rates, 2001).

The test plant *Hyptis spicigera* is commonly found in the Northern part of Nigeria. The choice of this plant for the present study is based principally on the result of a survey (Abubakar and Abdulrahman, 1998) carried out on some plants in Kaduna State, Nigeria, for pesticidal properties of which *Hyptis spicigera* showed a remarkable repellency against some weevils that attack grains. Also, claims by local farmers and traditional herbal healers on the medicinal properties of this plant with excellent results have further added interest on the need to study the biological properties of this plant.

Insect pests form a major component of adverse environmental factors militating

against sufficient food production in Nigeria. They reduce crop yields in the field and equally destroy stored food. Rural farmers suffer especially from the destructive tendency of these insect pests as they cannot afford the high cost of pesticides available in the market. These pests are vectors of many human diseases and need to be eradicated. Nigeria currently depends heavily on the importation of synthetic insecticides. There is no single indigenous company as far as we know that is involved in the manufacture of bio-insecticides in Nigeria. Synthetic insecticides are not environmentally friendly and their massive importation affects our foreign exchange earnings.

Several varieties of plants have been reported to possess insecticidal properties (Pushpalatha and MuthuKrishna, 1995). The active compounds responsible for the insecticidal property in some of these plants have also been isolated and characterized and the mechanisms of action elucidated (Chauret *et al.,* 1976; Dwuma Badu *et al.,* 1976; Madrigal *et al.,* 1979; Gbewonyo and Candy, 1992). There is little or no detailed scientific report or study on the secondary metabolites and the biological properties of *Hyptis spicigera.* (NAPRELERT, 2005). This study is aimed at providing some scientific data on the secondary metabolites and the biological properties of the plant *Hyptis spicigera.*

1.6 Statement of research hypothesis/research question.

All the various extracts of the leaf part of the plant exhibit biological activity against some microorganisms. The claim by the local users of this plant will be established. Scientific methods will be used to isolate some of the bio-active compounds of the leaf part for structural elucidation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botanical Description of *Hyptis spicigera* Lam

Hyptis spicigera belongs to the family Lamiaceae. It is commonly known as **Black beniseed, or Black sesame** (Burkill, 1995). It is an erect aromatic herb, up to 1metre in height, with a terminal inflorescence in which the seeds are packed in quadruplets or more in the flowers. The seeds are very small and light (Burkill, 1995).The plant is found around Senegal to western Cameroon, possibly native to Brazil, now widely naturalized in tropical Africa and Asia as well as Nigeria. It grows naturally and commonly as a weed. It prefers roadsides, waste places, cultivated places and often damp places (Burkill, 1995).The seeds are used for oil production while the leaves are eaten as vegetables. Generally, the whole plant is used in traditional stores to protect cowpea against damage by *Callosobruchus* species (Lambert *et al.*, 1985).It is locally used as mosquito repellant by burning of the whole plant (Dalziel, 1937).

2.2 Plant Classiffication

Kingdom: *plantae* – Plants

Subkingdom: *Tracheobionta* – Vascular plants

Superdivision: *Spermatophyta* – Seed plants

Division: *Magnoliophyta* – Flowering Plant

Class: *Magnoliopsida* – Dicotyledons

Subclass: *Asteridae*

Order: *Lamiales*

Family: *Lamiaceae* – Mint family

Genus: *Hyptis* Jacq – Bushmint

Species: *Hyptis spicigera* Lam – Marubio

Growth Habitat: Subshrub

2.3 Morphological Description:

The plant is wild, but in some parts of Central and West Africa, it is cultivated like sesamum (Beniseed). *Hyptis spicigera* is an erect aromatic herb, which grows up to 1metre, sometimes more, and possesses green lanceolated leaves with a terminal inflorescence. The terminal inflorescence is dense cylindrical or ovoid with cylindrical spike up to 9cm long(Burkill, 1995).The plant possesses very tiny brown/black seeds that cluster in groups of fours, fives or even more, which are encased in each flower that make up the inflorescence(Dalziel,1937).

2.4 Uses of *Hyptis spicigera*

Hyptis spicigera plant is locally known as, "**Bunsuru fadama**" or "**Dai fadama**"by Hausas in northern Nigeria.Part of the plant commonly used traditionally is the leaves. When the plant is crushed and applied to the head, it relieves head colds and headaches (Dalziel, 1937). The Bajju and Tyapp people of Kaduna state, Nigeria, make use of the inflorescence (where the seeds are packed) to cure headaches by sniffing it.



Fig 1: A typical mature *Hyptis spicigera* plant.

Traditionally, in the northern part of Nigeria, it is used by the local herbalist for the treatment of cold and stomach congestion in children. Also, it is used to massage boils and cuts or burns that have pus. It is also used for cough in addition with some other spices like garlic, ginger and olive oil. The plant has several medicinal uses as claimed by traditional practitioners.The whole plant is used for making a local concoction known as, "**Agbo**"in Yoruba which could be drank or the steam from the leaves inhaled for the treatment of cold and feverish conditions.The plant with other items added is also used for the cure of chesty cough by massaging the concoction on the chest of the person with such a condition. It is also said to be used for rheumatism when the plant extract is mixed with other items.

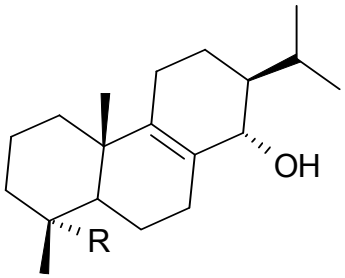
The seeds are eaten and used for oil production, which has a yield of about 24-37% of drying oil. The leaves are eaten as vegetables (Burkill, 1995).In the Upper Volta, the whole plant is used in traditional stores to protect cowpeas against damage by insects (Lambert *et al.*, 1985). It is also used to treat granaries before storing grains and it is usually placed at the bottom before filling the grains for example, groundnut, bambaranut and cowpea and this protects the grains from birds and insects that would normally feed on them. When the whole plant is burnt it has been found to be repellant to mosquitoes.

2.5 Terpenoidal constituents

The genus *Hyptis,* family Lamiaceae (Labiatae) comprises about 400 species, and is one of the largest genera of the family. It is native of Central and South America, including the southern part of United States (Willis, 1966; Falcao and Menezes, 2003). Now several species were introduced in Western and Central Africa (for instance

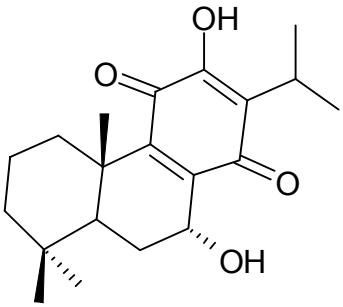
Cameroon, Nigeria, Burkina Faso, Ivory Coast), and even in India, China, Thailand and Fiji Islands, where they are cultivated specially for the production of essential oils. Moreover, they are important for the ethno pharmacologic point of view. For this reason a large number of species has been investigated for their content of essential oils, and minor attention was given to other constituents. For instance, the occurrence of diterpenoids has been reported only in nine species.

A total of 66 diterpenoids occurs in the genus, and 26 of them show heterocyclic structures. The first paper concerning this investigation was published in 1974 and reported the isolation of two novel diterpenoids with the abietane skeleton from the aerial parts of *Hyptis suaveolens* collected in Trinidad (Manchand *et al*., 1974): suaveolic acid **[1]** and suavelol **[2]**.Two years later, three diterpenoids were isolated from the root extract of *Hyptis fruiticosa* harvested in Brazil (Marletti *et al.*, 1976). The first one was identified with the already known horminone **[3]**, **[5]** while the second product was new and was attributed the structure **[4]** of 14-methoxy-taxodione. The structure of the third product was elucidated the following year (Delle Monache *et al*., 1990) as **[5]**: it is a new natural compound and was given the name hyptol. All these three diterpenoids have the abietane skeleton, and hyptol **[5]** shows α, β-unsaturated furanic ring fused with the C-ring of the abietane skeleton.



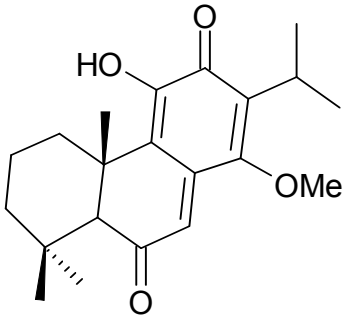
[1]: R = CO_2H

[2]: R = CH_2OH

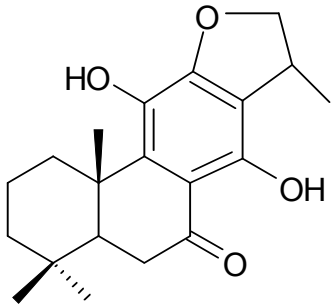


[3]

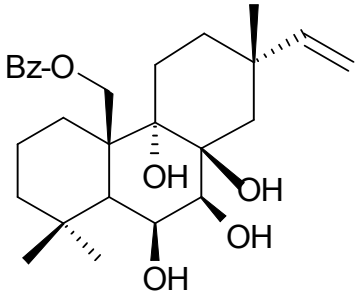
Suaveolic acid [1], Suavelol [2]



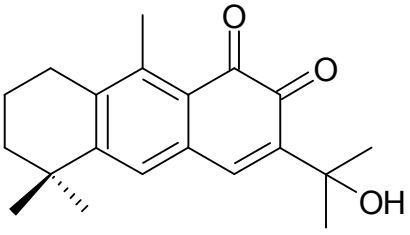
[4]



[5]



[6]

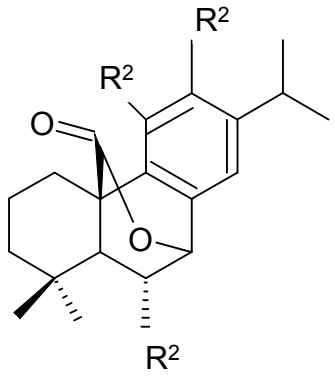


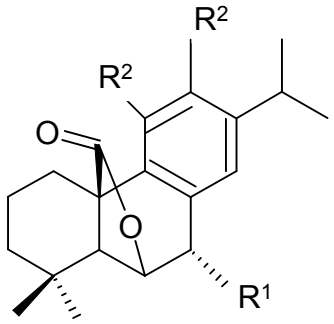
[7]

A diterpenoid with a different skeleton was isolated in 1990 from the leaves of *Hyptis salzmanii,* growing in Brazil (Messana *et al*., 1990). It was given the name salzol (6,7,8-trihydroxy-20-benzoyloxy-isopimarane),**[6]**.In the same year the roots of *Hyptis umbrosa* from Brazil (Delle Monache *et al*., 1990) yielded an unusual product, with a rearranged abietane skeleton, named umbrosone. The elucidation of the structure **[7]** rested on deep NMR investigations. The aerial parts of *Hyptis dilatata,* collected in Panama (Urones *et al., 1998)* proved to be very rich in diterpenoids. The paper published in 1998, based on extensive chromatographic fractioning, reported the isolation of three known abietane compounds from the first fraction: carnosal **[8]** (Brieskorn *et al*., 1964), rosmanol **[9]** (Inatani *et al*., 1982; Fraga *et al*.,1985) methyl-rosmanol **[10]** (Arisawa *et al.*, 1987; Gonzalez *et al.*, 1989).The treatment of the following fractions required acetylation and diazomethane treatment: many known abietane derivatives were isolated: diacetyl-carnosol **[11]**.(Brieskorn *et al*., 1964; Inatani *et al.,1982)* triacetyl-rosmanol **[12]** (Inatani *et al.,1982;* Arisawa *et al.*, 1987) diacetyl-methylrosmanol **[13]** (Arisawa *et al.*, 1987)diacetyl-ethylrosmanol **[14]** (Gonzalez *et al.*, 1989) triacetyl-isorosmanol **[15]**,(Nakatani and Inatani,1984) triacetyl-epirosmanol **[16] (** Fraga *et al*.,1985) diacetyl-carnosic acid **[17] (**Gonzalez *et al.*, 1989) diacetyl-carnosic acid methylester **[18]** (Al-Hazimi, 1986) acetyl-pisiferic acid methylester **[19] (**Gonzalez *et al*.,1989;Geiwiz and Haslinger,1995).Two previously undescribed derivatives were identified as: diacetyl-epimethylrosmanol **[20]** and diacetyl-epiethylrosmanol **[21]** respectively.

It must be evidenced that the nine abietane structures **[11]-[21]** are not authentic natural products, but derivatives originating from the acetylation/diazomethane treatment: the diterpenes originally occurring before the treatment are carnosol **[8]**, rosmanol **[9]**,

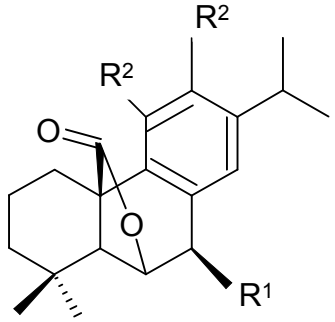
methylrosmanol **[10]**, but evidently also ethylrosmanol **[22]**, isorosmanol **[23]**, epirosrnanol 2-t, epimethylrosrnanol **[25]**, epiethylrosmanol **26**, carnosic acid **[27]**, pisifericacid **[28]**, all not acetylated or partially acetylated products. These true natural products **[22]-[28]** were not isolated.

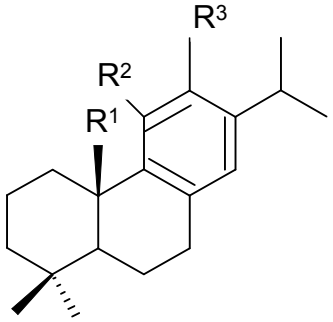




[8]	R^1 = H	R^2 = OH
[11]	R^1 = H	R^2 = OAc
[15]	R^1 = OAc	R^2 = OAc
[23]	R^1 = OH	R^2 = OH

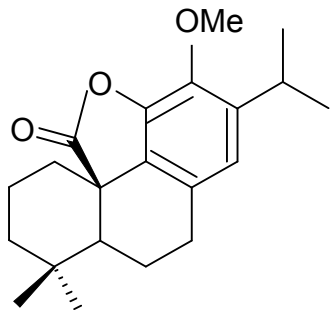
[9]	R^1 = OH	R^2 = OH
[10]	R^1= OMe	R^2 = OH
[12]	R^1 = OAc	R^2 = OAc
[13]	R^1 = OMe	R^2 = OAc
[14]	R^1 = OEt	R^2 = OAc
[22]	R^1 = OEt	R^2= OH



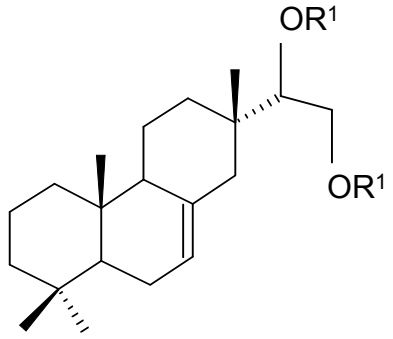


[16]	R^1 = OAc	R^2 = OAc
[20]	R^1= OMe	R^2 = OAc
[21]	R^1 = OEt	R^2 = OAc
[24]	R^1 = OH	R^2 = OH
[25]	R^1 = OMe	R^2 = OH
[26]	R^1 = OEt	R^2= OH

[17]	R^1 = CO_2H	R^2 = OAc	R^3 = OAc
[18]	R^1= CO_2Me	R^2 = OAc	R^3 = OAc
[19]	R^1 = CO_2Me	R^2 = H	R^3 = OAc
[27]	R^1 = CO_2H	R^2 = OH	R^3 = OH
[28]	R^1 = CO_2H	R^2 = H	R^3 = OH

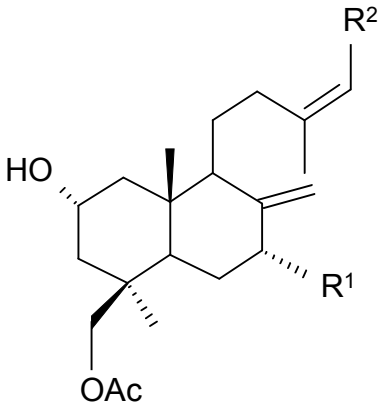


[29]

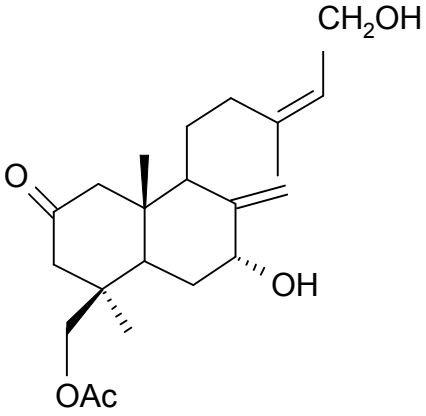


[30] R^1 = Ac

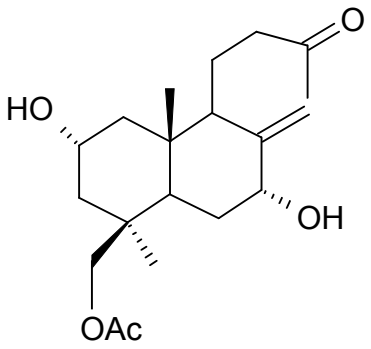
[31] R^1 = H



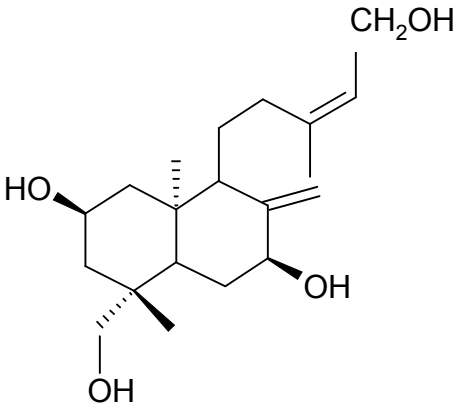
[32]	R^1 = OH	R^2 = CH_2OH
[33]	R^1 = OH	R^2 = CH_2OAc
[34]	R^1 = OAc	R^2 = CH_2OAc
[35]	R^1 = OH	R^2 = CHO



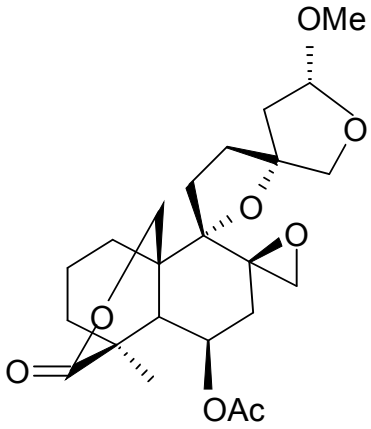
[36]



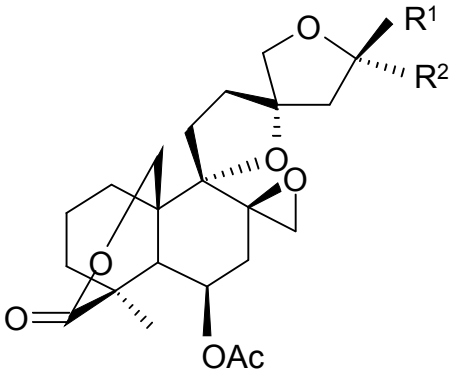
[37]



[38]



[39]

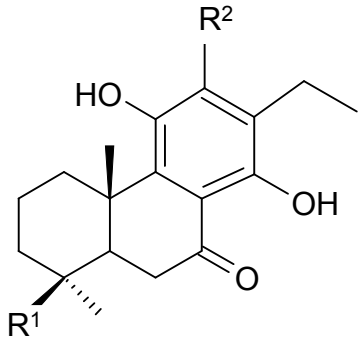


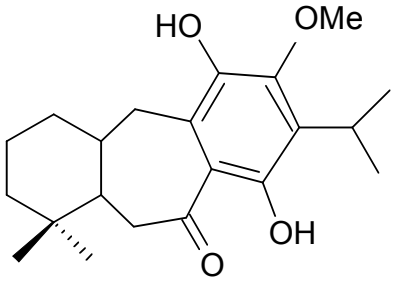
[40] R^1 = OMe R^2 = H

[41] R^1 = H R^2 = OMe

Many of the diterpenoids isolated from *Hyptis dilatata* show heterocyclic rings of 20,6- and 20,7-1actones. Also the known l9 heterocyclic **[20]**, 1-lactone **[29]** was isolated. All the **[8]-[29]** products show the abietane skeleton.

Seven new products have been isolated from the aerial parts of *Hyptis spicigera* occurring in Mexico (Fragoso-Serrano *et al*., 1999) and popularly known as "hierba del burro". It is remarkable that these compounds show different skeletons, as they are labdane derivatives.The first product has the structure **[32]** of 19-acetoxy-2α,7 α, 15-trihydroxy-labda-8(17),(13Z)-diene. The other new products are 15.19-d iacetoxy-2α,7α-dihydroxy-labda-8(17),(13Z)-diene **[33]**: 7α,15.19-triacetoxy-2α-hydroxy-labda-8(17),(13Z)-diene 34; 19-acetoxy-2α,7α-dihydroxy- labda 8(17),(13Z)-dien-15-al 35: 19-acetoxy-7α,15-dihydroxy-labda-8(17),(13Z)-dien-2-one **[36]**; 19-acetoxy 2α,7α-d ihydroxy-labda-14, 15-dinor-labd-8(17)-en-13-one **[37]**; 2α,7α, 15, 19-tetrahydroxy-ent-labda 8(17),(13Z)-diene **[38]**. It is remarkable that product **[38]** has the labdane skeleton, whereas the [**33]-**[**37]** products have the normal labdane backbone.Three diterpenoids, also in this case with the labdane skeleton, have been found in the aerial parts of *Hyptis fasciculata* collected in Brazil (Ohsaki *et al*.,2005).The first product was identified as the known (Manchand *et al*, 1974) methoxynepetaefolin **[39]**, showing four oxigenated rings, whilst the others are new natural products, 15 β-methoxyfasciculatin **[40]** and 15α-methoxyfasciculatin **[41]**, both also rich in oxygenated rings.



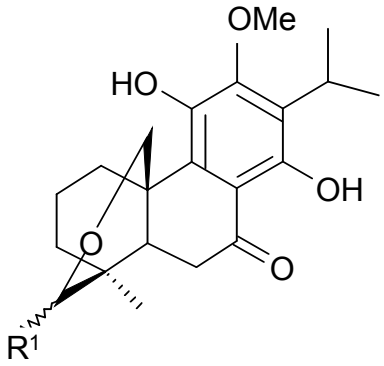


[42] R^1 = Me R^2 = OMe

[43]

[44] R^1 = CHO R^2 = OMe

[47] R^1 = Me R^2 = H

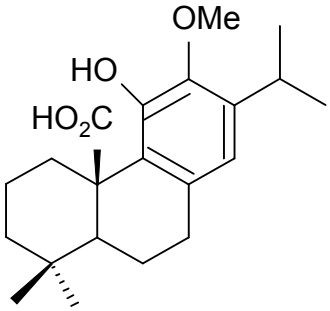


[45] R^1 = O

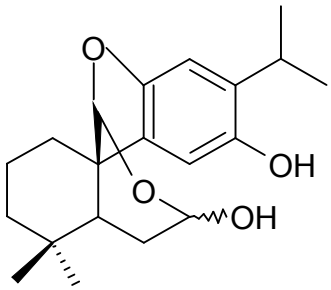
[46] R^1 = OH

A recent paper (Araujo, 2003) reported on the investigation of the roots of *Hyptis platanfolia,* again from Brazil. Five abietane derivatives were isolated: two of them are the known inuroyleanol **[42]** and coulterone **[43]** (Frontana *et al*., 1994) whilst new products are 19-oxo-inuroyleanol **[44]**. 11, 14-dihydroxy-12-methoxy-7-oxo-8, 11, 13-abidatrien-19,20β-olide **[45]** and 19,20-epoxy-12-melhoxy-11, 14, 19-trihydroxy-7-oxo-8, 11,13-abietatriene [**46]**, the last as a diastereoisomeric mixture at the 19-hydroxy group. No trivial names were given to these new products. Both products **[45]** and **[46]** have a lactone or lactol 20, 19-ring.

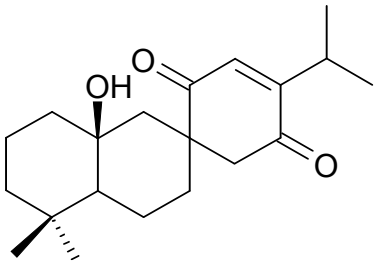
Another species from North eastern Brazil is *Hyptis martillsii,* popularly known as "cidreira-do-mato", whose roots were investigated. A paper reported the isolation of two known diterpenoids: carnosol **[8]** (Brieskorn *et al*., 1964) and 11,14-dihydroxy-8, 11,13-abietatrien-7-one **[47]**.Some new diterpenes have been described: 12-O-methyl-carnosic acid **[48]** and the structurally interesting 7 -seco-7(20), II (20)-diepoxy-7,14-dihydroxy-abieta-8,11,13-triene **[49]**(Araujo, 2003) as well as a product **[50]** with rearranged abietane skeleton, designated as martiusane. Two more abietane diterpenes have been found: the known 7α-acetoxy-royleanone **[51]** and the new 7β-hydroxy-11, 14-dioxo-abieta-8, 12-diene **[52]** (Araujo, 2003).



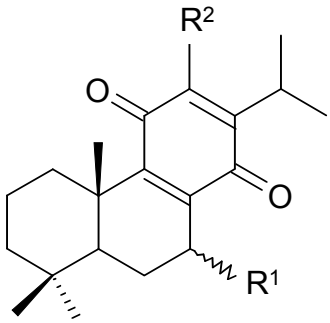
[48]



[49]

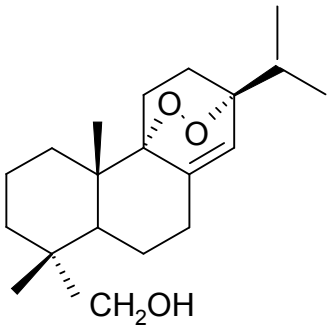


[50]



[51] R^1 = αOAc R^2 = OH

[52] R^1 = βOH R^2 = H

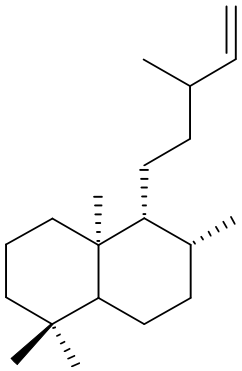


[53]

An abietane diterpenoid with high anti plasmodial activity has been isolated (Chukwujekwu *et al*., 2005) from the leaves of *Hyptis suaveolens* collected in Nigeria. It has the structure **[53]** of 9α,13α-epi-dioxiabict-8(14)-en-18-ol. The same product had been isolated from *Cedrus atlantica* (Barrera *et al*., 2005).

The **[1]-[53]** diterpenoids listed below have been isolated from the aerial parts or from the roots or from the whole plant. A large number of investigations on the essential oils of this genus have been carried out for the purpose of identification of their components, almost exclusively mono- and sesquiterpenes. However, in one case also the occurrence of minor amounts of some diterpenes have been detected. In the case of the oil from *Hyptis suaveolens,* studies showed the presence of three unidentified diterpenes (Flores and Medina, 1970). In a sample of the oil from Malaysia the GC analysis indicated the occurrence of six unidentified diterpenes (Din *et al*., 1988). In a sample of oil from Nigeria (Iwu *et al*., 1990) showed the occurrence of a diterpene hydrocarbon indicated as 5βH,8βH,9βH, 10α-labd-14-ene 54 but no structural formula was given. Two diterpenes, 8.11,13-abietatriene **[55]** and 8, 11, 13-abietatrien-18-ol **[56]** (syn. dehydroabietol, dehydroabietinol) were identified in the oil from Kumaon, India (Pant *et al*., 1992).Particularly rich in diterpenoids was the oil from Cameroon (Ngassoum *et al*.,1999): biformene **[57]**, atisirene **[58]**, rimuene **[59]**, abietatriene **[55]**, phyllocladene **[60]**, abietinol **[61]** (syn. abietol), dehydroabietol **[56]** and the linear diterpene phytol **62** were identified. The oil from North India (Rana *et al*., 2004) contained abietadiene **[63]**, abietatriene **[55]** and dehydroabietol **[56]**. Many products occurred in the oil from Alfenas (Brazil) :(Martins *et al.*, 2006,) abietatriene **[55]**, abietadiene **[63]**, abieta-7,l3-dien-3-one **[64]**. abietinol 61. 4-epi-abietinol 65,dehydroabietol **[56]**, manoyloxide **[66]**.

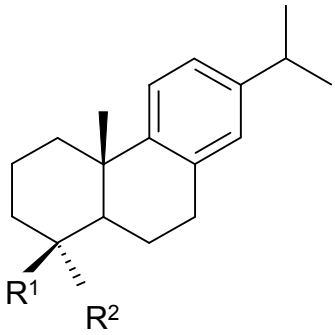
Remarkable differences were observed in the content of diterpenes (and also of monoterpenes and sesquiterpenes) depending on the geographical origin of the oil and on the season of harvesting.



[54]

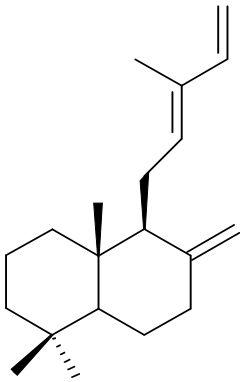
R^2 = Me

R^2 = CH_2OH

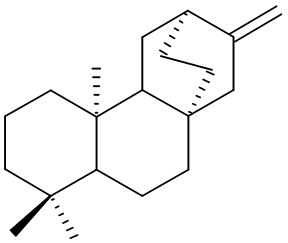


[55] R^1 = Me

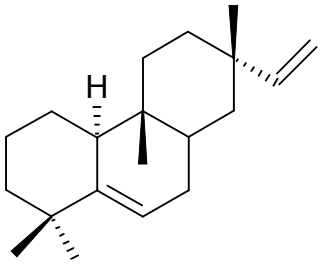
[56] R^1 = Me



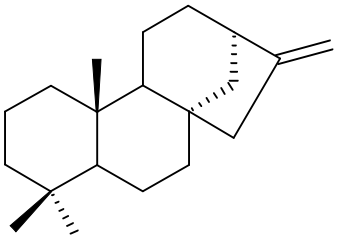
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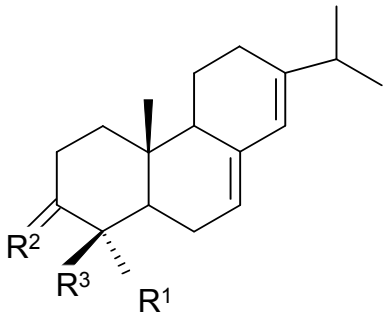
[58]

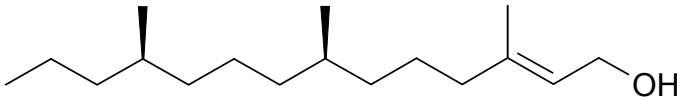


[59]



[60]



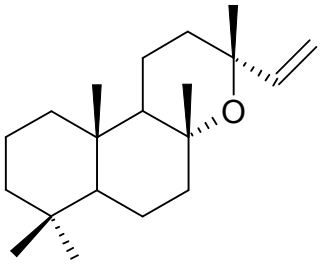


[61] $R^1 = CH_2OH$ $R^2 = H.H$ $R^3 = Me$ **[62]**

[63] $R^1 = Me$ $R^2 = H.H$ $R^3 = Me$

[64] $R^1 = Me$ $R^2 = O$ $R^3 = Me$

[65] $R^1 = Me$ $R^2 = H.H$ $R^3 = CH_2OH$



[66]

There is no report on the occurrence of diterpenoids in other oils in the literature as far we know. Concerning the structure of these diterpenoids, it can be remarked that they show the abietane or the labdane skeleton prevalently only few have different skeletons.

2.6 Biological and ethnopharmacological applications

2.7 Biological Significance of Terpenoids and Other Secondary Metabolites

The need to explain the *raison d'être* of the enormously rich structural variations of the secondary metabolites that are encountered rather in plants than in animals, brought up some arguments claiming that they can not be simply "waste products" of primary metabolism accumulated in the plant cell because of the absence of an efficient excretory system in these organisms. Beginning with the latter half of the last century, reasons for their existence have been confirmed in many studies in the field of chemical ecology.

The immobile living style of plants has forced them to develop a self-defense system against herbivores, insects or microorganisms, as well as an intraspecific communication system and exchange of information with other living organisms that would support their production and the continuity of their race. Secondary metabolites as terpenoids, flavonoids, alkaloids and many other compounds are responsible for data transfer between different kinds of living organisms.For example, a group of these metabolites called phytoalexines, in which isoflavonoids, sesquiterpenes, furanoterpenes, polyacetylenes and dihydrophenanthrenes can be encountered, exhibit antimicrobial activities and act in the plant body against phytopathogenic microorganisms such as fungi

and viruses. The production of these compounds in the cell is induced as a response to infections in the plant, to stress situations like drought, cold, severe exposure to UV-light or to herbivorous attack.

In this context, terpenoids are very valuable sources for plants because of their toxic and deterrent properties which are useful in defense systems and because of their volatility which renders them ideal signals in communication systems.Terpenoids, from volatile monoterpenoids to nonvolatile triterpenoids, may be broadly deterrent against herbivory on plants. Not only individual compounds but also mixtures of related structures often contribute to produce antifeedent and toxic effects. The pine bark beetle, *Dendroctonus brevicomis* avoids feeding on pine trees which are high in limonene content, although α- and β-pinene, myrcene and 3-carene do not disturb its feeding. Most sesquiterpene lactones and some diterpenes appear to have repellent effects towards insect herbivores. The triterpenoid azadirachtin is a well known natural insecticidal agent. Slugs and snails are also known to feed on a wide variety of plant species. But two sesquiterpenes, petasin and furanopetasin which occur in *Petasites hybridus* repel snails feeding on this plant.

The most diffused utilization of species belonging to the *Hyptis* genus is related to their essential oils, obtained, also in industrial scale, from several species. Such oils are largely used in cosmetics and flavouring and as insecticides and insect repellents. Dried whole plants are used for pest control. Oils, parts of plants and extracts find diffused application in ethno pharmacology of tropical countries. About the biological activities and related pharmacology, a lot of information is reported in a review (Falcao and Menezes, 2003) and the references therein. Rarely such pharmacological activities have

been attributed to a single diterpenoid (Falcao and Menezes, 2003; Fragoso-Serrano *et al*, 1999; Costa-Lotufo *et al*., 2004 ;Araujo, 2003) or to the whole mixtures of terpenoids, but usually to the oils or to the extracts in different solvents. In the case of *H. martiusii,* the cytotoxic and antimitotic activity have been tested on the pure diterpenoids (Costa-Lotufo *et al*., 2004; Araujo 2003).The diterpenoids from *H. spicigera* have been shown to inhibit the larval growth of the European corn borer (Fragoso-Serrano *et al*, 1999).Ladan *et al*., 2009 have also reported the potency of *H.spicigera* leaf extract on broad spectrum of pathogenic microorganisms that are resistant to ampicillin drugs.

Therefore, many reports of this plant are concerned with activities of its extracts as expectorant, antihelmintic, antibacteric, antimicrobic, antiviral antibiotic, antimicotic, antiseptic, antimalaric, antidolorific, anticonvulsive, antioxidant, cytotoxic, anti-tumour. Preparations are used against gastrointestinal disorders, fevers, cough, asthma, cramps, conjunctivitis, depression and other ailments. The extract of *H spicigera* shows antimicrobial, phytotoxic and antifungal properties, and cytotoxic activity on KB cells 45 (Fragoso-Serrano *et al*., 1999). The powdered plant is used widely as insecticide in Africa (Ayeard *et al*., 1993).

The ethanol extract of *H.umbrosa* has a significant antimicrobic activity and also the methanol extract of *H.salzmanii* is active against *Staphylococcus aureus, Bacillus subtilis, Candica albicans* and *Mycobacterium smegmatis* (Messana *et al.*, 1990).The methanol extract of *H albida* is active against C. *albicans* and other Gram-positive and Gram-negative bacteria (Rojas *et al*., 1992) and used in Mexico against gastroenteric diseases (Pereda-Mirinda *et al*., 1993). Some *Hyptis* species are active against schistosomiasis (Gilbert *et al.*, 1970).

The essential oil of *H.mutabilis* has antiulcerogenic activity (Barbosa and Barbosa, 1992) and the essential oil of *H lanceolata* shows antifungine properties (Zollo *et al*., 1998). Extracts of leaves of *H.Ilabilis* are used in Mexico and Brazil against malaria and a lot of ailments (Barbosa and Barbosa,1992; Pereda-Mirinda and Delgado, 1990).The oil from *H.pectinata* has insecticide (Pietschmann *et al*.,1998) and antiseptic activity while the extracts of *H.pectinatu* have antimicrobic, antibacteric activity (Pereda-Mirinda and Delgado, 1990) and are cytotoxic against various cancer cells.The aqueous extract is antinociceptive and anti-edematogenic (Bispo *et al*.,2001) it is widely used in Mexico and West Africa. The methanol extract of the roots of *H.fructicosa* showed antibacterial and anti-tumour activity (Araujo, 2003) while the terpenoids from *H.emoryi* have been investigated as antifertilizing agents (Tanowitz *et al.*, 1984) and tumour-inhibitors. The extracts of the whole plant of *H. capitata* have been reported to be active against HIV, leukemia, and cancer.It also exhibited anti-inflammatory, anti-arthritic, anti-diabetic, anti-ulcer, anti-artheriosclerosy properties while *H. verlicillata* has various activities such as insecticide, acaricide, anticancer, anti-inflammatory, cytotoxic, antimitotic, antimicrobial (Lee *et al*.,1988).

Four species of Lamiaceae, namely, *Mentha cordifolia* Opiz ex Fresen, *Ocimum basilicum*, *Fornia citratum* Back and *H. suaveolen* (L.) Poit were examined individually for their antibacterial properties and the synergistic effect against drugs-susceptible and drugs-resistant clinical isolates of bacteria (Chitsomboon *et al.*, 2003). All of them showed antibacterial activities against the clinical isolates tested. *H. suaveolens* individually displayed the best antibacterial activity against methicilin-resistant *Staphylococcus aureus*. *H. suaveolens* in combination with *O. basillicum* showed

synergistic effect against ciprofloxacin-resistant *Pseudomonas aeruginosa*. Dehydroabietinol isolated from *H. suaveolens* was found to inhibit growth of chloroquine-sensitive as well as chloroquine-resistant strains of *P. falciparum* cultivated in erythrocytes *in vitro* (IC_{50} 26-27 μm) (Christensen , 2002). However, erythrocytes exposed to dehydroabietinol were transformed in a dose- dependent manner towards spherostomatocytic forms with concomitant formation of endovesicles, as disclosed by transmission electron microscopy. Thus, dehydroabietinol incorporates into the erythrocyte membrane effecting the survival of *Plasmodium* parasites. The observed antiplasmodial effect of dehydroabietinol is presumably an indirect effect on the host cell. Because of these findings, microscopic investigations should be generally used to support claims of antimalarial effects of apolar natural products.

The most investigated species is H. *suaveolens,* which is largely employed in ethno-medicine in Mexico, Brazil, Central and South America, West Africa, India (Rao and Adinarayana, 1970; Asekun *et al*, 1999). The roots, leaves, flowers are commonly used in ethno-medicine, the oil is active as antimicrobial, antibacterial, fungicidal (Singh and Handique, 1992). It has been reported that suaveolic acid **[1]** and suavelol **[2]** occurring in *Hyptis suaveolens* collected in EI-Salvador have anti-inflammatory activity and has been previously isolated from the same species.

Table 2.0: Diterpenes from Genus *Hyptis*

Compound	Hyptis specie	Structure No.
Suaveolic acid	*H. suaveolens* (*A*)	3
Suavelol	*H. suaveolens* (A)	3
Horminone	*H. fruticosa* (R)	4
14-methoxy-taxodione	*H. fruticosa* (R)	4
Hyptol	*H. fruticosa* (R)	6
Salzol	*H. salzmanii* (A)	7
Umbrosone	*H. umbrosa* (R)	8
Carnosol	*H. dilatata* (A)	9
	H. martiusii (R)	26
Rosmanol	*H. dilatata* (A)	9
Methylrosmanol	*H. dilatata* (A)	9
Diacetyl-carnosol	*H. dilatata* (A)	9
Triacetyl-rosmanol	*H. dilatata* (A)	9
Diacetyl-methylrosmanol	*H. dilatata* (A)	9
Diacetyl-ethylrosmanol	*H. dilatata* (A)	9
Triacetyl-isorosmanol	*H. dilatata* (A)	9
Triacetyl-epirosmanol	*H. dilatata* (A)	9
Diacetyl-carnosic acid	*H. dilatata* (A)	9
Diacetyl-carnosic acid methyl ester	*H. dilatata* (A)	9
Acetyl-pisiferic acid methyl ester	*H. dilatata* (A)	9
Diacetyl-epimethylrosmanol	*H. dilatata* (A)	9

Diacetyl-epiethylrosmanol	*H. dilatata* (A)	9
Ethylrosmanol	*H. dilatata* (A)	9
Isorosmanol	*H. dilatata* (A)	9
Epirosmanol	*H. dilatata* (A)	9
Epimethylrosmanol	*H. dilatata* (A)	9
Epiethylrosmanol	*H. dilatata* (A)	9
Carnosic acid	*H. dilatata* (A)	9
Pisiferic acid	*H. dilatata* (A)	9
Lactone	*H. dilatata* (A)	9
Diacetyl-esquirolin B	*H. dilatata* (A)	9
Esquirolin B	*H. dilatata* (A)	9
19-acetoxy-2α,7α,15-trihydroxy-labda-8(17),(13Z)-diene	*H. spicigera* (A)	21
15,19-diacetoxy-2α,7α,15-dihydroxy-labda-8(17),(13Z)-diene	*H. spicigera* (A)	21
19,7α,15-acetoxy-2α-hydroxy-labda-8(17),(13Z)-diene	*H. spicigera* (A)	21
19-acetoxy-2α,7α,15-dihydroxy-labda-8(17),(13Z)-diene-15-al	*H. spicigera* (A)	21
19-acetoxy-7α,15-dihydroxy-labda-8(17),(13Z)-diene-2-one	*H. spicigera* (A)	21
19-acetoxy-2α,7α,15-dihydroxy-labda-14,15-dinor-labd-8(17),(13Z)-en-13-one	*H. spicigera* (A)	21

2α,7α,15,19-tetrahydroxy-ent-labda-8(17),(13Z)-diene	*H. spicigera* (A)	21
Methylnepetaefolin	*H. fasciculate* (A)	22
15β-methoxfasiculatin	*H. fasciculate* (A)	22
15α-methoxyfasciculatin	*H. fasciculate* (A)	22
Inuroyleanol	*H. platanifolia* (R)	24
Coulterone	*H. platanifolia* (R)	24
19-oxo-inuroyleanol	*H. platanifolia* (R)	24
11,14-dihydroxy-12-methoxy-7-oxo-8,11,13-abietatriene-19,20-β-olide	*H. platanifolia* (R)	24
19,20-epoxy-12-methoxy-11,14,19-trihydroxy-7-oxo-8,11,13-abietatriene	*H. platanifolia* (R)	24
11,14-dihydroxy-8,11,13-abietatriene-7-one	*H.martiusii* (R)	26
12-O-methyl-carnosic acid	*H.martiusii* (R)	28
7-seco-7(20)-diepoxy-7,14-dihydroxy-abieta-8,11,13-triene	*H.martiusii* (R)	28
Martuisane	*H.martiusii* (R)	28
7α-acetoxy-royleanone	*H.martiusii* (R)	29
7β-hydroxy-11,14-dioxo-abieta-8,12-diene	*H.martiusii* (R)	29
9α,13α-epi-dioxiabiet-8(14)-en-18-ol	*H. suaveolens* (A)	31
5βH,8βH,9βH,10α-labd-14-ene	*H. suaveolens* (EO)	35
8,11,13-abietatriene	*H. suaveolens* (EO)	36-40
8,11,13-abietatriene-18-ol	*H. suaveolens* (EO)	36,38-40

Biformene	*H. suaveolens* (EO)	37
Atisirene	*H. suaveolens* (EO)	37
Rimuene	*H. suaveolens* (EO)	37
Phyllocladene	*H. suaveolens* (EO)	37
Abietinol	*H. suaveolens* (EO)	37,39-40
Phytol	*H. suaveolens* (EO)	37
Abietadiene	*H. suaveolens* (EO)	38,39,40
Abieta-7,13-dien-3-one	*H. suaveolens* (EO)	39,40
4-epi-abietinol	*H. suaveolens* (EO)	39,40
Manoyloxide	*H. suaveolens* (EO)	39,40

R = roots. A = aerial parts. EO = essential oil.

2.8 Pesticidal properties

Pesticides have made great contribution for quick and effective management of plant diseases and microbial contaminations in several agricultural commodities. In spite of use of all available means of plant protection, about 1/3 of the yearly harvest of the world is destroyed by pests and losses due to this is expected to be nearly $300 billion per year (Chandler, 2005). Incessant and extensive use of synthetic pesticides is posing serious environmental problems due to their residual toxicity (Campos *et al*., 2005). It is estimated that hardly 0.1% of the agro-chemicals used in crop protection reaches the target pest, leaving the remaining 99.9% to the environment to cause hazards to non target organisms including humans. In recent years a large number of synthetic pesticides have been banned in the Western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain and extension of their ability to destroy both useful and harmful pests (Ortelli *et al*., 2005). With constant use, many pathogenic microorganisms and pests have acquired resistance to synthetic pesticides (White *et al*., 2002). Pathovars of *Xanthomonas* are known to cause diseases on several vegetable and cash crops and are reported to have developed resistance to kanamycin, ampicillin, penicillin and streptomycin (Mandavia *et al*., 1999). This seriously hinders the management of diseases of crops and agriculture products. Considering the deleterious effects of synthetic pesticides on life supporting systems, there is an urgent need for alternative agents for the management of pathogenic microorganisms and pests (Mahajan and Das, 2003). A green plant represents a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Cowan, 1999; Newman *et al*., 2000; Gibbons, 2005). Reports are available on the use of

active agents from higher plants, in place of chemical fungicides, that are non-phytotoxic, more systemic and easily biodegradable (Gottlieb *et al*., 2002). This has led scientists to screen large number of plants for antibacterial, antifungal, insecticidal activity against important seed borne phytopathogenic microorganisms and crop pests with the ultimate aim of developing plant based formulations for plant disease management (Kiran and Raveesha, 2006; Raghavendra *et al*., 2006).

Insects are the major pests of cereals grain or flour during storage. Damages due to insects affect the quality, the quantity, the commercial and agronomic value of the product. Many pests of stored products are Coleopterans and the most destructive tropical species for cereals belong to the genus *Sitophilus* and *Tribolium* (Ahn *et al*., 1998). The control of pests in stored products, principally cereal grains, by use of the chemicals, a common strategy for post harvest loss avoidance, leads to the apparition of many problems like the pollution of environment, toxicity to human being, emergence of resistant pest strains and many others damages (Cimanga *et al*., 2002).These synthetic pesticides are expensive for the users and may cause potential risk due to lack of technical knowledge related to their safe use. Among these unexpected effects, the development of resistance by some pests occurred and the lethal effect on non-target species are possibilities. It is the case of *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae) which is nowadays one of the most resistant insects to industrial pesticides. Moreover, *T. castaneum* is a major pest of cereal flour depreciating the quantity and quality of the food (Delobel and Tran, 1993) *T. castaneum* in grain storage appears after the implementation of major grain pest as *Sitophilus* species. Any treatment to cure attacks of this pest potentially leads to direct poisoning of consumers because the

flour or main components of animal food treated by chemical pesticides are directly consumed or used for the formulation of provender. Chemical insecticides cannot be mixed with flour or provender because of their adverse effect on food quality (Schumutterer, 1990).Very little is reported on the use of chemical natural or synthetic to *T. castaneum* in flour or provender destined to the alimentation. It becomes, therefore, useful to build up alternative methods of controlling pest by methods that are user-friendly as the use of agents with high efficacy on the pest and low persistence in the food. There are needs to develop and popularise such control techniques that are clean and user-friendly as the use of natural essential oils. These natural products as essential oils are often highly specific and biodegradable of low persistence. In the early seventies, an alternative was the use of natural products as pesticides to control pests during storage; some of these natural products protect grain without any observed effects on their germination, smell and taste. Ethnobotany has therefore, played a very important role in the protection of crops against pests in Africa and Asia where plants and plant products have been used in granaries by the farmers naturally to protect their farm produce from insect attack. Most of the essential oils or vegetable oils used in crop protection are extracted from plants formerly known to have insecticidal activity. A popular figure is that of neem seeds (*Azadirachta indica*) and cotton seeds (*Gossypium hirsutum*). Recent research report on this topic focused on the oils which are environment and consumer-friendly.The active ingredients of neem tree belongs to a general class of natural products called triterpenes or more specifically, “Limonoids” which are concentrated in the seed. These limonoids otherwise known as tetranortriterpenoids are mainly azadirachtin, nimbin, nimbidin, salanin, meliantriol, di-acetyllazadiracthtin of which azadirachtin is the

most active. These limonoids are known to have distinct antifeedant and growth inhibitory effects (Schummutterer, 1990).Neem products have shown activity on a wide range of insect pests of many crops worldwide (Schmutterer, 1985; Jacobson,1986; Saxena,1989). Insecticidal effect of neem is closely related to the larvicidal, acaricidal and nematicidal properties.It has been observed that ethanol extract of seeds of *A.indica* mixed with beans protected the grain against pest infestation during storage without affecting instrumental hardness (% hard to cook/mean gram force) and consumer acceptabilitgy (Dunkel *et al*., 1995).Neem seed oil and a fraction containing volatile components have been demonstrated to adversely affect the gonotrophic cycle and oviposition of female *Anopheles stephensis* and *Anopheles* culicifacies (Diptera:culicidae), causing irreversible impairment of vitellogenesis (Dhar *et al*., 1996).Similarly, a 1% azadirachtin formulation commercially called ‘’Neemazal’’ has been found effective against root sucking (*Aphis fabae* Hom.Apididae) and free-feeding pest insects (*Heliothis armigera* Lep.Noctuidae) in a concentration and time-dependant manner (Hummel and Kleeberg,2002).Insecticidal activity of neem extracts have also been reported against *Clavigralla scutellaris,Gryon fulviventre* (Mitchell *et al.,*2004),cowpea bruchid,*Callosobruchus maculatus* (Lale and Mustapha, 2000) and female boll weevil (Shower *et al*., 2004).A comparative assessement of the insecticidal effect of *Azadirachta indica, Hyptis suaveolens* and *Ocimum gratissimum* on *Sitophilus zeamais* and *Callosobruchus maculatus* (Iloba and Ekrakene,2006) showed that *H.suaveolens* performed better in reducing emergence trend of new adults than the other plants tested.

2.9 Other essential oils from plants

Volatile oils are very complex mixtures of compounds. The constituents of the oils are mainly monoterpenes and sesquiterpenes which are hydrocarbons with the general formula $(C_5H_8)n$. Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters,ethers, ketones, phenols and oxides. It is estimated that there are more than 1000 monoterpene and 3000 sesquiterpene compounds. Other compounds include phenylpropenes and specific compounds containing sulphur or nitrogen. Hundreds of new natural substances are being isolated and identified every year, but data concerning their biological activities areknown for only some. In certain plants, one main constituent may predominate. In basil, for example, methyl chavicol makes up 75% of the oil. In other species, there is no single component which predominates. Instead, there is a balance of various components, as for example in the oil of sweet marjoram where the individual chemicals are represented by 0.1 –10% of total oil volume. The presence of trace components, even those as yet unidentified, can influence the odour, flavour and possibly also the biological activity of the oil to a significant extent.

Various industries are now looking into sources of alternative, more natural and environmentally friendly antimicrobials, antibiotics, antioxidants and crop protection agents.The possibility of utilising volatile oils is now being investigated as, although their biological activity has been known for centuries, their mode of action was not fully understood. The biological activity of the oils can be compared with the activity of synthetically produced pharmacological preparations and should be investigated in the same way (Colgate, 1993; Svoboda *et al*., 1998; Svoboda and Deans 1995; Baratta *et al.,*

1998a, b). Generally, their action is the result of the combined effect of both their active and inactive compounds. These inactive compounds might influence resorption, rate of reactions and bioavailability of the active compounds. Several active components might have a synergistic effect. To add to the complexity of volatile oils, there is evidence that the time of harvest influences the oil composition and consequently the potency of their biological activity (Deans and Svoboda, 1988; Lis-Balchin *et al.,* 1992; Galambosi *et al*., 1993; Marotti *et al*., 1994a, 1994b). Other factors such as genotype, chemotype, geographical origin and environmental and agronomic conditions, can all influence the composition of the final natural product (Svoboda *et al*., 1992, 1995; Collins *et al.,* 1994; Galambosi *et al*., 1999). In addition, enantiomeric composition of various monoterpenes in different species can further complicate the biological activity of given oil (Ravid *et al.*, 1987; 1992a, b; 1994a – e, 1996). This complexity of the final product explains why pharmaceutical firms prefer synthetic or semisynthetic compounds. They are mainly interested in the discovery of active chemical structures from which they can develop and prepare synthetic analogues. These are more controllable from the point of reproducibility, patentability, safety, and are more economically viable. Consequently, individual components of essential oils are at present under specific investigation to elucidate their particular activity (Table 4). For example, carvacrol, citronellol, eugenol, geraniol and limonene were tested as efficient food preservatives, without showing any mutagenicity.

An excellent survey on the uses of fragrances and essential oils as medicaments was published by Buchbauer and Jirovetz (1994). It has been suggested that volatile oils, either inhaled or applied to the skin, act by means of their lipophilic fraction reacting with

the lipid parts of the cell membranes, and as a result, modify the activity of the calcium ion channels. At certain levels of dosage, the volatile oils saturate the membranes and show effects similar to those of local anaesthetics. They can interact with the cell membranes by means of their physiochemical properties and molecular shapes, and can influence their enzymes, carriers, ion channels and receptors. The authors describe various studies concerning the physiological effects on humans. These include brain stimulation, anxiety-relieving, sedation and antidepressant activities, as well as increasing the cerebral blood flow. The studies also describe the effects of odours on cognition, memory, and mood. The fragrance compounds are absorbed by inhalation and are able to cross the blood-brain barrier and interact with receptors in the central nervous system. Bioassays used for the description and explanation of volatile oil action, are usually carried out on mice, rats and toads e.g. the influence of peppermint oil on intestinal transport (Beesley *et al*., 1996); the effect of volatile oils on the skin penetration (Abdullah *et al*., 1996); the effect on skeletal muscle fibres (Fogaca *et al*., 1997); the screening for analgesic properties (Aydin *et al.*, 1996). Increasing numbers of aromatherapists and physiotherapists are using essential oils both in private practice and 3 within NHS hospitals and hospices, and their reports in all the main aromatherapy journals stress the positive effects of oils.

The most important suggested areas of essential oil use are in urology, dermatology, sleep and nervous disorders, laxatives, erosive gastritis, cardiac and vascular systems, immunomodulating drugs, colds and coughs. Various plant species are being investigated in detail and are being thoroughly tested for their pharmacological properties. The oils are tested in behavioural pharmacological experiments, their effects

being monitored for use over a wide range of conditions as well as for any signs of toxicity. These conditions include stress-related illness, formation of stomach ulcers and tumor growth. Their effects on blood circulation, nerve growth, nucleic acid, liver, protein, lipid, carbohydrate and cholesterol metabolisms are also monitored, as well as any effects on the activity of the adrenal gland and the body's immune system. Antiviral activity of the volatile oil from *Houttuynia cordata* was tested against herpes simplex virus, influenza and HIV-1 (Hayashi *et al*., 1995). It was suggested that the antiviral activity of the oil may be due to interference with the virus envelope. In another experiment (Tkachenko *et al.*, 1995) essential oil from several species of the genus Heracleum showed promising activity against influenza virus. Further experiments are required to substantiate claims of antiviral activities and to elucidate the mode of action. Only 3 references were found from the last 5 years concersning detailed investigation of anticancer activities (Aruna and Sivaramakrishnan, 1996; Hailat *et al*., 1995 and Saenz *et al*., 1997), where the authors used enzymes and cellular polypeptides assays to assess various cancer cell lines. *Nigella sativa, Cuminum cyminum, Papaver somniferum* and *Ocimum sanctum* volatile oils can be considered as protective agents against carcinogenesis.

Plant essential oils as antioxidants were researched in detail with the view to investigating their protective role for highly unsaturated lipids in animal tissues (Deans *et al.*, 1993). The oils have shown their action as those of hepatoprotective agents in ageing mammals and these studies described the beneficial impact of volatile oils upon the PUFA's, in particular the long chain C_{20} and C_{22} acids. In addition, volatile oils also demonstrated a positive effect upon docosahexaenoic acid (DHA) levels in ageing rodent

retinas. The reason that antioxidants are important to human physical well being comes from the fact that oxygen is a potentially toxic element since it can be transformed by metabolic activity into more reactive forms such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, collectively known as active oxygen. These molecules are formed in living cells by various metabolic pathways. Specific molecules, pollution from tobacco smoke and burning of fossil fuels, together with UV radiation and pollutants such as ozone, nitrogen oxide and sulphur dioxide, add to the formation of free radicals. Superoxide is converted by an enzyme, superoxide dismutase, into H_2O_2. H_2O_2 is able to cross all biological membranes. Much of the damage done by these compounds is thought to be due to their conversion to highly reactive oxidants such as the hydroxyl radical. Formation of OH from O_2 requires traces of catalytic metal ions, mainly iron and copper. The ability of the copper ion / H_2O_2 system to do severe damage to proteins and DNA is well established. The hydroxyl radical is very reactive as it combines with almost all molecules found in living cells. Proteins, lipids, carbohydrates and DNA in living cells represent oxidizable substrates. The secondary events include changes in membrane structure, permeability and fluidity, lysosomal destabilization and stimulation of apoptosis. Lipid peroxidation finally leads to loss of membrane function and integrity leading to cell necrosis and death. Hydroxyl radicals can also react with bases in the DNA and cause mutations. Oxygen free radicals appear to be an important factor in chronic inflammatory joint disease such as rheumatoid arthritis. There are several known reactions of oxygen centred free radicals which are relevant to tissue injury in inflamed joints. Single oxygen can also be generated in the lens of the eye and contribute to the development of cataracts. Superoxide and hydrogen peroxide can stimulate growth in a

variety of malignant mammalian cell types. They may have an important role as extracellular messengers for cell growth and viability.Several substances have been proposed to act as antioxidants in vivo. They include beta carotene, albumin, uric acid, oestrogens, polyamines, flavonoids, ascorbic acid, plant phenolics, vitamin E and some drugs such as non-steroidal anti-inflammatories. They can stabilise the membranes by decreasing their permeability and they also have an ability to bind free fatty acids. It has been suggested that volatile oils could act as such agents. It has been found that certain volatile oils and their components are cytostatic to tumour cell lines and can offer potential as novel antiproliferative agents. Dorman *et al*. (1995) screened Pelargonium sp., *Monarda citriodora var.* citriodora*, Myristica fragrans, Origanum vulgare ssp.* hirtum and *Thymus vulgaris* for their antioxidative effect using a thiobarbituric acid (TBA) assay. The oils showed active antioxidant capacities at extremely low levels of dilution. Rosemary has long been recognised as having antioxidant molecules and these have been identified as carnosic acid, carnasol, carsolic acid, rosmaridiphenol and rosmarinic acid, found in ethanol-soluble fraction. Antioxidant properties are also found in the volatile oil fraction. However, it is very important to realise that in certain cases, antioxidants can be pro-oxidant and can stimulate free radical reactions.

Volatile oils of many plants are known to have antimicrobial activity (Deans *et al.*, 1992; Piccaglia *et al.*, 1993). This activity could act as chemical defence against plant pathogens. Pathogens can readily penetrate at wound sites caused, for example, by herbivores.Wounding of leaves which are covered with volatile oil glands results in the rupture of glands causing the oil to flow over the wound. The existence, therefore, of antimicrobial activity in the oil, would be of considerable benefit to the plant. Indeed, a

good majority of aromatic and medicinal plants do not succumb to many of the commonest diseases. It is also suggested that complex oil presents a greater barrier to pathogen adaptation than would a more simple mixture of monoterpenes. This theory is well documented in the detailed study of *Myrica gale* volatile oil and its inhibitory properties against a broad spectrum of fungal species (Carlton *et al*., 1992; Svoboda *et al.*, 1998b). The complicated mixtures of monoterpenes and sesquiterpenes in the whole oil represented the strongest barrier to fungal infection. Deans and Ritchie (1987) examined 50 plant volatile oils for their antibacterial properties against 25 genera of bacteria, using an agar diffusion technique. The main plant species and microorganisms investigated by other researchers over subsequent years using this same technique, with or without modifications, are listed in Tables 6 and 7. Volatile oils exhibited various reductions in growth of microorganisms, depending on the oil concentration and chemical composition. Food microorganisms (e.g. Salmonella enteritidis and Listeria monocytogenes) are of particular interest (Fyfe *et al*., 1998; Tassou *et al*., 1995).

A lot of attention has been focused on the pesticidal properties of the *Hyptis spicigera* plant and other species of *Hyptis*. The essential oils from the plant have shown high mortality on the first larval instars and on young adults with doses 436.04 and 548.18ppb (Kouninki *et al*., 2005).At this last dose, even aged adults were highly sensitive, the mortality observed was above 95%.The fifth larval instars were resistant at all the doses tested, and no important mortality was observed (Kouninki *et al*,2005).The genus *Hyptis* is very large (400 species), and many of its reported species are known for their medicinal and pesticidal use by local herbal practitioners and have been evaluated by scientists as well (Almtorp *et al*.,1991; Kuhnt *et al*., 1994; Raja *et al.,*1990).Some of

the species have antifungal, antibacterial, and anticonvulsant (Akah and Nwambi, 1993; Asekun *et al*., 1999) properties and can be used in the treatment of gastrointestinal ailments and malaria (Pereda-Miranda and Gascon-Figueroa, 1988). The leaves of those species are also largely used as potent insect repellents traditionally in many parts of the world (Aycard *et al*.,1993;Pereda-Miranda and Delgado, 1990).The bioactivity of the essential oils from *Hyptis suaveolens* against storage mycoflora have been carried out on stored food grains (Sharma,2002).This study showed that the volatile oils of *H.suaveolens* contain 1,8-cineole which possessed fungitoxic activity and can be exploited for the management of spoilage of stored commodities.

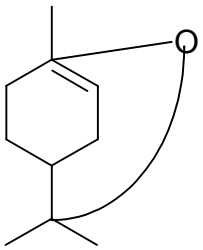
Azevedo *et al*., (2001) investigated the essential oils of *H. suaveolens* collected from eleven localities in the Brazilian Credo region by GC-MS. It was found that sabinene, limonene, bicyclogermacrene, β-phellandrene and 1,8-cineole were the principal constituents. Three groups of essential oils were distinguished with respect to the content of p-mentha-2,4-diene, limonene, β-phellandrene,γ-terpinene and bicyclogermacrene. Geographically, variation in essential oil composition indicated that the sesquiterpenes are mainly produced in the plant samples grown at lower latitudes. According to the study of some workers (Aguirre*,* 2004), a novel trypsin inhibitor purified from the seeds was purified and characterized. Its N-terminal sequence showed a high content of acidic amino acids (seven out of 18 residues). Its inhibitory activity was potent toward all typsin-like proteases extracted from the gut of the insect *Prostehanus truncatus* (Coleoptera: Bostrichidae), a very important pest of maize. This activity was highly specific, because among proteases from seven different insects, only those from *P. truncatus* and *Manduca sexta* (Lepidotera: Sphingidae) were inhibited. It has been found

that the essential oil of *H. suaveolens* displayed significant inhibitory activity against two gram-positive and four gram-negative bacteria and was also very active against the fungus *Candida albicans* (Adeniyi *et al.*, 1999).

One interesting research work about *H. suaveolens* has been done by Peerzada (1997). It was reported that twenty three compounds were found and abundant enough to be identified by GC-MS.

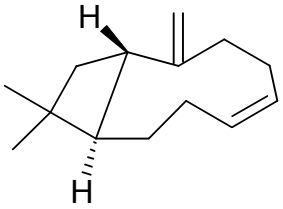
Table 2.1: The chemical composition of essential oils from *H. suaveolens*.

Component	Percentage (%)
α-Thujene	0.3
A-Pinene	2.5
Camphene	0.02
Sabinene	3.9
β-Pinene	4.2
Myrcene	0.6
A-Phellandrene	2.0
1,8-Cineole	32
γ-Terpinene	0.7
α-Terpino1ene	0.3
Linalool	0.06
Fenchol	0.3
4-Terpinenol	2.3
α-Terpineol	0.2
Eugenol	1 .2
α-Copaene	1 .8
β-Elemene	1 .0
β-Caryophyllene	29
α-Bergamotene	1 .6
A-Bergamotene	2.0
Aromadendrene	0.5
γ-cadinene	0.1
δ-cadinene	0.5



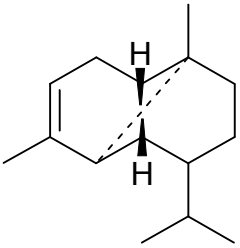
1,8- Cineole

[67]



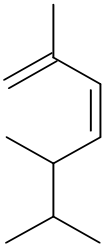
β- Caryophyllene

[68]



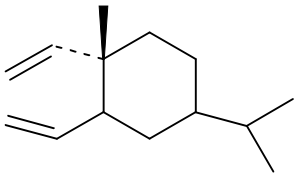
α- Copaene

[69]



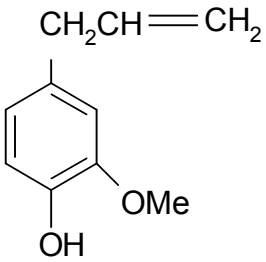
α- Phellandrene

[70]



β- Elemene

[71]



Eugenol

[72]

Major components, 1-8-cineole **[67]** and β-caryophyllene **[68]** and others were only found in *H.suaveolens*, Peerzada (1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1 General Experimentals

All solvents used for TLC and for final elution thereafter were purchased from Aldrich Chemicals Ltd. For other chromatographic techniques, commercially available solvents were used after distillation.

3.2 Instrumentation

3.2.1 Physical constants

All melting points were recorded in glass capillary tubes on Gallenkamp melting point apparatus and are uncorrected. Purity of samples was established by TLC.

3.2.2 Spectroscopy

Infrared spectra were measured in CCl_4 and Kbr as case may be on Shimadzu 8400S spectrophotometer. Nuclear magnetic resonance spectra (both ID and 2D) were recorded in $CDCl_3$ and DMSO on FT-NMR spectrometers operating at 300 and 400 MHz. respectively for ^{1}H NMR and at 75 and 100 MHz respectively for ^{13}C NMR. The chemical shifts are recorded in ppm (δ) and coupling constants (J) are in Hz.

3.2.3 Chromatography

Column chromatography was performed with silica gel (E-Merck, type 60,70-230 mesh) Pre-coated silica gel GF_{254} preparative plates (20x20cm,0.2mm thick) (E-Merck) were used for preparative TL**C.**

Silical gel (E. Merck 60 GF_{254}) coated on glass plates were used for preparative TLC. HPLC (LC-20AB, Prominence Shimadzu) was performed in the reverse phase mode [(column: C_{18}, Bondapak, ODS, 250mm x 4.6mmI.D.x 5µm)] using isocratic mode [(mobile phase: 50% MeOH-H_2O v/v)], injection volume (5µl) and flow rates (0.2ml/min, 0.3ml/min,0.4ml/min,0.6ml/min 0.8ml/min.and 1.0ml/min) respectively. The results are presented in Table 4.10.

3.2.4 Spray reagents

3.2.5 Ceric sulphate

Saturated solution of ceric sulphate in 65% sulphuric acid was used for detection of terpenes which give a pink/blue colouration after spraying the spots on TLC plates and upon heating for 10-20minutes at 120^{o}C in the oven.

3.2.6 Iodine

A few iodine crystals were placed in a TLC tank and warmed for a couple of minutes on a water bath (40-50^{o}C). Spots appeared on the TLC plate when kept inside the tank for a minute.

3.3 Collection of the plant material.

The fresh leaves (15kg) of *Hyptis spicigera* were collected in Basawa Village, Zaria, Kaduna state, Nigeria on the 26th November, 2007. It was taxonomically identified and authenticated by Mallam U. S. Gallah of the Herbarium Section, Department of

Biological Science, Ahmadu Bello University, Zaria, and a sample Voucher No.528 was deposited at the Herbarium Section.

3.3.1 Extraction and isolation.

The plant was dried in the shade for 14 days and pulverized to powder using pestle and mortar in the Laboratory. Approximately 5kg of the powdered plant material was macerated sequentially with hexane (6L), ethyl acetate (6L) and methanol (6L) at room temperature and concentrated *in vacuo* to afford the various crude extracts which were stored in the refrigerator (4^oC) until needed for further analysis. The various yields are presented in Table 4.0.

3.3.2 Lipid extraction:

The seed of *H.spicigera* was powdered using a pestle and mortar.About 200g of the powdered material was weighed into a soxhlet thimble and extracted with petroleum ether ($40\text{-}60^oC$), (500ml) according to the methods of Horowitz (1984) and Omoti and Okyi (1987). The solvent was concentrated *in vacuo* and allowed to dry in the fume hood to afford the oil which was kept in the fridge at 20^oC for the next analysis.

3.3.3 Physicochemical analysis:

The physicochemical properties of the oil (Refractive index, specific gravity, colour, Iodine value, saponification value, acid value, free aftty acid value, peroxide value amongst others) were determined using standard procedures of AOAC (1997).

Tocopherol and vitamin A were determined using IUPAC (1979) and Pearson (1981) methods respectively.The results are presented in Table 4.5.

3.3.4 Preparation of the fatty acids for GC-MS analysis:

About 0.2g of the oil was converted to fatty acid methyl esters (FAME) using methanolic potassium hydroxide solution method (Nikolaos B. K,. and Geige D., 1983).The FAME was washed with water and extracted with hexane in a separatory funnel .The upper layer was drained off and dried over anhydrous sodium sulphate,about 5µl was injected on GC-MS spectrometer.

3.3.5 GC-MS analysis of the derivatised oil:

The fatty acid methyl esters of the oil was analysed by GC-MS (Shimadzu QP2010 Series) adopting the following analytical conditions:Column;Cross -linked 5% phenyl methyl silicone (DB-WAX, 30m X 0.25mm X 0.25µm).Carrier gas;helium at flow rate 0.90ml/min, injector temperature program 100^{o}C for 2min., then increased to 190^{o}C at 4^{o}C/min. Identification of the fatty acids was achieved by retention times when compared with authentic standards analysed under the same conditions and relative percentages of each fatty acid was determined based on peak area measurements. The results are presented in Table 4.6.

3.3.6 Extraction of the volatile oils and GC-MS analysis

About 500g of the fresh leaf parts was chopped into pieces and weighed into a distillation flask fitted with condensers. Steam was supplied to the flask through a steam

generator at constant flow. The essential oil which vaporizes with the steam was condensed into a collecting separatory funnel and the oil separated by gravity and dried over anhydrous sodium sulphate.

The GC-MS of *H.spicigera* essential oil was analysed on a Shimadzu QP-2010 Instrument at 70 eV and 250^{o}C. GC Column: ULBON HR-1 equivalent to OV-1, fused silica capillary –0.25mm_50M with film thickness-0.25 μ. The GC-MS was operated under the following conditions- the initial temperature was 60^{o}C for 5 minutes and then heated at the rate of 5^{o}C per minute to 250^{o}C. Carrier gas (helium) flow was 2 ml per minute. The identification of components was based on comparison of their mass spectra with those present in the National Institute for Standard Technology computer data bank (NIST:2009s. LIB). The results are presented in Table 4.11.

3.4 Microorganisms and Media

The antimicrobial activities of hexane, ethylacetate and methanol crude extracts of *Hyptis spicigera* were determined using ten pathogenic microorganisms (*Staphylococcus aureus, Salmonella pyogens*, *C. ulkins, Salmollena typhi, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia,Corynebacterium ulcerans, Neisseria gonorrhea and Candida albicans*). Pure strains of the microorganisms were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Shika, Zaria. All isolates were of high purity and maintained in a slant of blood agar medium at 4^{o}C until when needed.

3.4.1 Antimicrobial studies

The paper disc method of was used to determine the antimicrobial activities from the plant. The Petri dishes were sterilized in an oven at 160^0C for one hour. Each of crude leaf extracts (0.05g) were dissolved in 10ml of the appropriate solvent of extraction to give a concentration of $5x10^{-3}$ µg/ml. The solutions were used for the antimicrobial screening of the extracts. Blood agar was the growth medium used for the pathogenic microorganisms. 40g of the agar base was weighed and dissolved in a liter of distilled water in a flask capped with cotton wool and sterilized at 120^oC for 15mins.The medium was allowed to cool, and 20ml of the sterilized medium was added to the sterilized petri dishes, the plates were covered and allowed to solidify. The plates were then inoculated with the test microorganisms by spread plate technique and incubated at 37^oC for 30min. Filter paper discs were cut and sterilized at 160^oC for 30min. and the sterilized filter papers were then soaked in the solution of the extract from the plant. The discs were then dried at 45^oC and planted on the plates seeded with the test microorganisms and then incubated at 37^oC for 24hrs.They were then inspected for the zones of inhibition or growth zones which were measured and recorded in millimeters with the use of a transparent ruler. The results are presented in Table 4.3.

3.4.2 Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was carried out as previously described (Linda and Clyde ,1978). The plant extract (0.1g) was dissolved in each of the solvent (solvents were analar grade and obtained from Sigma) of extraction and used for the study. Plant extract were pre-incubated in nutrient broth (Sigma) at different

concentrations (10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml and 0.625mg/ml). Normal saline was used to make a turbid suspension of the microorganisms and the solution was diluted to that of the Mc-Farlands scale 150×106 cfu/ml and was used for the determination of the MBC. The results are presented in Table 4.4.

3.5 Phytochemical screening:

Tests and reagents preparations were carried out according to the methods described by Sofowora (1993) and Plummer, (1983). The results are presented in Table 4.1.

3.5.1 Dragendroff's reagent:

3.5.2 Bismuth nitrate (2.5g) was dissolved in ethanoic acid ($10cm^3$) and distilled water ($40cm^3$) was added to give stock solution (A). Potassium Iodide (6.75g) was dissolved in distilled water ($10cm^3$) to give stock solution (B). Stock solution A (5ml) and B (5ml) were mixed with 33% ethanoic acid ($20cm^3$) and distilled water (20ml) to give Drangendroff's reagent.

3.5.3 Fehling's solution:

$CuSO_4$ crystals (34.64g) was dissolved in a minimum amount of water containing a few drops of tetraoxosulphate (VI) acid and the solution made up to $500cm^3$ with distilled water.

3.5.4 Fehling's solution B:

Sodium hydroxide **(**60g) and Sodium potassium tartate (173g) were dissolved in water. The solution was filtered and the filtrate made up to 500cm^3 with distilled water.

3.5.5 Mayer's reagent:

Mercuric Chloride (13.35g) was added to distilled water (60cm^3) to give solution A. Potassium iodide (5g) was added to distilled water (20cm^3) to make solution B. The two solutions (A and B) were then mixed and made up to 100cm^3 with water.

3.6 Tests for carbohydrate:

3.6.1 Molisch's test (**standard test for carbohydrates**):

A small quantity of each extract was added to a test tube containing distilled water (1cm) and the solution mixed with 4 drops of Molisch's reagent. This was then shaken carefully on adding concentrated tetraoxosulphate (VI) acid (1cm).The formation of a red ring at the inter-phase of the liquid indicates the presence of carbohydrate. The results are shown in Table 4.1.

3.6.2 Fehling's test (**Standard Test for Free Reducing Sugars**):

Mixture of an equal volume of Fehling's solutions A and B (5cm^3) was added to a small quantity of each extract and the solution boiled for 5 minutes. Formation of a red precipitate indicates the presence of free reducing sugars. The results are presented in Table 4.1.

3.6.3 Standard test for combined reducing sugars:

Boiling with dilute hydrochloric acid ($1cm^3$) hydrolyzed a small quantity of each extract. The solution was neutralized with 10% sodium hydroxide solution and a mixture of equal volume of Fehling's solution A and B ($5cm^3$) added and then boiled. A red colour indicates the presence of combined reducing sugars. The results are presented in Table 4.1.

3.6.4 Resorcinol or Selivanoff's test (Standard test for ketones):

A crystal of resorcinol and hydrochloric acid ($2cm^3$) was added to a small quantity of each extract and the solution was boiled for 5 minutes. A red colour indicates the presence of ketones. The results are presented in Table 4.1.

3.6.5 Test for Pentoses:

To a small quantity of the extract was added concentrated hydrochloric acid ($1cm^{3)}$ and a little phloroglucinol. The mixture was heated on a low flame. A red colour indicates the presence of Pentose. The results are presented in Table 4.1.

3.7 Tests for glycosides:

3.7.1 General test for glycosides:

A small quantity of each extract was boiled with 2.5M tetraoxosulphate (VI) acid ($2.5cm^3$) for 15 minutes the resulting solution was cooled and neutralized with 20% potassium hydroxide and then boiled again with a mixture of Fehling's solution A and B

($5cm^3$) formation of a brick red precipitate indicates the presence of glycosides. The results are presented in Table 4.1.

3.7.2 Test for Anthraquinones (Borntrager's test):

A small quantity of each extract and trichloromethane ($5cm^3$) was shaken for 5 minutes the resulting solution was filtered and the filtrate was shaken with equal volume of 10% NH_3 solution, a pink red or violet colour in the ammoniacal upper phase indicates the presence of free hydroxy anthraquinones. The results are presented in Table 4.1.

3.7.3 Test for cardiac glycosides:

3.7.3.1 *Keller kiliani test*:

About 0.05g of each extract was boiled with 5ml of 70% ethanol for 2-3 mins and filtered.The filtrate was diluted with an equal volume of water.The solution was shaken with 1-2 drops of lead acetate solution to precipitate other pigments and filtered.The filtrate was shaken with 5ml chloroform in a 50ml separatory funnel and the chloroform layer transferred into a porcelain dish and gently evaporated to dryness on a water-bath.The cooled reisdue was dissolved in 3ml of glacial acetic acid containing 2 drops of 5% Ferric chloride solution.This solution was transferred into a test-tube containing 2ml of concentrated sulphuric acid, a reddish-brown ring at the interface between the two liquid indicates the presence of deoxy sugar, a pale green or blue colour at the upper layer indicates the presence of a steroidal ring as the aglycone portion of the cardiac glycosides.The results are presented on Table 4.1.

3.7.3.2 Salkowski test:

Small quantity of each extract was dissolved in trichloromethane ($2cm^3$) and tetraoxosulphate (VI) acid ($1cm^3$) was carefully added to form a lower layer. A reddish brown colour at the interphase indicated the presence of a steroidal ring as the aglycone portion of the cardiac glycosides. The results are presented on Table 4.1.

3.7.4 Test for Saponin glycosides:

Small quantity of each extract was boiled with $10cm^3$ of distilled water. The solution was filtered into a portion of the filtrate.$2.5cm^3$ of a mixture of equal volume of Fehling's solution A and B was added. A brick red precipitate indicates the presence of saponin glycosides. The results are presented on Table 4.1.

3.7.4.1 Test for Saponins:

Ability of saponins to produce frothing in aqueous solution was used as a screening test for the extracts.A small quantity of each extract was shaken with water in a test tube. Frothing which persists was taken as a preliminary evidence for the presence of saponins. The results are presented on Table 4.1.

3.7.4.2 Keddes test for cardinodines:

To the extract (chloroform), 2% 3, 5-dinitrobenzoic acid was added and 5.7% of NaOH solution also added. The formation of purple or pink colour indicates the presence of Cardinolines. The results are presented on Table 4.1.

3.8 Tests for tannins:

A small quantity of each extract was boiled with 10cm^3 of distilled water and the solution was cooled and filtered:

A few drops of lead acetate were added to 1cm^3 of each filtrate. Formation of a white precipitate indicates the presence of tannins.To another portion of the filtrate, few drops of 1% Iron (III) chloride were added. A blue-black, green or blue-green precipitate was taken as evidence for the presence of tannins. The results are presented in Table 4.1.

3.8.1 Test for resin:

A small quantity of the extract was dissolved in the solvent of extraction,and equal volume of copper acetate solution was added, it was shaken vigorously and allowed to separate.The lower layer was separated into a test-tube and a drop of concentrated tetraoxosulphate (VI) acid added. A purple violet colouration confirms the presence of resins.

3.8.2 Tests for alkaloids:

A small quantity of each extract was added to 1cm^3 of 1% HCl, it was heated for 20 minutes, after which it was cooled and filtered. To the filtrate the following tests were carried out and the results are presented in Table 4.1.

3.8.2.1 Mayer's test:

Mayer's reagent (2drops) was added to the filtrate (1cm^3). Observation of a creamy precipitate indicates the presence of alkaloids.

3.8.2.2 Wagner's test:

Wagner's reagent (2drops) was added to the extract (1cm^3). A reddish brown precipitate indicates the presence of alkaloids.

3.8.2.3 Dragendoff's test:

Dragendorff's reagent (2 drops) was added to the extracts (1cm^3).Turbidity or precipitation was taken as evidence for the presence of alkaloids in extracts.

3.9 Tests for flavonoids:

10% NaOH (1cm^3) was added to the extracts (3cm^3). A yellow colouration indicates the presence of flavonoids. The results are presented on Table 4.1.

3.9.1 Shinoda test:

About 0.02g of the extract was dissolved in a 1cm^3 of water and a few drops of ammoniacal silver nitrate and allowed to cool.The colour was observed before and after heating for 5 mins.A brown,blue or black colour before heating with the blue turning black or brownish-black and black still remaining black is indication of the presence of flavonol glycosides.The only exception in this class of flanoid is hesperidin which turns black after heating.Areddish-brown colour before heating which still remains the same colour after heating indicates flavones aglycones.Chalcones do not form colour before heating except 2,3,4-trihydroxychalcones,it forms a reddish-brown or black colour after heating.

About 0.02g of each of the extract was dissolved in 1cm^3 of 50% ethyl acetate solution and the colour in the upper layer observed as a pale yellow in visible light indicating the presence of flavonol glycosides. The results are presented on Table 4.1.

3.10 Biological activity of ursolic acid derivative:

The ursolic acid and its derivative isolated from this work (Table 4.12-4.16) was subjected to further test in order to establish its biological activity using trypanosomes (*T.brucei brucei*) that affect both humans and animals. The parasites were maintained in the laboratory by continuous passage in rats until required. The passage was done when parasiteamia was in the range of 16–32 parasites per field with infected blood containing 1×103.Parasites were introduced intraperitoneally into healthy rats in 0.1–0.2mL blood/physiological saline solution. The numbers of parasites were determined microscopically at magnification (×400) and parasitaemia was monitored daily. The results are presented in Table 4.17.

3.10.1 *In-vitro* Test:

The Drug Incubation Infectivity test with wet and thick blood films method (Vincent *et al*., 2008) to detect any motile trypanosomes was done in triplicates in a 96 well microtitre plate with 50 μL of infected blood was incubated with 20 μL of ursolic acid derivative from 50%, 25% and 1% (previously prepared) giving a final concentration of 114 mg/mL, 57 mg/mL and 28.6mg/mL, respectively. Parasitaemia was monitored between 1–20 minutes of incubation at 30◦C. About 0.5 μL–1.0μL of test mixtures were observed every one minute under a microscope ×400. A set of Positive Control (PC), Negative Control (NC) and Diluent Control (DC) were set up to stand for 4hrs with the

infected blood containing the parasites. The PC contained 25mg/mL of DIMINAVETOR (1.05 g of Diminazene diaceturate and 1.31g Antipyrine), the NC contained only the infected blood suspended in heparin and Phosphate Buffer Saline Glucose (pH 7.2) and the DC contained only the pure vegetable oil (100%).

3.10.2 Determination of LD_{50} of *Hyptis spicigera* extracts:

Toxicity studies of the methanolic and dichloromethane leaf extracts of *H.spicigera* were carried out on mice intraperitoneally.The LD_{50} was calculated using the methods of Karber.

3.10.3 Animals used:

Swiss albino mice (18-30g) of either sex maintained at the animal house of the Department of Pharmacology and Clinical Pharmacy, Ahmadu Bello University,Zaria were used for the determination of the $LD_{50.}$ The animals were housed in a well ventilated room at a temperature of 27 ± 2^{o}C and fed with standard formulated feed and water *ad libitum.*

3.10.4 Preparation of the extract:

About 0.3g of the crude extracts of dichloromethane and methanol were weighed and homogenized in a mortar with two drops of tween 80.About 3ml of distilled water was added and properly mixed to give a 100mg/ml solution which served as the stock solution. From the stock solutions of ethylacetate and methanol extracts, serial dilutions were made to give 10mg/ml and 1mg/ml concentrations respectively.

3.10.5 Acute toxicity:

Different doses (100mg/ml, 10mg/ml and 1mg/ml) of the extracts were injected intraperitoneally into six groups of four mice each. The number of death was recorded at 24h after treatment and $LD_{50\ values}$ determined using standard methods as modified by Aliu and Nwude, 1982.Results are presented in Table

3.11 Insecticidal evaluation:

The cowpea was fumigated with phostoxin to clear any initial infestation like eggs and larvae. After the fumigation, the grains were dried for 72hrs to remove any residue of the phostoxin on the grains. In the experimental design, there were sixty-six (66) treatments and three replicates. Concentrations of 1000ppm, 100ppm and 10ppm each of the crude extracts and standard check (Azadirachtin) namely: hexane, ethylacetate and methanol were prepared.The cowpeas were carefully soaked in the concentrations prepared in a plastic jar (500ml capacity) to ensure good coating. The soaked seeds were exposed to air to allow for dryness and complete removal of the solvents. Ten pairs of *Callosobruchus maculatus* were infested with 100g of grains. The set-up was left for F1 progeny emergence. Mortality count was carried out at 24, 48 and 72hrs respectively while oviposition count was taken after one week (7days).Progeny emergence was taken at eight weeks (2 months). The results are presented in Tables 4.7-4.9 respectively.

3.12 Chromatographic separations:

3.12.1 Thin Layer Chromatography:

The outcome of the antimicrobial and insecticidal studies led to the choice of ethylacetate extract for the chromatographic separations. Five components from ethylaceate extract were obtained using hexane: acetone (4:1v/v) after series of trials of other extracts with other solvent systems.

3.12.2 Packing of Column:

The crude ethylacetate extract (25g) was dissolved in 10ml of ethylacetate and 5g of silica-gel and macerated very well using pestle and mortar to make fine powder. The powdered material was fractionated through a silica-gel (60-200 mesh) column chromatography (4cm x 60cm) using the combination of hexane: acetone (95:5, 90:10, 85:15, 80:20 etc) yielding twenty-five fractions. Some of the fractions collected were pooled together based on their similar R_f-values to give a total of twelve fractions. These were further purified for GC-MS, FT-IR, MS and full NMR analyses. The results are shown on Table 4.13.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Yields of crude extracts

Table 4.0: Percentage extraction of the leaf part of the plant material

Sample	Extracts	% Yield	Texture/Color
Leaf	Hexane	7.44	Viscous/Light yellow
	Ethylacetate	16.77	Solid/greenish
	Methanol	19.54	Solid/greenish
Seed	Hexane	20.87	Liquid/yellow
	Ethylacetate	18.16	Solid/greenish
	Methanol	22.25	Solid/greenish
Hyrodistillation of leaf part	Volatile oil	0.65	Liquid/colourless

The various yields of the crude extracts for the leaf and seed parts of *Hyptis spicigera* are shown in **Table 4.0.** Yields of methanol extracts for the leaf and seed (19.54%; 22.25%) were the highest showing that methanol was the best extracting solvent amongst the solvents used. Generally, methanol extracted the highest quantity of the plant material followed by ethyl acetate (16.77%; 18.16%) for both leaf and seed parts of the plant while hexane being a non polar solvent extracted the lowest quantity (7.44%) in the leaf and high quantity of lipid (20.87%) in the seed **(Table 4.0)**. Different solvents extract different types of plant constituents and this is due to their different polarities. This feature enables the extraction of plant constituents to be more selective with optimal results.

4.2 Phytochemical screening

Table 4.1: Phytochemical screening of the extracts of *Hyptis spicigera.*

Constituents	Leaves			Seed		
	Hexane	Ethylacetate	Methanol	Hexane	Ethylacetate	Methanol
Carbohydrate:						
Molisch test	-	++	+++	-	++	++
Fehling's test	-	+++	+++	-	-	+++
Pentose test	-	+	+	-	-	++
Ketose test	-	+	+	-	+	+

Key:

- **No growth**
+ **Scanty growth**
++ **Moderate growth**
+++ **Heavy growth**

Table 4.1: Phytochemical screening of the extracts of *Hyptis spicigera* continued

	Hexane	Ethylacetate	Methanol	Hexane	Ethylacetate	Methanol
Glycosides:						
Saponin	-	++	+++	-	+	++
Cardiac glycosides	+	++	+++	-	++	++
- Kellic Kaliani test						
Steroids	-	++	+++	-	++	+++
Triterpenes	-	+++	++	-	++	++
Anthaquinones						
Pre-A	-	+	+	-	+	+
Combined A	-	-	+	-	+	+

Key:

- **No growth**
+ **Scanty growth**
++ **Moderate growth**
+++ **Heavy growth**

Table 4.1: Phytochemical screening of the extracts of *Hyptis spicigera* continued.

	Hexane	Ethylacetate	Methanol	Hexane	Ethylacetate	Methanol
Flavonoids:						
10% NaOH + dil. HCl	-	++	+++	-	++	++
Schinoda test	-	+	++	-	++	+
Tannins:						
Ferric chloride test	-	++	+	-	++	-
Lead sub acetate	-	-	++	-	++	++
Alkaloids:						
Dragendoff's reagent	+	++	++	+	-	++
Wagner reagent	-	-	-	-	++	-
Mayer's reagent	-	++	++	-	++	-

Resin:						
Copper acetate	+	++	+++	+	+	++
Acetic anhydride	+	+	++	+	++	+

Key:

- **No growth**
+ **Scanty growth**
++ **Moderate growth**
+++ **Heavy growth**

The extracts of the leaves of *H.spicigera* were screened for the presence of secondary metabolites, alkaloids, glycosides, flavonoids, carbohydrates tannins, sterols, terpenoids and resins **(Table 4.1).**The results of the phytochemical screening showed that all the extracts with the exception of hexane extract contained the secondary metabolites analysed in high and moderate concentrations.

The results showed that carbohydrate is present in the extracts of both the leaves and seeds in high and moderate amounts. Methanol extract showed high amounts of carbohydrates in both the leaves and seed while ethylacetate extract showed moderate amounts of the carbohydrates in the leaves of the plant **(Table 4.1)**. The phytochemical content was similar to that obtained by other authors (Onayade *et al*., 1991) although different extracts revealed the different partitioning abilities of the different solvents.

Ethylacetate and methanol extracts showed moderate and high amounts of cardiac glycosides. A lot of plants contain non toxic glycosides that can be hydrolysed to give phenolic compounds that are toxic to microbial pathogens (Aboaba and Efuwape, 2001). The saponin content in the extracts was found to be moderate and high in the ethylacetate and methanol extracts respectively. Saponins possess the property of precipitating and coagulating red blood cells (Sodipo *et al.,* 1991). It also foamed in aqueous solution, has haemolytic effect and bind on cholesterol sites. These properties make saponins present in the plant to exhibit medicinal properties (Sodipo *et al*.,1991) and this therefore supports the findings in this present study that extracts of the plants may be useful in chemotherapy of mycotic infections which the antimicrobial studies revealed. Alkaloids were present in moderate amount from the ethylacetate and methanol extracts respectively. Reports indicate that naturally occurring alkaloids and their synthetic

derivatives have analgesic, antispasmodic and bactericidal activities (Okwu and Okwu, 2004).They exhibit marked physiological activity when administered to animals. Classes of alkaloids are among the major powerful poisons known and despite being poisonous, some of the alkaloids are known to be useful in correcting renal disorders (Konkwara, 1979). The use of some plants for medicinal purpose, in the traditional treatment of diseases is due to the presence of flavonoids and saponins (Zwadyk, 1992; Othira *et al*, 2009)), hence the use of *H. spicigera* for the treatment of diarrhea, dysentery, colds and several other diseases by local herbalists or traditional healers is not surprising. The methanol and ethylacetate extracts gave high concentrations of flavonoids. Flavonoid containing plants have been used as diuretic, laxative, emollient and poultice (Baba-Mousa *et al*, 1999) therefore; the use of *H.spicigera* rich in saponins and other Hyptis species in traditional medicine lent credence to the medicinal potentials of the plant. Tannins in some medicinal plants have been found to be responsible for the antiviral and antibacterial activities exhibited by such plants (De-Ruiz *et al*, 2001; Fyhrquist *et al.*, 2002; Elegani *et al.*, 2002). Therefore, *H. spicigera* with high tannin content could probably be a source of phytochemicals for the treatment of bacterial infections. Phenolic compounds like tannins present in plant cells are inhibitors of many enzymes (proteolytic and hydrolytic) used by plant pathogens. Other compounds such as saponins have antifungal properties (Aboaba and Efuwape,2001;Mohanta *et al*.,2007).Therefore, these phytochemicals detected in this study may be responsible for the antimicrobial potency of the leaf extracts of *Hyptis spicigera* and also lend credence to the claims of traditional application of the plant as remedies for various ailments.

Table 4.2: Sensitivity tests of the extracts against Some Selected Pathogenic Micro organisms

Test Organism	Leaf Extract			Seed Extract		
	LHE	**LEA**	**LME**	**SHE**	**SEA**	**SME**
Staphylococcus aureus	S	S	S	S	S	S
Streptococcus pyogens	S	S	S	S	S	S
Corynebacterium ulcerous	S	S	S	S	S	S
Bacillus subtilis	R	S	R	R	R	S
Escherichia coli	S	S	S	S	S	S
Salmonella typhi	S	S	S	S	S	S
Klebsiella pneumonia	S	S	S	S	S	S
Pseudomonas aeruginosa	S	S	S	S	S	S
Neisseria gonorrhea	S	S	S	S	S	S
Candida albicans	S	S	S	S	S	S

KEY: S = Sensitivity, R = Resistance,
Leaf part: LHE=Hexane Extract, LEA=Ethylacetate Extract, LME=Methanol extract,
Seed part: SHE=Hexane Extract, SEA=Ethylacetate Extract, SME=Methanol Extract

Table 4.3: Zone of Inhibition of the extracts against the Microorganism (mm)

Test Organism	Types of Extracts						Control	S.E.M
	Leaves			Seeds				
	Hexane	Ethylacetate	Methanol	Hexane	Ethylacetate	Methanol		
Staphylococcus aureus	23	34	32	20	29	30	0	5.692
Streptoccus pyogens	25	30	19	7	20	17	0	4.468
Basillus subtillis	22	24	27	20	23	22	0	4.695
Corynebacterium ulcerous	30	28	27	32	14	20	0	5.814
Salmonella typhi	21	22	31	0	10	21	0	6.234
Escherichia coli	23	18	19	7	30	14	0	4.130
Klebsiella pneumonia	25	15	17	16	11	12	0	4.087
Pseudomonas aeruginosa	0	33	37	0	15	21	0	7.540
Neisseria gonorrhea	9	40	17	8	26	14	0	2.909
Candida albicans	7	26	14	0	22	10	0	2.764

Table 4.4: Minimum Inhibitory Concentration of the extracts against microorganisms

Organisms	Hexane Extract					Ethylacetate Extract					Methanol Extract				
	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.625mg/ml	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.625mg/ml	10mg/ml	5mg/ml	2.5mg/ml	1.25mg/ml	0.625mg/ml
S.aureus	-	+	+	++	+++	-	-	*	+	++	-	-	*	+	++
S.pyogenes	++	++	+++	+++	+++	-	-	*	+	++	-	-	*	+	++
C.ulcekins	+	++	-	-	+++	-	-	*	+	++	-	-	*	+	++
B.subtilis	+	++	+++	-	+++	-	-	*	+	++	-	*	-	-	+
S.typhi	++	++	-	-	++	-	-	*	+	++	-	-	*	+	++
E.coli	+	+	++	-	+++	-	-	*	+	++	-	-	*	+	++
P.aeruginosa	+++	++	+	-	+++	-	-	*	+	++	-	-	*	+	++
K.pneumoniae	+	*	+	++	+++	-	-	*	++	++	-	*	+	+	++
N.gonorrhoea	++	++	++	+++	++	-	-	-	*	++	-	*	+	++	+++
C.albicans	+	*	+	++	++	-	-	*	+	++	-	*	+	++	++

Key: **- No growth**
*** MBC or MIC**
+ Scanty growth
++ Moderate growth
+++ Heavy growth

4.3 Antimicrobial activities of *H. spicigera*

The antimicrobial data of the leaf extracts using hexane, ethylacetate and methanol is shown **(Tables 4.2-4.4).**The solvent extracts of *H.spicigera* leaves showed significant bactericidal activity at very low concentrations of 2.5 mg/ml for the methanol extract although *S. aureus*, *N. gonnorhae* and *C. albicans* were most resistant to these solvent extracts with MBC of 5.0 mg/ml for the methanol extract **(Table 4.2)**. *P. aeruginosa* showed the greatest sensitivity to the plant extract with MBC of 2.5 mg/ml of the leaf extract for all the solvent extracts tested, while *N. gonorrhea* indicated resistance to the extracts at MBC of 5.0 mg/ml **(Table 4.2)**.

The antimicrobial activities of the crude extracts of *H. spicigera* **(Table 4.2)** showed the leaf extracts to have a broad spectrum activity against the micro organisms tested.

All the extracts showed bactericidal activity against all the antibiotic resistant microorganisms tested, though with some variations as against the standard drug penicillin. The methanol extract of *H. spicigera* exhibited highest activity against the micro organisms tested. This could probably be correlated with its high content of saponin and glycosides. The methanol extract has previously been shown to exhibit low insecticidal potency as demonstrated by Othira *et al*. (2009). The membrane lytic abilities of saponin glycosides (Babayi *et al*., 2004) could account for the high bactericidal activity of the methanol extract as compared to the other extracts. The extracts were found to be bactericidal at low concentrations of 2.5 – 5 mg/ml against the microorganisms tested. This is significant in the application of this extract as a possible potent chemotherapeutic agent. Antibiotic resistance in beta-lactamase producers like *K.*

pneumoniae and *N. gonorrhaea* is of growing concern clinically. In this study, *H. spicigera* leaf extract showed strong bactericidal potency against antibiotic resistant strains of these organisms *in vitro* with methanol extract showing the highest activity at 2.5 mg/ml.

4.4 Physicochemical properties of *Hyptis spicigera* seed oil

Table 4.5: Physicochemical properties of *Hyptis spicigera* seed oil.

S/No.	Analysis	
1	Colour	Golden yellow
2	Texture @ 27^{o}C	Liquid
3	Specific Gravity @ 20^{o}C	0.9261
4	Refractive Index	1.4950
5	Lipid content	21%
6	Iodine value	81.22g I_2/100g
7	Saponification value	154.10mgKOH/g
8	Acid value,	2.25mg/g
9	Free fatty acid	3.50%
10	Peroxide value	1.95meq H_2O_2
11	Tocopherol(Vitamin E)	186.15mg/ml
12	Vitamin A	Absent

Table 4.6: **Fatty acid profile of *Hyptis spicigera* seed oil**

Name	**Rt(mins)**	**% Area**
Palmitic acid	15.36	16.06
Myristic acid	15.79	6.40
Stearic acid	16.94	3.53
Linoleic acid	17.06	71.85
Oleic acid	17.27	1.02
6-Octadecenoic acid	17.71	1.34

Table 4.5 showed the physicochemical properties of the oil with the following properties: refractive index (1.4950) which is in close agreement with other drying oils that exhibit values of 1.48 and 1.49 (Deul,1951) respectively. Iodine value of 81.22g I_2/100g is an indication that the oil contains unsaturated fatty acids which is close to the iodine value of peanut oil of 86g I_2/100g. The peroxide value (1.95millieq.H_2O_2) showed the oil to be stable to oxidative degradation cause by over exposure to oxygen, heating and improper storage. Peroxide value depends on a number of factors such as oxidation by oxygen, extraction methods and storage. It is a measure of degree of lipid prone to oxidation in fats/oil.Acid and free fatty acid values (2.25% and 3.5%) of the oil are within tolerable limits required for oil that will be lost during refining steps designed to remove fatty acids. These values are relatively low compared to almond (7.6mg KOH/g) and similar to that of fluted pumpkin (3.5mg KOH/g) (Dhellot *et al.*, 2006).

4.5 Fatty acid profile of *H.spicigera* seed oil

The fatty acid composition of the fatty acid methyl ester (FAME) of *Hyptis spicigera* seed oil was analysed and compared with their fatty acid standards analysed under same analytical conditions by GC-MS **(Figure 12)** is shown **(Table 4.6).**The oil has four main fatty acids namely: palmitic acid (16.0%), Stearic acid (3.53%), Linoleic acid (71.85%) and Myristic acid (6.40%) with linoleic acid being the most abundant. Its high content of linoleic acid is of particular interest especially in the fight against cardiovascular diseases and also as a good source of edible oil. Linoleic acid contents in sesame oil (35-50%), palm oil (10.5%) and palm kernel oil (2.3%) and other oils used as food additives have health promotive properties (Kapseu and Parmentier, 1997;

Rocquelin *et al.,* 1998). *Hyptis spicigera* seed oil with high linoleic levels is also potentially useful as food additive and can be converted to gamma-linolenic acid that could be used as a dietary supplement to increase the production of anti-inflammatory l-series prostaglandins (Wretenjo, 2002).

Industrial trans fatty acid(TFA)consumption is believed to increase cardiovascular risk in multiple ways such as having adverse effects on blood lipoprotein profiles and coronary heart disease(CHD) risk impacting in individuals and population (Uauy *et al.*, 2009). Oils rich in linoleic acid are more effective in enhancing contents and yields of vaccenic acid and conjugated fatty acids in fats than oils (Bu *et al.*, 2007). Globally, current dietary recommendations are geared towards assisting consumers choose low fat low saturated fat diets that are beneficial for cardiovascular health (FAO/WHO, 1994).

The vitamin E content of *Hyptis spicigera* oil is relatively high (186.15mg/ml) than in sesame oil (1.40mg/g) and sunflower oil (41.08mg/g).Vitamin E is an antioxidant which has been implicated with lowering of cholesterol levels. Though the oil is rich in vitamin E, there is an insufficient research finding on the medicinal properties of *Hyptis spicigera* oil. Vitamin A was absent in the oil of *H.spicigera*,which is similar to other oils like cottonseed, sesame, sunflower oils but present in soybean oil, palm oil amongst others.

4.6 Insecticidal evaluation of extracts

Table 4.7: Percent mortality and adult emergence of *Callosobrochus maculatus* treated with extracts of *Hyptis spicigera.*

(a) Mortality:

Treatments	**24hrs**	**48hrs**	**72hrs**
Hexane extract	(40%)	(55%)	(87%)
Ethylacetate extract	(60%)	(80%)	(98%)
Methanol extract	(35%)	(47%)	(70%)
Azadirachtin Std.	(70%)	(88%)	(98%)
Control	(8%)	(15%)	(21%)

(b) Emergence:

Treatments:

Hexane extract	(30%)	(18%)	(15%)
Ethylacetate extract	(20%)	(16%)	(10%)
Methanol extract	(25%)	(28%)	(23%)
Azadirachtin Std.	(15%)	(10%)	(6%)
Control	(55%)	(70%)	(87%)

Table 4.8: Oviposition deterrent of extracts of *Hyptis spicigera* on *Callosobruchus maculatus*

(c) Oviposition deterrent:

Treatments	No. of eggs laid	% egg laid
Hexane extract	8.0	11.11
Ethylacetate extract	3.0	4.16
Methanol extract	4.0	5.56
Azadirachtin Std.	2.0	2.78
Control	55.0	76.39

Table 4.9: Percent seed damage to cowpea treated with extracts of *Hyptis spicigera* infested with *Callosobruchus maculates.*

Treatments	**No. of weeks**		
	8	**12**	**16**
Hexane extract	0.00	1.00	2.00
Ethylacetate extract	0.00	3.00	5.00
Methanol extract	9.00	15.00	23.00
Azadirachtin Std.	0.00	2.00	10.00
Control	40.00	60.00	88.00

The insecticidal efficacy of the plant leaf extracts of *Hyptis spicigera* is shown **(Tables 4.7-4.9).** All the extracts tested at 1000ppm, 100ppm and 10ppm against azadirachtin as a standard check showed insecticidal activity relative to the control. Azadirachtin from neem was used as a standard check because of its broad spectrum activity against many insect pests and also being a biologiacally active limonoid isolated from plant.The extracts showed more adult emergence of the *C.maculatus* with percent mortality ranging from 8% to 98% **(Table 4.7)**, while the control showed 8% to 21% mortality for *C.maculatus.* After 72 hours post treatment, adult emergence indicated that more *C.maculatus* ranging from 6% to 30% emerged while the control ranged from 55% to 87% respectively **(Table 4.7)**. Neem leaves have been used as an effective postharvest protectant for some grains prone to insecticidal attack.Village farmers usually mix powdered neem leaves with their grains in bags and keep in barnsneem and this has been found to be effective in the control of cowpea weevil (*Callosobruchus maculates,* F.), a major pest of cowpeas. Some workers (Jenkins *et al*., 2003) have found that neem kernel extract has reduced infestations of *C. maculatus*, after the neem kernel extract had been stored at high temperatures, including 2 weeks at 50°C followed by up to 5 months storage at 28°C. However, the number of neem kernel extract-treated eggs that survive to become adults was significantly reduced even when the neem kernel extract was exposed to 50°C for 2 wk. This was attributed to the mortality observed, due to the presence of other limonoids other than azadirachtin in neem kernel extract (Schmutterer, 1985).

There is no literature report found on the insecticidal activity of the extracts of *Hyptis spicigera* but some workers have shown the essential oils from *Hyptis spicigera* to possess high mortality on the first larval instars and on young adults of some insects

with doses 436.04 and 548.18ppb (Kouninki *et al*., 2005).They found that the fifth larval instars were resistant at all the doses tested, and no important mortality was observed. From the present study it showed that the essential oils of *H.spicigera* as reported by some of these workers has more insecticidal potency than the leaf extracts used for similar purpose. This could probably be due to the presence of various components in the volatile oils which work in synergy to boost the potency in controlling pests unlike the phytochemicals from the leaf extracts. In the present study, the extracts of the leaves of *Hyptis spicigera* was found to possess insecticidal activity in controlling *Callosobuchus maculatus* Fab. infesting stored cowpea seeds (*Vigna unguiculata*).

Hyptis is widely used against stored pests, other pest insects and against mosquitoes (Palsson and Jaeson, 1999). Some of the leaves of the *Hyptis* species especially *Hyptis suaveolens* and *H.spicigera* are largely used as potent insect repellents, by native populations of many parts of the world (Ayeard *et al.*, 1993; Pereda-Miranda and Delgado, 1990). The bioactivity of the essential oils from *Hyptis suaveolens* against storage mycoflora have been carried out on stored food grains (Sharma ,2002). This study showed that the volatile oils of *H.spicigera* contain 1,8-cineole which possessed fungitoxic activity and can be exploited for the management of spoilage of stored commodities.

The insecticidal activity of Hyptis species using its volatile oils is well documented (Othira *et al*, 2009; Sanon *et al.*, 2006, Raja *et al.*, 1990, Sharma, 2002). The genus *Hyptis* is a very large (400 species), and many of its reported species are known for their medicinal and insecticidal use by local herbal practitioners and have been evaluated by scientists (Almtorp *et al*., 1991; Kuhnt *et al*., 1994; Raja *et al.,* 1990).

Essential oils are secondary metabolites abundant in aromatic plants families such as Lamiaceae and Annonaceae, and contain a large number of compounds such as monoterpenes and sesquiterpenes. Essential oils of aromatic plants have been tested for their potential as protective agents for human and/or livestock feeds. Many researchers have pointed out the use of essential oils from aromatic plants as the best way to control pests since it does not lead to human and animal toxicity (Isman, 2000; Shaaya and Kostyukovysky, 2006). Many aromatic plants are known to possess insecticidal activity, ovi-positing deterrent, and reduce the progeny. Essential oils are known to exhibit low toxicity to mammals, and the most important constituents are terpenoids and phenols found in plant essential oils which have minimal toxicity and have even been approved as flavouring agents in food (Isman, 2000; Shaaya and Kostyukovysky, 2006). Previous reports have shown that a lot of attention has been focused on the pesticidal properties of the *Hyptis* and other plant species with the sole purpose of developing soft natural biodegradable natural insecticide that will replace chemical insecticides (Kouninki *et al*., 2007; Othira *et al*, 2009; Sanon *et al*, 2006; Raja *et al*, 2005; Sharma, 2002). This study has shown that *Hyptis spicigera* extracts can be formulated to soft natural botanicals for post harvest storage thereby replacing chemical insecticide used for the same purpose.

4.7 Purification of ethylacetate fraction from column chromatography by HPLC

Table 4.10: Purification of ethylacetate fraction from column chromatography.

Flow rate (ml/min)	Retention Time Peak 1	Retention Time Peak 2	Peak Area 1	Peak Area 2
0.2	7.901	12.120	13834420	7475793
0.3	7.769	9.715	14730828	97741913
0.4	5.825	7.301	13271394	72688501
0.6	5.833	7.396	6933591	4225331
0.8*	5.826	7.450	3224545	27418453
1.0	2.347	2.967	5531341	23722762

*Best separation achieved

The High Performance Liquid chromatography (HPLC) was carried out using a reversed-phase column (Shimpack CLC-ODS:4.6mm I.D.x15cmx 5µm) eluted at different flow rates of 0.2, 0.4, 0.6, 0.8 and 1.0ml/min with mobile phase composition of acetonitrile:water (50%/50%,vol./vol) and detection wavelength set at 230nm.The chromatographic data obtained for the purification of the ethyl acetate fraction from column chromatography is shown (**Table 4.10** and **Figures 14-19)**.The significance of this purification using the analytical High Performance Liquid Chromagrphy (HPLC) was to obtain pure fractions for further spectroscopic characterisation. The analytical results displayed in **Table 4.10** and **Figure 18** showed that the best purification method was achieved with 0.8ml/min.with one sharp, symmetrical peak as a major component. The eluate of the prominent peak was collected and analyzed by GC-MS. The MS spectrum **(Figure 21)** of the eluate showed one major peak and with library matching suggesting 3-buten-2-ol, a secondary alcohol with retention index 571, and molecular weight of 72 respectively. The identity of the spectra above 95% was needed for the identification of the major peak.This MS data is a guide in structural information and can be further authenticated with full NMR data. Mass spectrometry is an accurate analytical tool for characterizations of organic compounds (known and unknown).It is capable of high precision in the identification of compounds based on its large spectral library of known organic compounds (about 300,000).The general strategy of any method development scheme is to first explore variables that cause a large effect on separations and then move progressively towards fine tuning of the method.The purification of the ethylacetate fraction was carried out by manipulating the flow rates and mobile phases as variables which was optimized for the purification of the fraction leading to the MS analysis.

4.8 GC-MS analysis of the volatile oils of *Hyptis spicigera*

Table 4.11: Percentage composition of th volatile oils from the leaf parts of *Hyptis spicigera.*

Peak No.	Std.R_t(mins)	Component	%
1	3.99	α-pinene	12.16
2	4.48	β-pinene	9.47
3	4.79	α-Phellandrene	10.19
4	5.13	Eucalyptol(Cineole)	4.11
5	5.36	γ –Terpinene	6.33
6	5.45	Bicyclo[4.1.0]hept-2-ene	7.60
7	5.85	Linalol	0.64
8	6.16	Octen-1-ol acetate	0.42
9	6.23	Bicyclo[3.2.1]oct-2-ene	0.29
10	6.52	Oxirane,2-(hexyn-1-yl)-3-methoxymethylene	0.50
11	6.94	3-Cyclohexen-1-ol	0.80
12	7.32	P-Cymen-8-ol	0.51
13	7.47	P-Menth-1-en-8-ol	0.48
14	7.55	7,7-Dimethyl-4-methylenebicyclo[4.1.0]heptan-3-ol	0.43
15	7.74	9-Decen-2-one,5-methylene	2.49
16	8.46	(4-Methyl-3-cyclohexen-1-yl)methanol	0.57
17	9.22	2-Oxabicyclo[2.2.2]octan-6-al	1.70
18	9.53	7-Oxabicyclo[4.1.0]heptanes	1.36
19	9.67	Bicyclo[7.2.0]undec-4-ene	7.92

20	10.40	Ocimene	2.22
21	10.81	Aromadendrene	0.89
22	11.23	8-Methylenedispiro[2.0.2.5]	1.29
23	11.50	11-Tridecyn-1-ol	3.48
24	12.09	Caryophyllene	2.61
25	12.69	Bicyclo[3.1.0]hexan-3-ol	0.60
26	13.40	Octadecanal	0.24
27	14.78	3-Bromo-1-cyclodecene	0.28
28	15.68	2,4-Diisopropenyl-1-methyl-vinylcyclohexane	1.52
29	16.46	2,5-Dimethyl-3-vinyl-1,4-hexadiene	1.15
30	16.89	1-Isopropenyl-3-propenylcyclopentane	0.47
31	17.11	13-Hexyloxacyclopentane	0.47
32	17.45	Methyl heneicosanoate	0.26
33	17.73	7-Oxabicyclo[4.1.0]heptanes	2.41
34	18.45	Hexadecanoic acid phosphine	0.87
35	19.34	Cyclohexanone ethylene acetal	2.64
36	19.88	Dodecanoyl chloride	1.06
37	20.59	9-Octadecenoic acid-1,2,3-propanetriylester	2.91
38	20.82	N-Dodecanoyl chloride	3.27
39	20.97	Spiro[1,3-dioxolane-2,21-[6,7]diazabicyclo[3.2.2]non-6-ene	2.58
40	22.07	γ –pinene	0.05

The hydro distillation of fresh leaves of *H. spicigera* gave a colourless essential oil of 0.65% yield **(Table 4.11)**. The value indicated a low content of the oil and is in the same range as those cited in the literature. Other workers have reported the yields of essential oils from other *Hyptis species* such as *H.suaveoleas* (0.15%), *H. pectinata* (0.60%), *H. spicigera* (0.12%) and *H. lanceolata* (0.50%). From their reports, it showed that the volatile oils content of *H. spicigera* in this work is higher than those reported by these workers (Tchoumbougnang *et al.*, 2005; Onayade *et al.*, 1990). The chemical composition of essential oils of the *Hyptis spicigera* was elucidated employing GC interphased with MS. The GC-MS of the components are shown **(Figure 13)**. There were 40 compounds identified in *H. spicigera* volatile oil **(Table 4.11)** according to their order of elution on Ov-101 column and compared with standard retention times under same analytical conditions (Adams, 1983).

The essential oil yields obtained (0.65%) from the leaf of *H. spicigera* had three main components: α-pinene (12.16%), α-phellandrene (10.19%) and β-pinene (9.47%), other compounds found in this essential oil were γ-Terpineol (6.33%), Bicyclo [4.1.0] hept-2-ene (7.60%), Bicyclo [7.2.0] undec-4-ene (7.92%). Other workers (Ngassoum *et al.*, 2007) have shown the essential oils of the flower of *H.spicigera* to contain two main components, namely: 1,8-cineole (24.0%) and (E) – caryophyllene (22.2%). Other compounds found in the essential oils were α-pinene (9.1%), β-pinene (5.7%), α-terpineol (8.3%) and linalol (8.4%). The essentials oils obtained from the leaves of *H.spicigera* in this study, showed low composition of cineole (4.11%) caryophyllene (2.61%) while α-pinene (12.16%), β-pinene (9.47%) and α-phellandrene (10.19%) were higher in the leaves than in the flowers as reported by Ngassoum *et al.*, 2007. Flowers are highly

scented and are attractive parts used by insects for cross-pollination. It is therefore expected that some high concentration of the essential oils may be found there than other parts of the plant.

Similarly, it has been reported (Onayade *et al.*, 1990) that the essential oil from aerial parts of *Hyptis spicigera* contained mainly sesquiterpene hydrocarbons (74%), β-caryophyllene (68%) being the major component while α-pinene (5%) occurred in substantial quantity. Essential oils from the leaf of *H.spicigera* in this study has higher amount of α-pinene (12.16%) than in the aerial part (6.8%) as reported by Onayade *et al.*,1990. The difference in the amount of these secondary metabolites is probably a manifestation of the intrinsic variability of the species due to climatic and soil difference as well as ecotypes. Geographically variation in essential oil composition indicated that the sesquiterpenes are mainly produced in the plant samples grown at lower latitudes (Onayade *et al.*, 1990). Also, Tchoumbougnag *et al.*, 2005 have shown the essential oil from the leaves of *Hyptis spicigera* from Cameroon to contain four major component α-pinene (28.3%), β -caryophyllene (19.1%), limonene (13.4%) and β-pinene (10.3%). The composition of these secondary metabolites from Cameroun is higher than the values obtained from *H. spicigera* specie found in Northern Nigeria for α-pinene (12.16%), caryophyllene (2.61%) while β-pinene (9.47%) content compared favorably from the two countries. Several research teams have shown interest in the volatile compounds of *Hyptis genus*, especially due to the potency of its phytochemicals (Campos *et al.*, 2002). The phytochemicals from the volatile oils of *Hyptis spicigera* is of medicinal relevance in the remedy of ailments affecting the rural poor Africans and also solving the problem of bio-agro inputs needed by the resource poor African farmer in his farming activities.

4.9 Chromatographic separation

Table 4.12: Chromatographic separations using thin layer techniques

S/No.	Extract	Solvent system	No. of components
1	Hexane	PE:CH(2:3,v/v)	2
2	Ethylacetate	HE:AC(4:1,v/v)	3
		HE:EA(4:1,v/v)	4
		PE:CH(4:1,v/v)	4
		HE:EA(7:3,v/v)	7
3	Methanol	HE:AC(7:3,v/v)	8
		HE:EA(7:3,v/v)	7

Table 4.13: Column chromatography of crude ethyl acetate fractions.

S/No.	Fraction	Code	R_f-value	Yield(g)
1	1-15	HF-1	0.83	0.68
2	16-25	HF-2	0.70	0.81
3	26-32	HF-3	0.66	0.53
4	33-40	HF-4	0.52	1.61
5	41-46	HF-5	0.43	0.72
6	47-53	HF-6	0.30	0.98
7	54-60	HF-7	0.28	1.00
8	61-66	HF-8	0.20	0.33
9	67-70	HF-9	0.14	0.87

Table 4.14: Column chromatography of fraction HF4 (1.61g)

S/No.	Fraction	Code	R_f-values	Yield(mg)
1	1-5	ZF-I	0.88	100
2	6-10	ZF-2	0.80	56
3	11-16	ZF-3	0.75	78
4	17-22	ZF-4	0.68	60
5	23-27	ZF-5	0.63	110
6	28-31	ZF-6	0.55	250
7	32-35	ZF-7	0.37	80

Thin layer and column chromatographic separations of the extracts was carried out and the data **(Tables 4.12-4.14)** showed the various solvent systems used. Good separation (eight components) was obtained with hexane and acetone in the ratio of 7:3 vol/vol. while the least separation (two components) was achieved with petroleum ether and chloroform in the ratio of 2:3vol/vol. respectively. Fractions from column chromatography of the ethyl acetate extract showed the various relative fraction (R_f) values by thin layer chromatography and the yields **(Table 4.13)**. Fraction HF-4 R_f value (0.43) gave the highest amount (1.61g) while fraction HF-8, R_f value (0.2) gave the lowest yield (0.33g). HF-4, R_f value (0.43) and yield of 1.61g was subjected to further column chromatography due to the crystalline nature of the product and its high yield. There were thirty-five fractions **(Table 4.14)** collected which were reduced to seven fractions based on their similar R_f -values monitored by thin layer chromatography (TLC).The fraction ZF-6, R_f (0.55) and yield (250mg) was coded ZL-14. The compound obtained was a pale yellow powder with a melting point of 282-284^oC which was recrysallised from methanol to give white needle crystals with melting point at 288-290^oC [Lit. For Ursolic acid: 291^oC, Irmawata *et al*., 1997; Birgit *et al*., 2008].The purity of the sample was further confirmed by thin layer chromatography in different solvent systems viz: hexane/chloroform (4:1 vol/vol) petroleum ether/ethylacetate (7:3 vol/vol) and hexane/acetone (7:3 vol/vol) gave one spot each with different R_f-values. The sample was finally submitted for full NMR analysis for structural elucidation.

4.10 Structural elucidation of Sample ZL-14

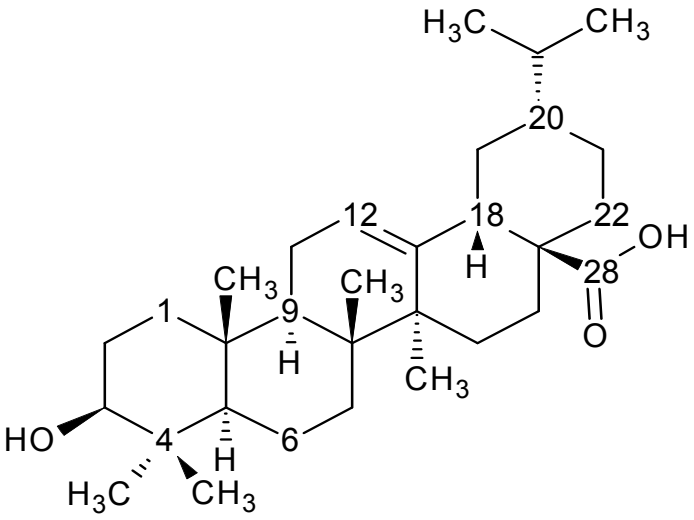
Table 4.15: ^{1}H and ^{13}C-NMR spectroscopic data of isolated compound **ZL-14** in $CDCl_3$ (ppm) compared with literature values for Ursolic acid (Guvenalp *et al*., 2009).

C-atom	δ_C Ursolic acid (Lit)	δ_C Isolated (ZL-14)	DEPT-135
1	39.2	38.83	CH_2
2	27.5	28.00	CH_2
3	78.5	79.07	CH
4	38.7	38.76	C
5	55.5	55.22	CH
6	18.60	18.29	CH_2
7	33.1	32.95	CH_2
8	39.6	39.49	C
9	47.8	47.54	CH
10	36.9	36.69	C
11	22.9	23.59	CH_2
12	125.7	125.87	CH
13	138.4	137.92	C
14	41.7	41.99	C
15	29.5	29.70	CH_2
16	24.1	24.15	CH_2
17	47.7	47.92	C
18	53.1	52.66	CH
19	39.2	39.05	CH
20	39.2	38.60	CH
21	30.5	30.61	CH_2
22	36.9	36.99	CH_2
23	28.0	28.13	CH_3
24	15.6	15.47	CH_3
25	15.3	15.59	CH_3
26	16.4	17.05	CH_3
27	23.1	23.29	CH_3
28	180.4	181.84	C
29	16.6	16.98	CH_3
30	22.8	22.60	CH_3
31	-	21.17	CH_3

The compound (ZL-14), white crystals showed IR (KBr disc) absorption bands **(Figure 10)** due to hydroxyl (O-$H_{str,}$ 3434 cm^{-1}), carboxyl (C=$O_{str,}$ 1696cm^{-1}), hydrocarbons (C-$H_{str,}$ 3000-2850cm^{-1}).The mass spectrum recorded gave a molecular ion peak (M^{+}) at m/z 470 **(Figure 11)** which corresponds to molecular formula of $C_{31}H_{50}O_3$. In the ^{1}H-NMR spectrum **(Figure 3)** an olefinic methine (δ_H 5.20) and an oxygenated methine (δ_H 3.20) were observed. Also in the high magnet field region, five singlets (δ_H 1.50-0.50) corresponding to methyl signals were observed.

In the ^{13}CNMR (100MHz, $CDCl_{3,}$ DEPT experiments) spectra **(Figures 4 and 5)**, thirty-one signals consisting of one carboxyl (δ_C 181.84), one olefinic quaternary carbon (δ_C 137.92) one olefinic methine (δ_C 125.87), one oxygenated methine (δ_C 79.07), [seven quaternary carbons, seven methines, ten methylenes, and seven methyls (δ_C 28.13, 23.29, 22.6,21.17, 17.05, 16.98, 15.59, 15.47] signals were observed.From the ^{13}CNMR spectrum (Figure 4),ursolic acid derivative **[73]** was found to be the major compound with thirty-two carbon atoms with thirty-one carbon atoms being accounted for while one carbon atom could not be accounted for and was presumed to be a noise signal.The distinctive characteristics of pentacyclic compounds of the ursane type showed the proposed structure (ursolic acid) from this work to have signals at 79.07 (C-3),125.87 (C-12),137.92 (C-13) and 181.84 (C-28) in $CDCl_3$ which is similar to other reported data of ursolic acid in the literature (Guvenalp *et al*., 2009;Moulisha *et al*., 2010;Pandurangam *et al*., 2010;Ramli *et al*., 1997;Silva *et al.*,2008;Klimek and Modnicki, 2005).The chemical shifts at these positions of ursolic acid in different solvents is comparable though with minor differences due to solvent effects.These data led to the conclusion for sample ZL-14 to be a pentacyclic triterpenoid of ursane type with one carboxyl, one double bond,

one hydroxyl. The sample was finally identified as 3β-hydroxy-20-isopropyl-urs-12-en-28-oic acid (ursolic acid derivative) through the comparison with several physical and spectral data reported in the literature (Houghton *et al*., 1986; Guvenalp *et al.,*2009;Thanakijcharoenpath and Theanphong, 2007). The comparison of the reported literature (Guvenalp *et al.,* 2009; Thanakijcharoenpath and Theanphong, 2007) values of ursolic acid derivative is shown **(Table 4.15)**, all the literature values compared very well with the experimental data obtained on sample ZL-14. The DEPT experiments **(Figure 5)** showed the various hydrocarbons and the quartenary carbons at the carbon positions of the compound. However, this appears to be the first report of ursolic acid derivative being isolated from *Hyptis spicigera* plant.



[73]

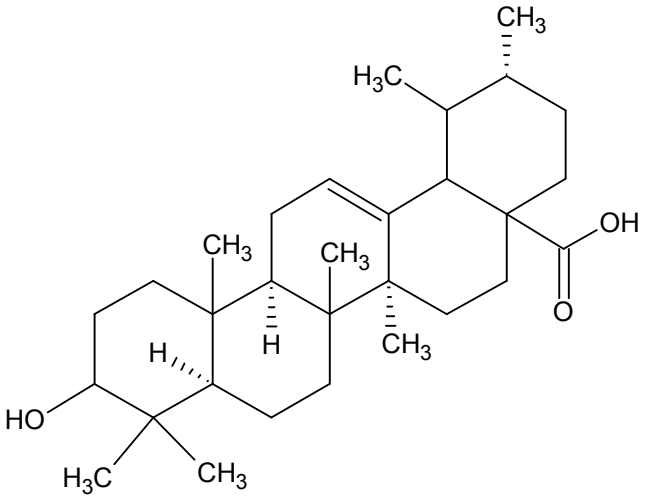
20-isopropyl- Ursolic acid (3β-hydroxy-20-isopropyl-urs-12-ene-28-oic acid) [73]

Other workers (Takagi *et al*., 1979; Seo *et al*., 1975; Romeo *et al*., 1977; Kriwacki and Pitney, 1989) have isolated ursolic acid from plant sources and reported its full NMR data: ^{13}C-NMR δ (125 MHz, CD_3OD, DEPT Experiment): 181.6 (C-28), 139.6 (C-13), 126.8 (C-12). 79.6 (C-3), 56.7 (C-5), 54.3 (C-l8), 47.6 (C-17), 47.6 (C-9), 42.8 (C-14), 40.7 (C-8), 40.4 (C-20), 40.4 (C-19), 39.9 (C-4), 39.8 (C-1), 38.1 (C-22), 38.1 (C-10), 34.3 (C-7), 31.7 (C-21), 29.2 (C-15), 28.7 (C-23), 27.8 (C-2), 25.3 (C-16), 24.3 (C-11), 24.0 (C-27), 21.5 (C-30), 19.4 (C-6), 17.8 (C-29), 17.6 (C-26), 16.3 (C-25), 16.0 (C-24). All these literature data and theoretical values confirmed the experimental data of the proposed structure of the sample analysed.

4.11 Structural elucidation of sample EL-2

Table 4.16: ^{1}H and ^{13}C-NMR spectroscopic data of isolated compound **EL-2** in DMSO-d_6 (ppm) compared with literature for saturated Ursolic acid (Thanakijcharoenpath and Theanphong, 2007.)

C-atom	**δ_C Ursolic acid (Lit.)**	**δ_C Isolated (EL-2)**	**DEPT-135**
1	38.2	38.88	CH_2
2	27.0	27.29	CH_2
3	76.8	77.46	CH
4	38.5	38.8	C
5	54.8	55.25	CH
6	18.0	18.40	CH_2
7	32.7	33.14	CH_2
8	39.1	39.22	C
9	47.0	47.53	CH
10	36.5	36.94	C
11	22.8	23.69	CH_2
12	124.6	125.00	CH
13	138.2	138.00	C
14	41.6	41.90	C
15	27.5	29.37	CH_2
16	23.8	24.23	CH_2
17	46.8	47.46	C
18	52.4	52.86	CH
19	38.5	38.94	CH
20	38.4	38.50	CH
21	30.2	30.81	CH_2
22	36.3	37.02	CH_2
23	28.2	27.96	CH_3
24	16.1	16.70	CH_3
25	15.2	15.60	CH_3
26	16.9	16.80	CH_3
27	23.3	23.78	CH_3
28	178.3	178.95	C
29	17.0	17.80	CH_3
30	21.0	20.00	CH_3



[74]

Ursolic acid (3β-hydroxy-urs-12-ene-28-oic acid) [74]

The compound **[74],** EL-2 (Yield: 25mg), a white powdery substance with a melting point of 280-282^{o}C showed absorption bands due to carbonyl stretching, C=O_{str} (1759cm^{-1}) and hydrocarbon stretching, C-H_{str} (3002cm^{-1}, 2980cm^{-1}) in the IR spectrum. The ^{1}HNMR spectrum (400MHz, $DMSO_d$) showed the presence of pentacyclic ring structures at signals of δ7.31 while all the methyl signals in the range 1.01-0.65 supported their attachement on the saturated carbons (**Figure 22**).

The ^{13}CNMR spectrum (400MHz, $DMSO_d$) indicated the presence of 30-carbon signals **(Table 4.16)** including a hydroxyl functional group located at the C-3 position showing a chemical shift of 77.66ppm and a carboxylic functional group at C-28 (178.95). The NMR spectra **(Figures 22 and 23)** and the DEPT spectrum **(Figure 24)** showed the following: methyl groups (seven number), methylene groups (nine number), methine groups (seven number), and quaternary carbons (seven number). The mass spectrum recorded gave a molecular ion peak (M+1) at m/z 457 **(Figure 25)** which corresponds to molecular formula of $C_{30}H_{48}O_3$ and molecular weight of 458g/mol.The proposed structure was identified as ursolic acid molecule of the ursane pentancyclic structure as can be seen from the ^{1}HNMR and ^{13}CNMR spectra **(Figures 23 and 24).** This proposed compound is being reported for the first time from *Hyptis spicigera* plant.The other compound isolated **[73]** is a derivative of the ursolic acid [**74]** found in the plant.

4.12 Toxicity studies on the ethylacetate and methanol extracts of *Hyptis spicigera.*

Table 4.17: Experimental results of toxicity tests.

Ethylacetate Extract				Methanol Extract			
Doses (mg/kg)	**Wt. of mice (Kg)**	**Vol.injected (ml)**	**Stock solution (mg/ml)**	**Doses (mg/kg**	**Wt. of mice (Kg)**	**Vol.injected (ml)**	**Stock solution (mg/ml)**
1200	0.020	0.20	120	1200	0.033	0.33	120
1600	0.027	0.27	160	1600	0.032	0.32	160
2900	0.028	0.28	290	2900	0.021	0.21	290
5000	0.028	0.48	290	5000	0.023	0.40	290

Table 4.17 showed the results of the tocixity test carried out using mice.After 24h of administering the extracts, two mice died at a dose of 2900mg/kg and 5000mg/kg. The LD_{50} was therefore calculated as follows using the Karber method.

LD_{50} = √Max. dose of survival X Min. dose of death.

√1600mg/kg X 2900mg/kg

2154mg/kg for both the methanol and dichloromethane extracts.

The acute toxicity established intraperitoneally LD_{50} of the extracts to be 2154mg/kg by the Karber method.This showed that the methanolic leaf extracts of *H.spicigera* is moderately toxic based on the WHO toxicity ratings (Gleason *et al*., 1969). It is assumed that substances more toxic than 1mg/kg are highly toxic and therefore, it is not important to calculate the LD_{50} and LD_{50} values greater than 5000mg/kg are of no practical interest.There was no histopathological studies carried out. Investigation of the acute toxicity of a material is the first step in the toxicological investigations of an unknown substance with LD_{50} as its index of acute toxicity.

4.13 Evaluation of the biological activity of ursolic acid derivative on trypanosome (*T.brucei brucei).*

The compound **[73]** was evaluated *in vitro* for activity against *Trypanosoma brucei brucei* (Tbb) and found to possess anti-trypanosoma activity *in vitro* in a dose dependent pattern in a short period of time. The drop in number of the parasites over time was achieved at a dose of 0.4µg/ml, 0.2µg/ml and 0.1µg/ml.The concentration of 0.4g/ml was more potent at 3 minutes and 2 minutes for the *T.brucei brucei,Tbb*. *Trypanosoma brucei brucei* (Tbb) causes African Trypanosomosis along with several other species of

trypanosomes although *T.brucei brucei,* Tbb has morphological similarities with *T. evansi*, the latter differs from related species by the absence of kinetoblast DNA minicircle (Nok and Williams,1996). Currently, 35 million people and 25 million cattle in Africa are at risk of contracting the disease which is fatal if untreated. Ironically, some registered trypanocides are frequently toxic, require lengthy administration, lack efficacy and are sometimes unaffordable for most of the patients (Hoet *et al.*, 2003).The potentials of ursolic acid derivative as a trypanocide can solve the problem associated with the use of toxic, and unaffordable synthetic trypanocides currently in the market.

The activities of pentacyclic triterpenoids of ursane,lupane and oleanone skeleton which accumulate in a number of terrestrial plants free or linked to carbohydrate moieties (Liu.1995) have been extensively reported (Ringbom *et al*., 1998; Saraswata *et al*., 2000).Ursolic acid has been shown to demonstrate activities such as hepatoprotective, ant-inflammatory,anti-viral and anti-tumor (Shishodia *et al*., 2003) as well as growth inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* with MIC values of 50µg/ml (Mallavadhi *et al*., 2004).Ursolic acid isolated from *Crataegus pinatifida* is active against HIV-1 protease at a concentration of 100µg/ml (Pengsuparp *et al*., 1994).The C-28 carboxylic acid moiety has been suggested and proved as the chemical group responsible for the enhanced pharmacological activity of ursolic acid (Kashiwada *et al*., 2000;Ma *et al*., 2000). The triterpenoid compounds have been reported to possess antifungal and antibacterial activity. Collins and Charles (1987) identified ursolic acid as one of the active components in rosemary claimed to inhibit growth of some food-associated bacteria and yeasts (Becker *et al*., 2005).Ursolic acid has been reported as the active constituent from a number of plant species used in traditional and folklore

medicine (Ringbom *et al*., 988; Saraswata *et al.*, 2000) and its derivatives also have shown inhibition to the growth of *S.aureus* and *Microsporium lenosum.* From this study, an ursolic acid derivative which is being reported for the first from this plant is a potential source of trypanocides which can be exploited.

CHAPTER FIVE

5.0 Summary, Conclusion and suggestions for further work

Natural products have revealed the ways to new therapeutic approaches, contributed to the understanding of numerous biochemical pathways and have established their worth as valuable tools in biological chemistry and molecular and cellular biology. The costs of drug discovery and drug development from natural products continue to increase at astronomical rates, yet despite these expenditures, there is a decrease in the number of new medicines introduced into the world market.

Natural products today are most likely going to continue to exist and grow to become even more valuable as sources of new drug leads. This is because the degree of chemical diversity found in natural products is broader than that from any other source, and the degree of novelty of molecular structure found in natural products is greater than that determined from any other source.

From ethnobotanical point of view the genus Hyptis from the family Lamiaceae is used mainly for post harvest storage of grains and as mosquito repellant. The aims of this work have been achieved as follows:

i. Phytochemical screening of the various extracts of leaves of *Hyptis spicigera* for secondary metabolites,

ii. antimicrobial study of the extracts of the leaves of *Hyptis spicigera* on some selected pathogens,

iii. evaluation of the insecticidal properties of the various extracts on grain weevils using cowpea,

iv. Isolation and purification of compounds from the extracts of *Hyptis spicigera* plant to elucidate structures of the isolated compounds using Nuclear Magnetic Resonance Spectroscopy (NMR), Mass Spectrometry (MS) and other spectroscopic techniques which have been compared with literature values.

v. The compounds isolated are Ursolic acid derivatives,

vi. Biological evaluation of one of the isolated compounds on *T.brucei bruce,*

In this study, the COOH group at C-28 and the C-3 hydroxyl group of triterpenoids are important for antibacterial and antifungal activity. The activity observed with the isolated compound **[73]** (Ursolic acid derivative) further validates the traditional uses of *Hyptis spicigera* for treating microbial infections.

The investigation on efficacy of the isolated compound on *Candida albicans* infected wounds in animal models could indicate the potential uses of this triterpene for topical treatment and prevention of infections in wounds.

For lack of time, other fractions were not considered for biological activities and further purification leading to structural studies, this can be pursued further.

From the analytical data obtained, it showed that *Hyptis spicigera* seed oil is rich in linoleic fatty acid and vitamin E and therefore is a potential source of vitamin E and linoleic acid which could find use in reducing the risk of coronary heart disease (CHD). Also the physicochemical properties of the oil showed the oil to be similar to other vegetable oils and is a potentially valuable non-conventional source for high quality oil needed by the oleochemical and pharmaceuticals industries. There is the need for further chronic toxicity evaluation of the oil and the leaves of *H.spicigera* plant to gurantee its safe consumption by humans.

Structural activity relationships (SAR) and synthesis of the compounds proposed in this work can be further pursued for pharmaceutical applications since the ethylacetate extract has shown remarkable antimicrobial activity against a broad spectrum of microorganisms. Conventional drugs used for the treatment of diseases caused by microorganisms are expensive and unaffordable.

The volatile oils and ursolic acid derivative isolated from this work showed potential application in the control of *Trypanosomiasis brucei brucei*.Further work on other trypanosomes as well as antimycobacterial activity of the volatie oils and ursolic acid can be pursued.

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APPENDICES

TEST OF HYPOTHESIS ON THE INSECTICIDAL POTENTIALS OF *H.SPICIGERA*

H_0: Mortality Rate is independent of the Treatment of Extract.

H_1: Mortality Rate is dependent of the Treatment of Extract.

DECISION

Reject the null hypothesis if **F_{cal}** is greater than **F_{tab}** (i.e. **$F_{cal} > F_{tab}$**) or reject the null hypothesis of independence if P value is less than 0.05 (i.e. $P \leq 0.05$).

Observation = 72

Degree of Freedom = v_1, v_2 = 7, 64

Confidence Interval = 95%

Significance level (α) = 0.05

$F_{tabulated}$ = 2.17

CONCLUSION

For the entire mortality rate,the null hypothesis is rejected which states that the mortality rate is independent of the treatment of extract and accept the alternative hypothesis that the mortality rate is dependent of the treatment of extract since the value of **F_{cal}** exceeds **F_{tab}**, which is for

M1: 69.718 > 2.17

M2: 69.718 > 2.17

M3: 3.466 > 2.17

M4: 16.027 > 2.17

M5: 27.261 > 2.17

M6: 7.635 > 2.17

M7: 4.295 > 2.17

M8: 2.526 > 2.17

M9: 3.694 > 2.17

M10: 3.827 > 2.17

```
REGRESSION
  /MISSING LISTWISE
  /STATISTICS COEFF OUTS R ANOVA
  /CRITERIA=PIN(.05) POUT(.10)
  /NOORIGIN
  /DEPENDENT Obs
  /METHOD=ENTER M1 M2 M3 M4 M5 M6 M7 M8 M9 M10 CONC
  /SCATTERPLOT=(Obs ,*ZPRED)
  /RESIDUALS NORM(ZRESID).
```

Regression

```
[DataSet1] C:\Documents and Settings\DR. LADAN\My Documents\MORTALITY READING.sav
```

Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	CONC, M3, M7, M6, M5, M8, M10, M2, M4, M9, M1[a]		Enter

a. All requested variables entered.

b. Dependent Variable: Obs

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.677[a]	.459	.359	16.750

a. Predictors: (Constant), CONC, M3, M7, M6, M5, M8, M10, M2, M4, M9, M1

b. Dependent Variable: Obs

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	14263.506	11	1296.682	4.622	.000[a]
	Residual	16834.494	60	280.575		
	Total	31098.000	71			

a. Predictors: (Constant), CONC, M3, M7, M6, M5, M8, M10, M2, M4, M9, M1

b. Dependent Variable: Obs

Pag

Fig 2: Regression data

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	93.914	187.670		.500	.619
	M1	1.297	13.547	.174	.096	.924
	M2	-3.092	13.396	-.414	-.231	.818
	M3	-1.429	1.970	-.106	-.725	.471
	M4	.931	2.323	.114	.401	.690
	M5	5.643	2.471	.400	2.284	.026
	M6	.832	2.145	.078	.388	.699
	M7	-1.606	1.586	-.132	-1.012	.315
	M8	-.009	.036	-.043	-.261	.795
	M9	-.591	1.268	-.675	-.466	.643
	M10	-.514	1.278	-.582	-.402	.689
	CONC	1.831	2.495	.072	.734	.466

a. Dependent Variable: Obs

Residuals Statistics[a]

	Minimum	Maximum	Mean	Std. Deviation	N
Predicted Value	9.52	64.52	36.50	14.174	72
Residual	-29.253	27.572	.000	15.398	72
Std. Predicted Value	-1.903	1.977	.000	1.000	72
Std. Residual	-1.746	1.646	.000	.919	72

a. Dependent Variable: Obs

Charts

Fig3: Coefficient constants

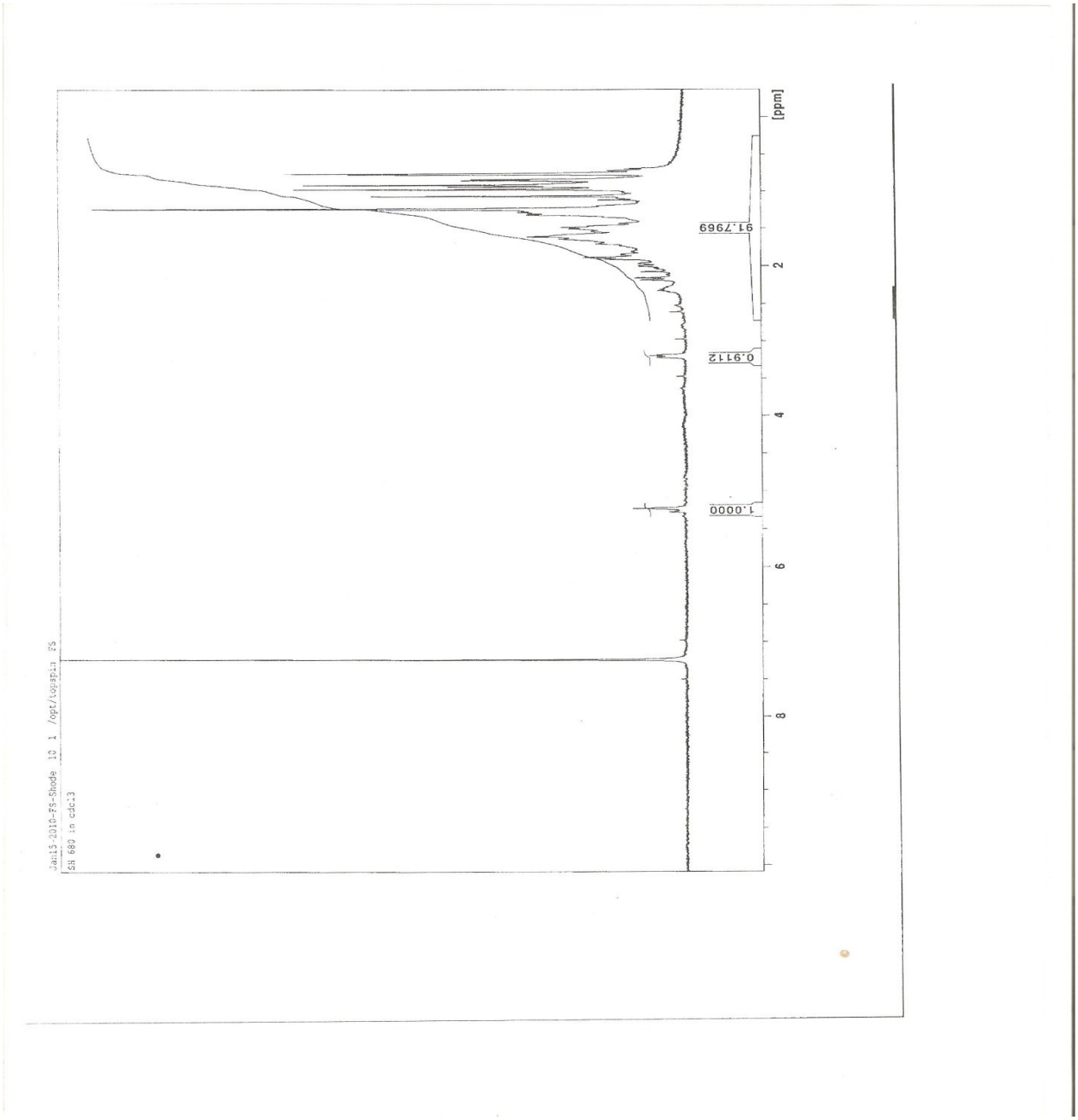


Fig 4: ^{1}HNMR spectrum of sample ZL-14

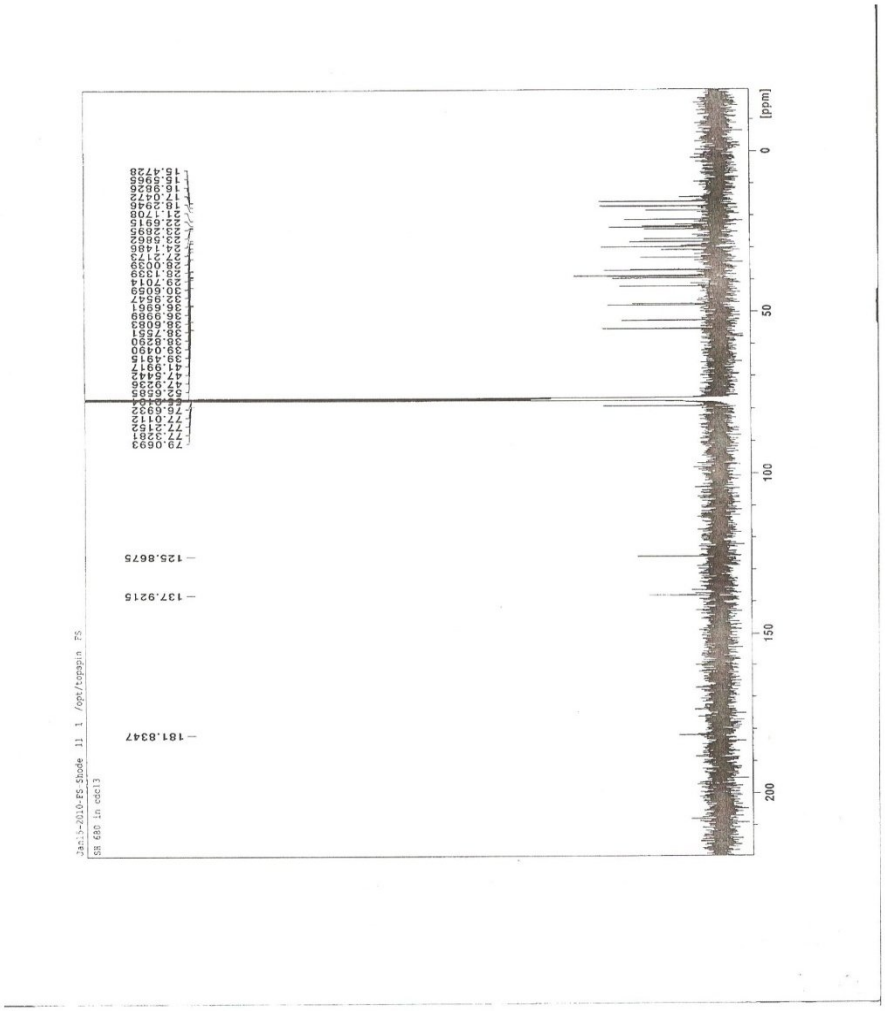


Fig 5: ^{13}CNMR [1] spectrum of sample ZL-14

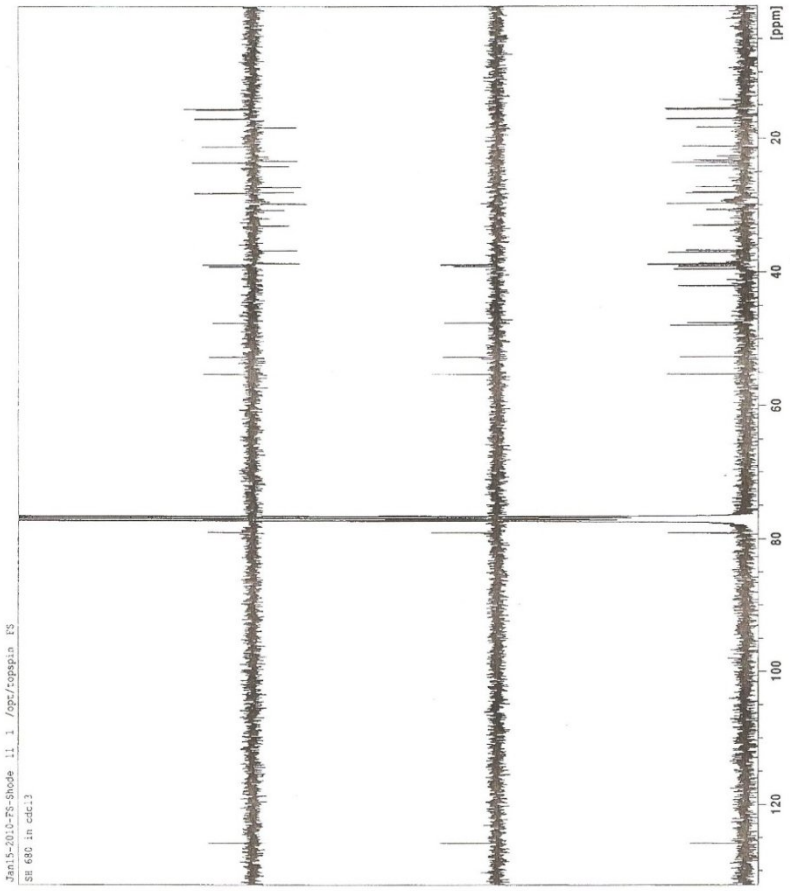


Fig 6: DEPT spectrum of sample ZL-14

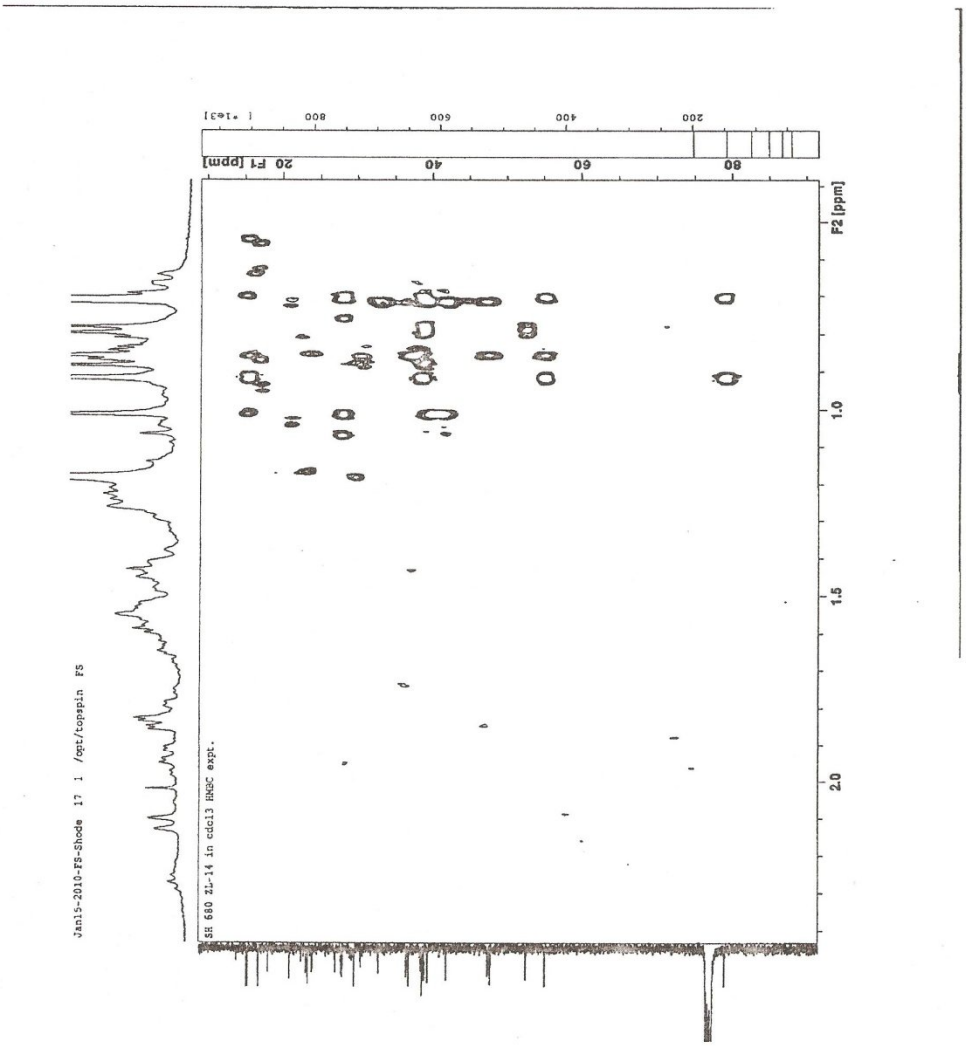


Fig 7: HMBC spectrum of sample ZL-14

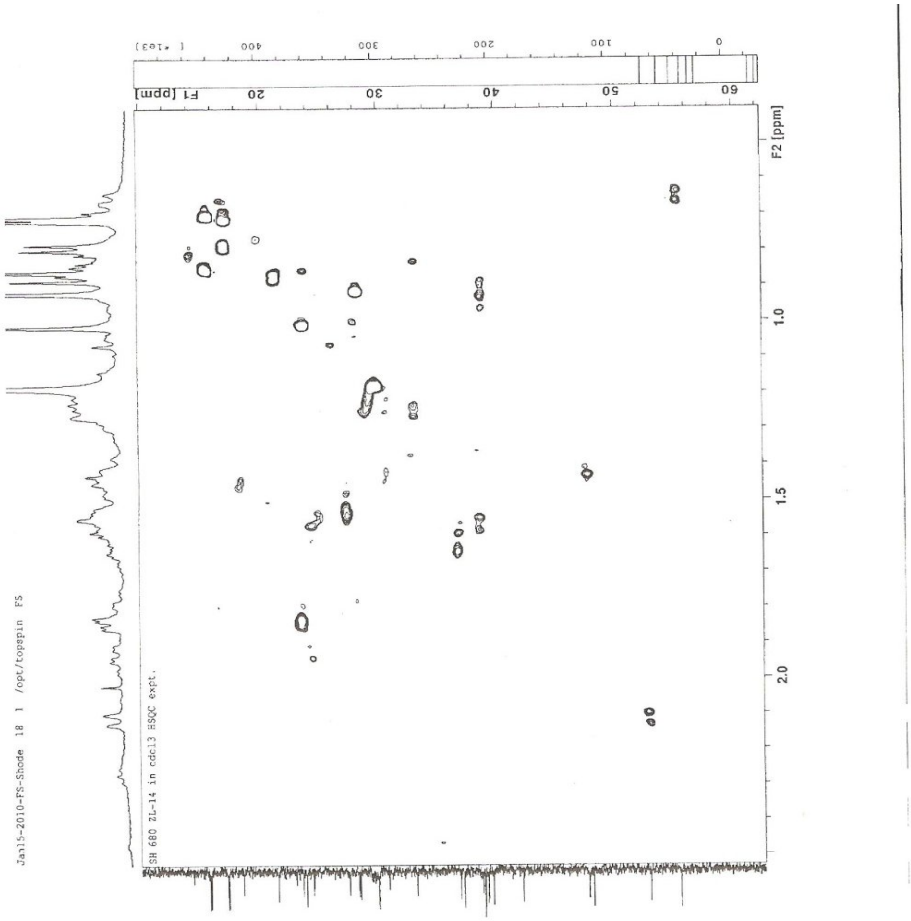


Fig 8: HSQC spectrum of sample ZL-14

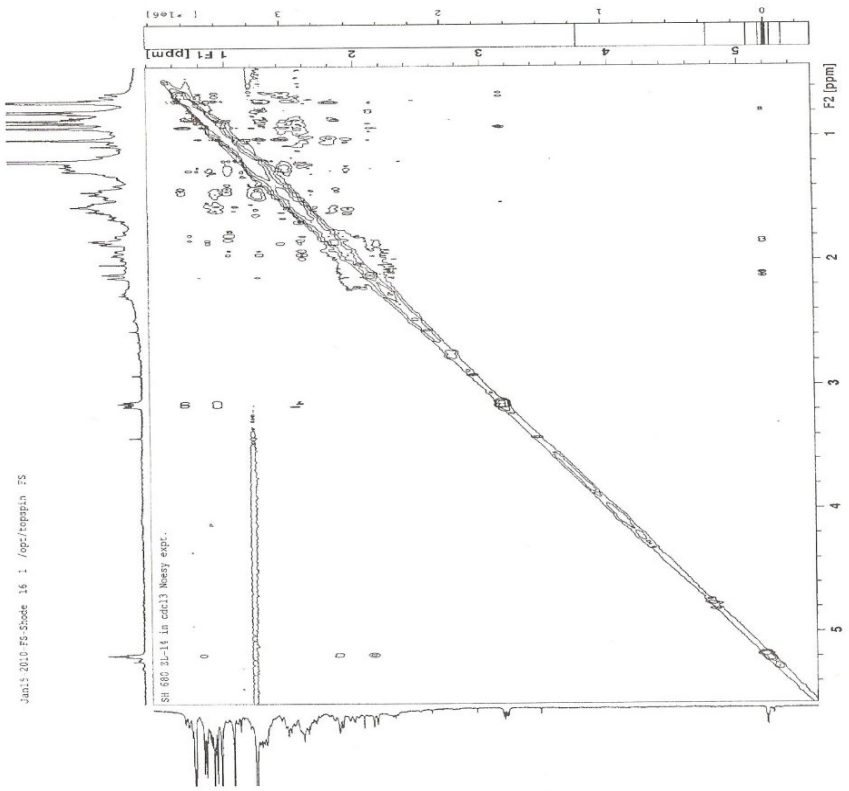


Fig 9: NOESY spectrum of sample ZL-14

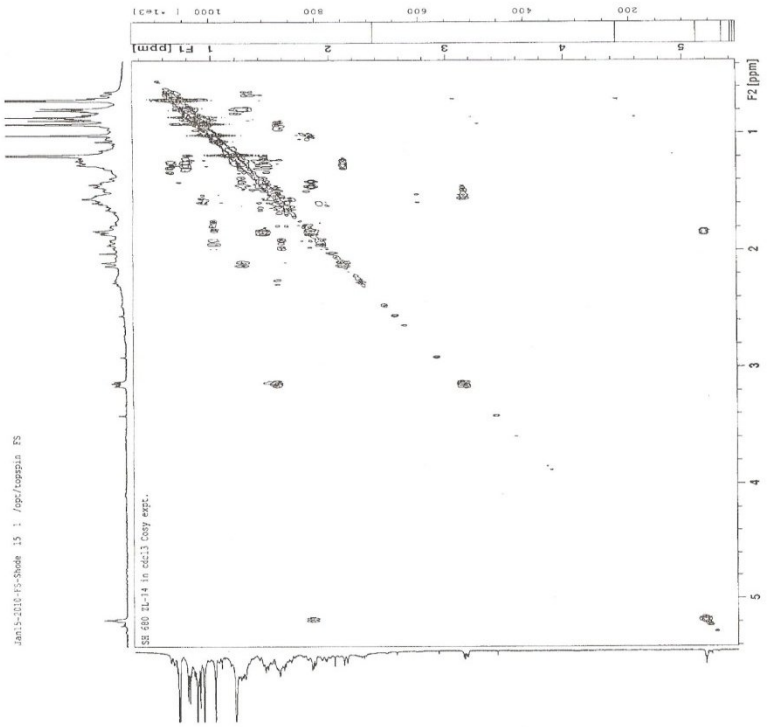
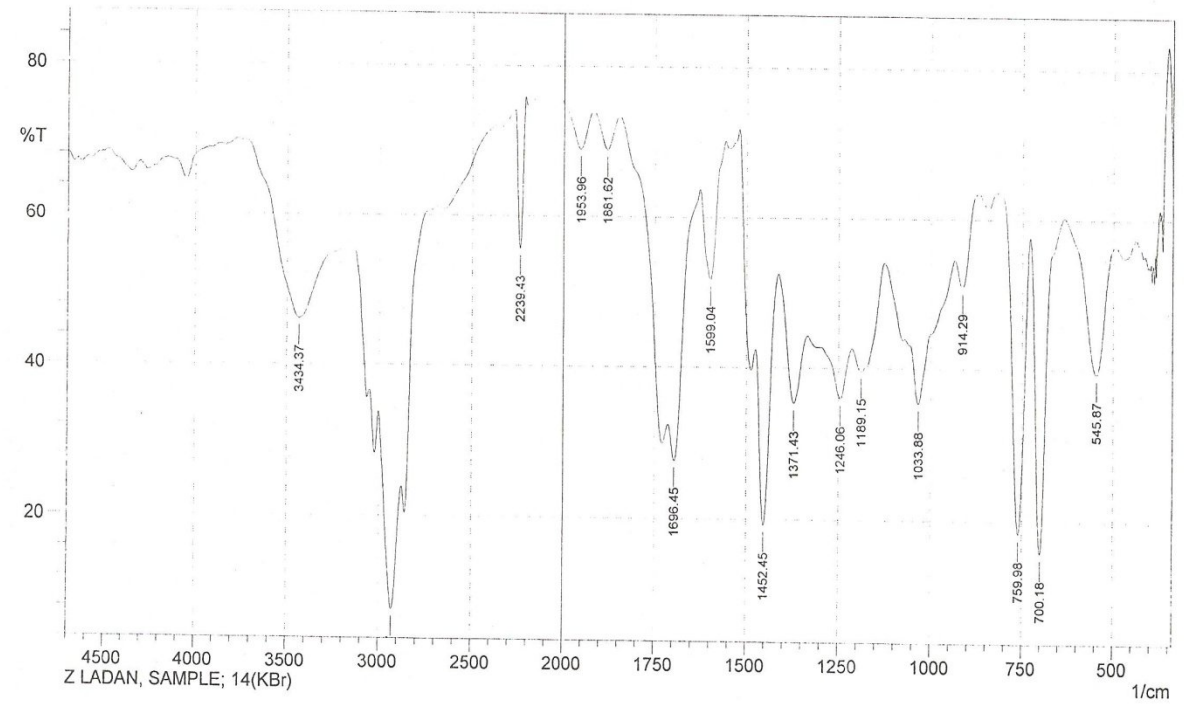


Fig 10: COSY spectrum of sample ZL-14



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	545.87	39.34	18.54	637.49	495.72	42.28	9.14
2	700.18	15.83	42.59	728.15	637.49	35.68	14.86
3	759.98	18.43	41.3	819.77	729.12	35.09	15.4
4	914.29	50.96	6.67	935.51	870.89	16.15	1.35
5	1033.88	35.29	11.63	1071.49	935.51	48.22	6.12
6	1189.15	39.54	6.44	1214.23	1127.43	31.36	3.75
7	1246.06	35.91	6.79	1298.14	1214.23	33.56	2.57
8	1371.43	35.25	12.89	1413.87	1334.78	29.91	4.89
9	1452.45	19.33	26.8	1474.63	1413.87	29.27	9.53
10	1599.04	51.74	14.74	1627.97	1558.54	15.51	3.4
11	1696.45	27.61	11.11	1713.81	1627.97	30.55	1.14
12	1881.62	68.92	4.76	1920.2	1848.83	10.45	1
13	1953.96	68.94	5.66	2017.61	1920.2	13.64	1.38
14	2239.43	55.69	19.31	2267.4	2210.5	10.8	3.7
15	2929.97	7.74	20.1	3004.23	2878.85	98.61	29.91
16	3434.37	46.03	15.1	3701.52	3241.48	121	25.08

Fig 11: FT-IR spectrum of sample ZL-14

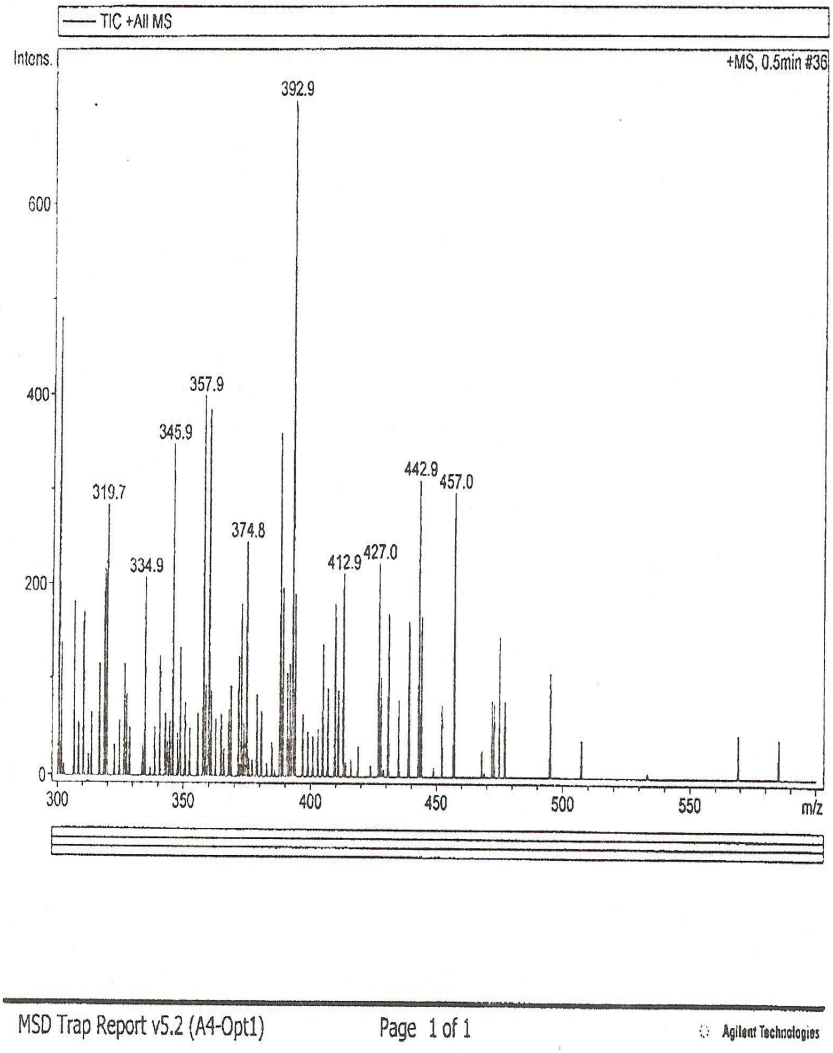


Fig 12: MS spectrum of ZL-14

GCMS-QP2010 PLUS
SHIMADZU,JAPAN

NARICT, ZARIA
GCMS ANALYSIS

FAME OF HYPTIS SEED OIL

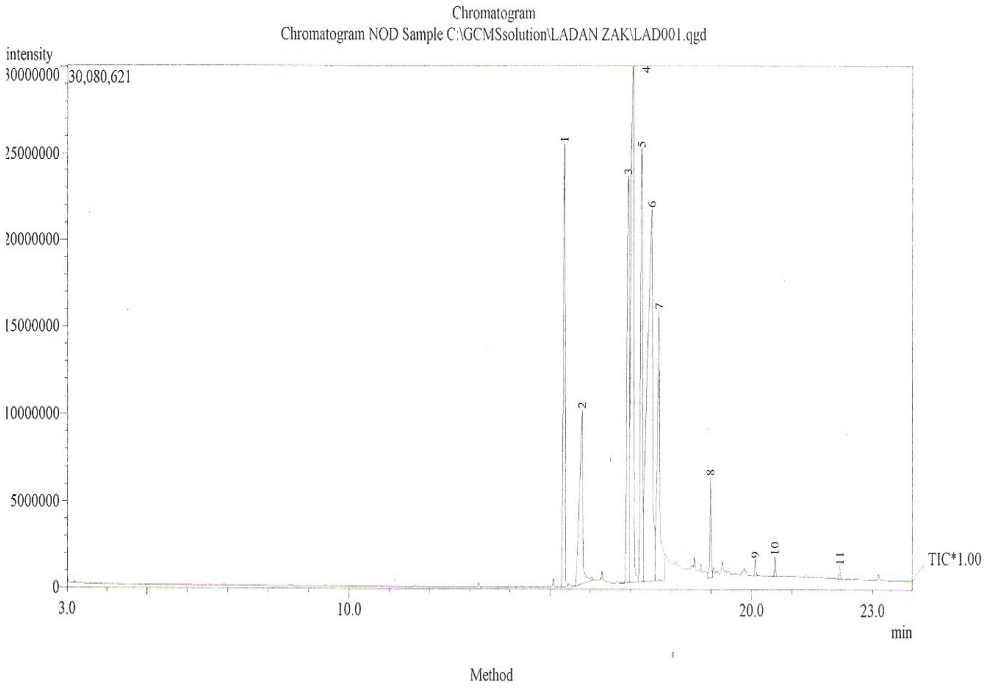


Fig 13: GC profile of *H.spicigera* seed oil FAME

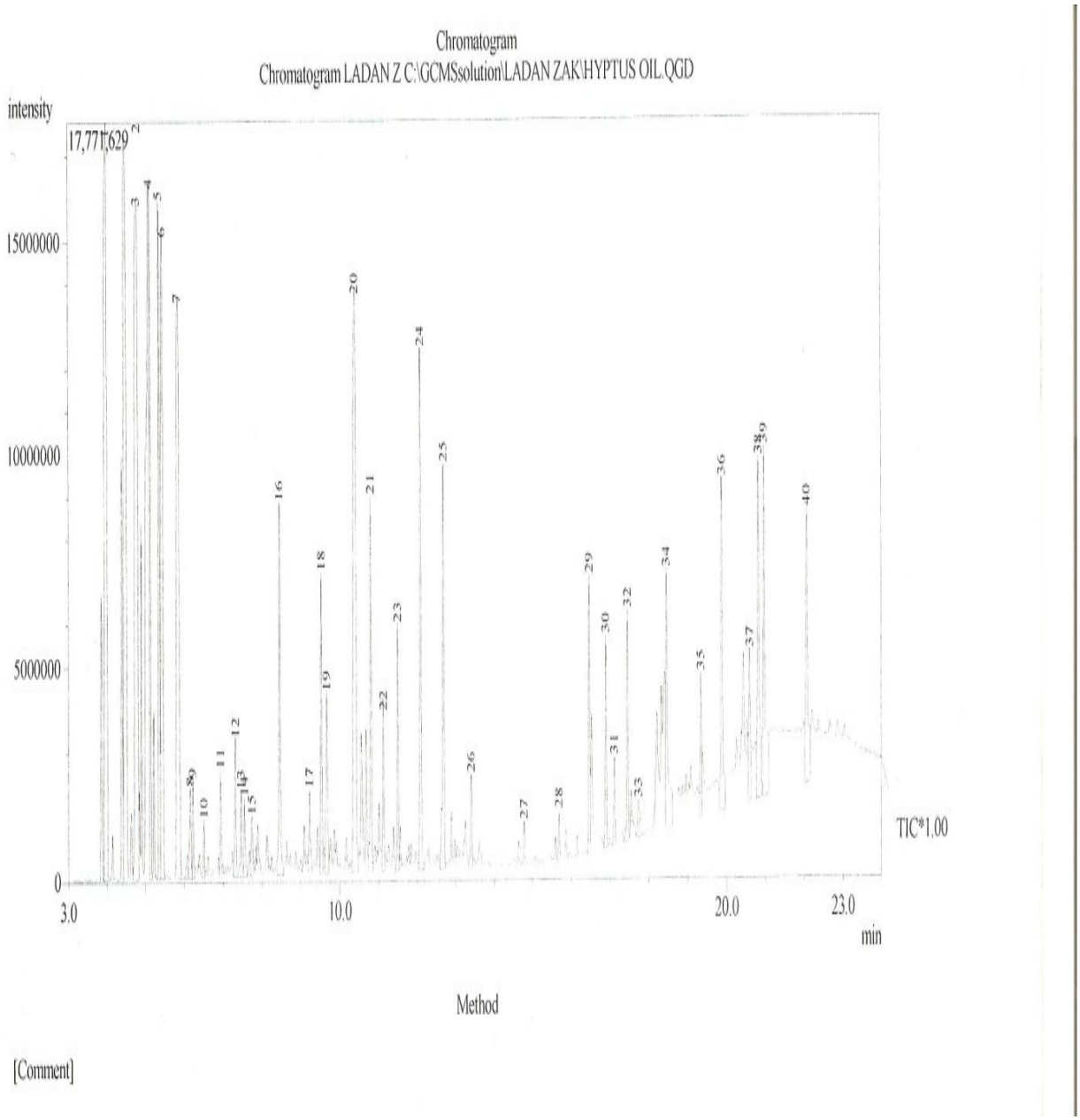


Fig.14: GC of essential oils of *H.spicigera* leaves

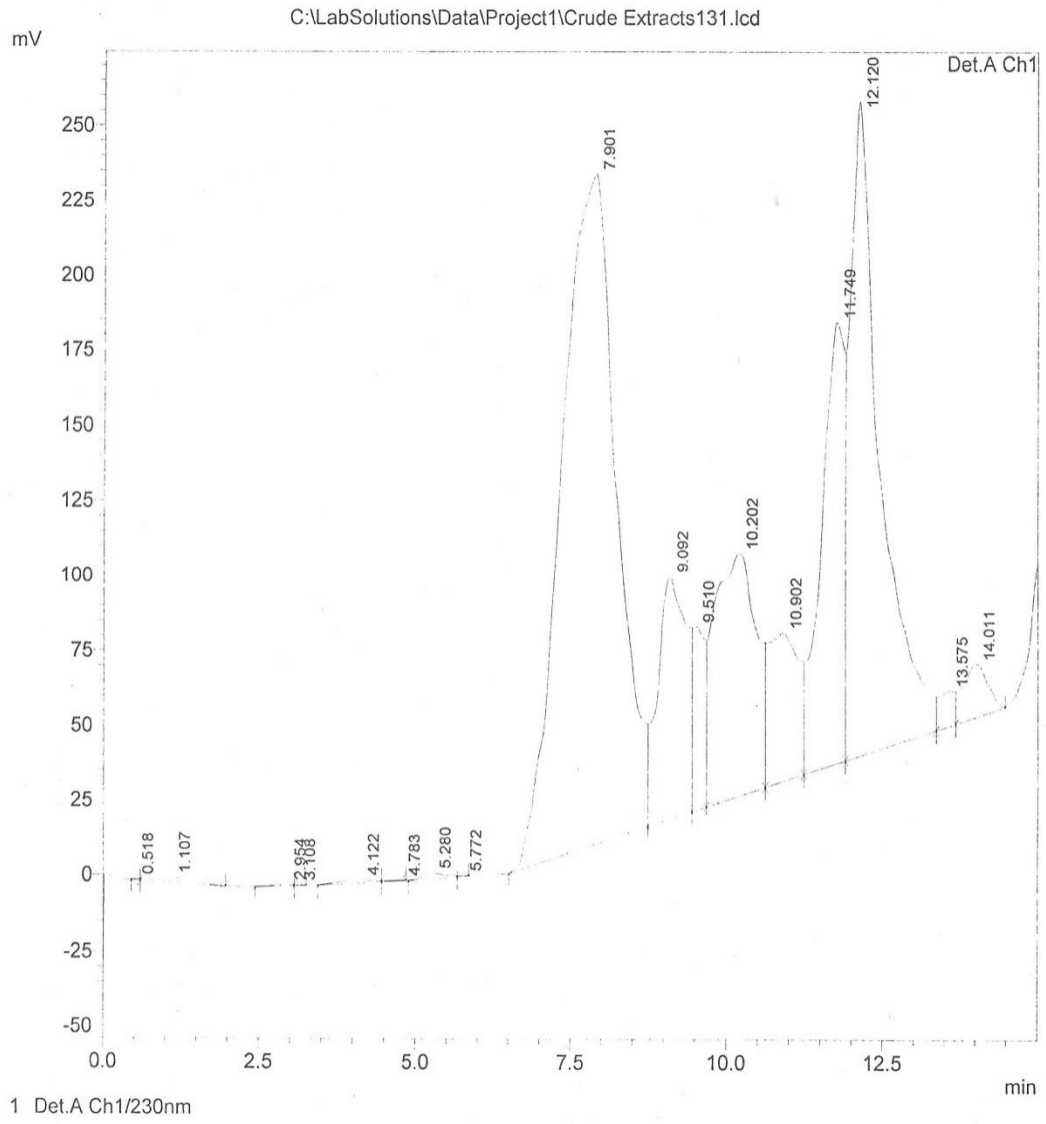


Fig 15: HPLC spectrum of ethylacetate fraction using a flow rate of 0.2ml/min

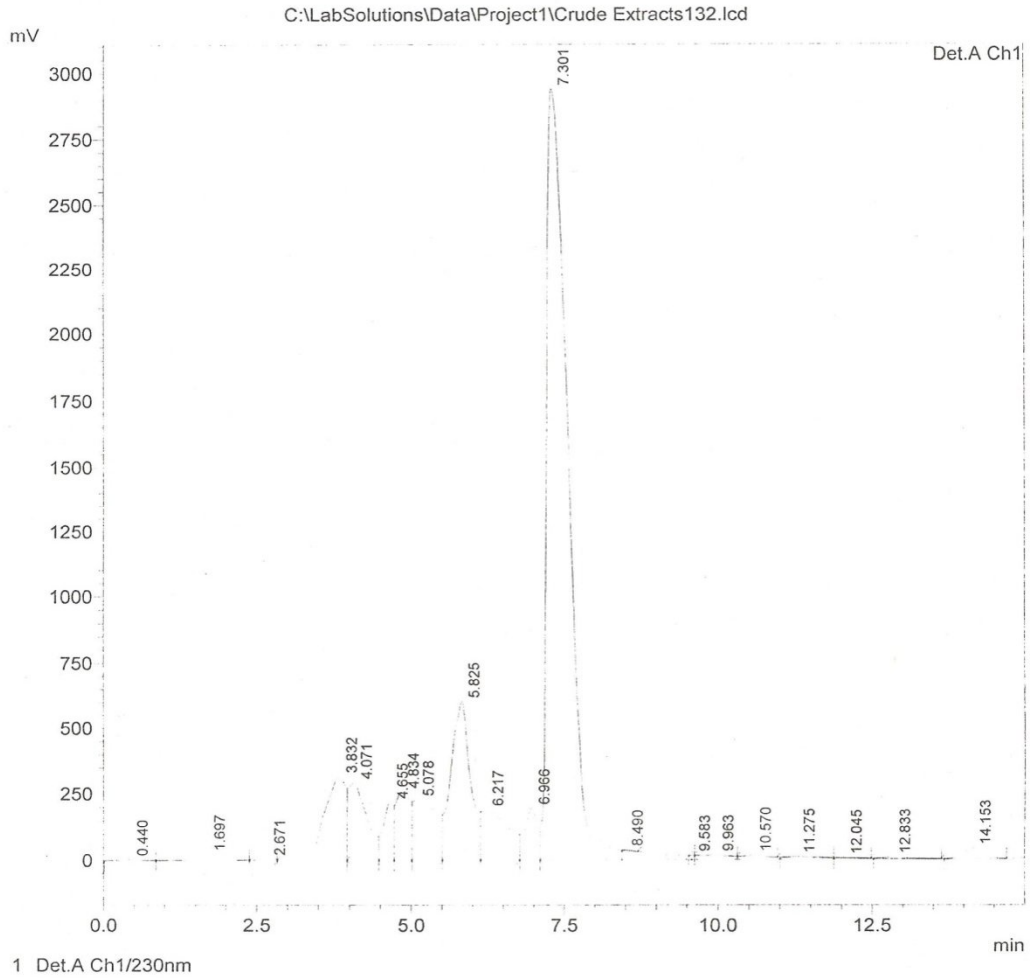


Fig 16: HPLC spectrum of ethylacetate fraction using a flow rate of 0.3ml/min

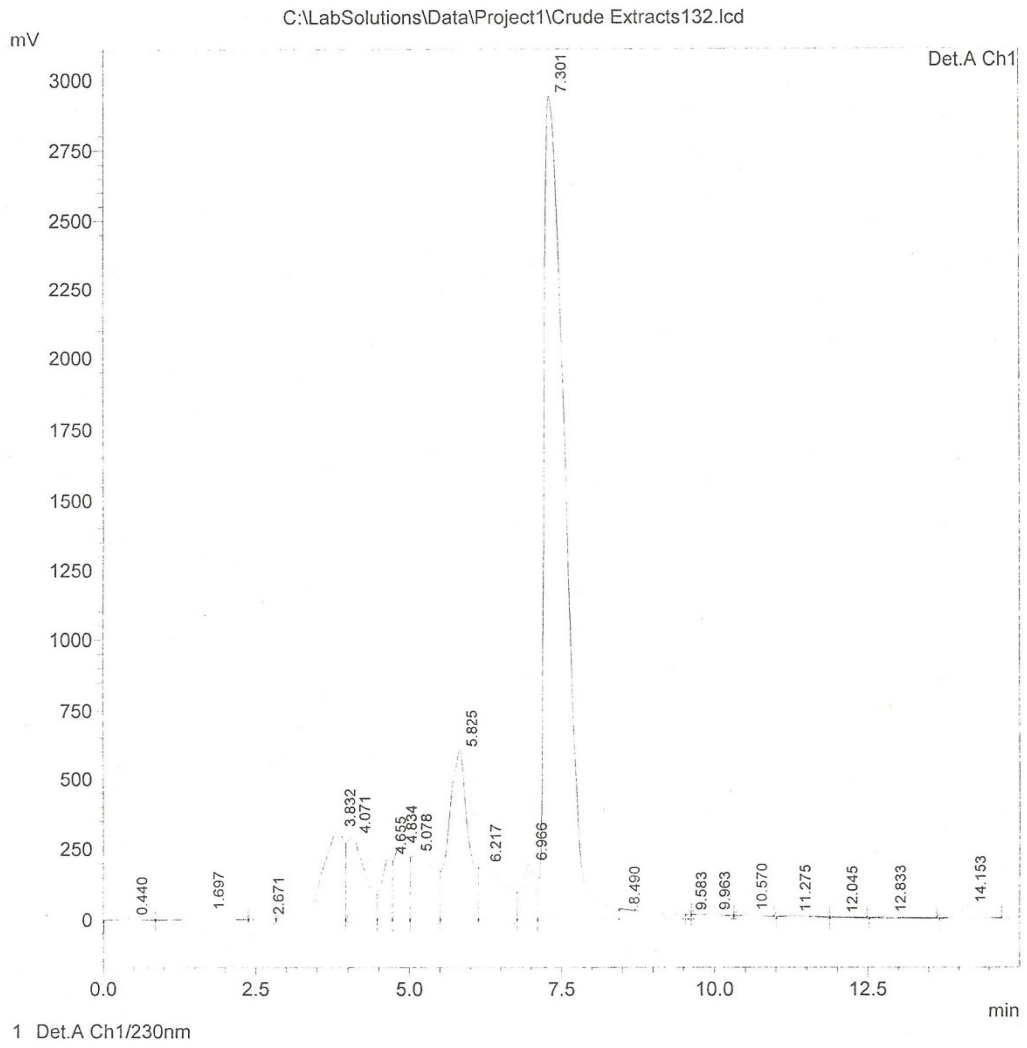


Fig 17: HPLC spectrum of ethylacetate fraction using a flow rate of 0.4ml/min

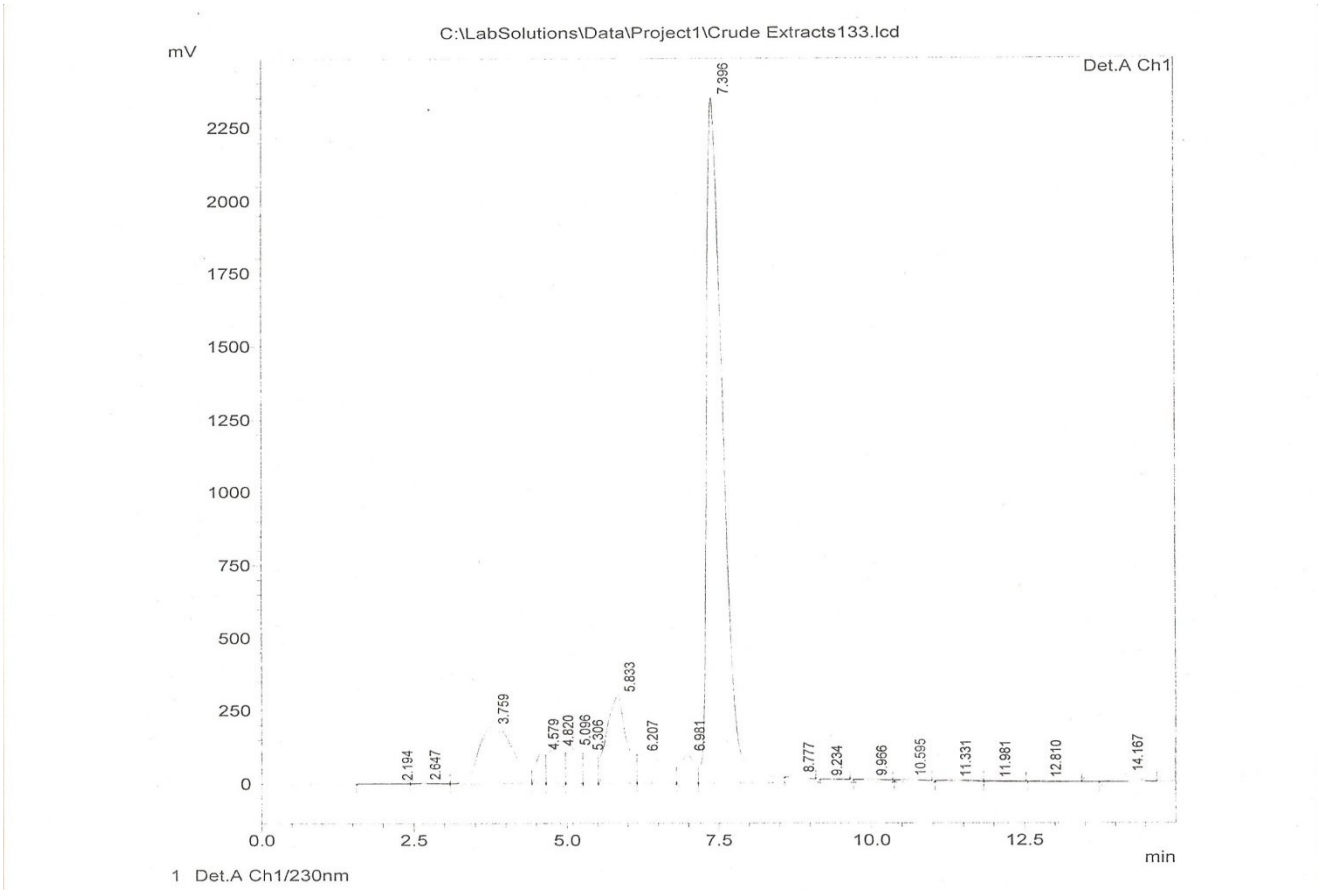


Fig 18: HPLC spectrum of ethylacetate fraction using a flow rate of 0.6ml/min

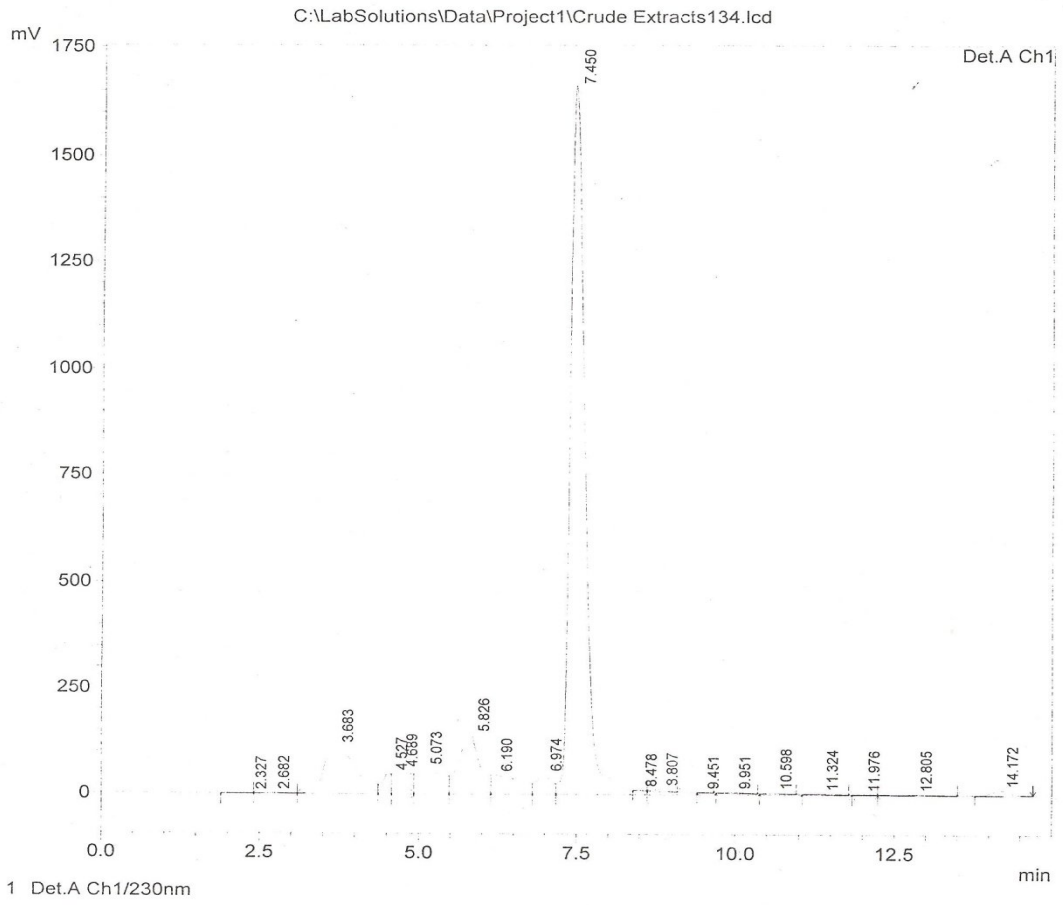


Fig 19: HPLC spectrum of ethylacetate fraction using a flow rate of 0.8ml/min

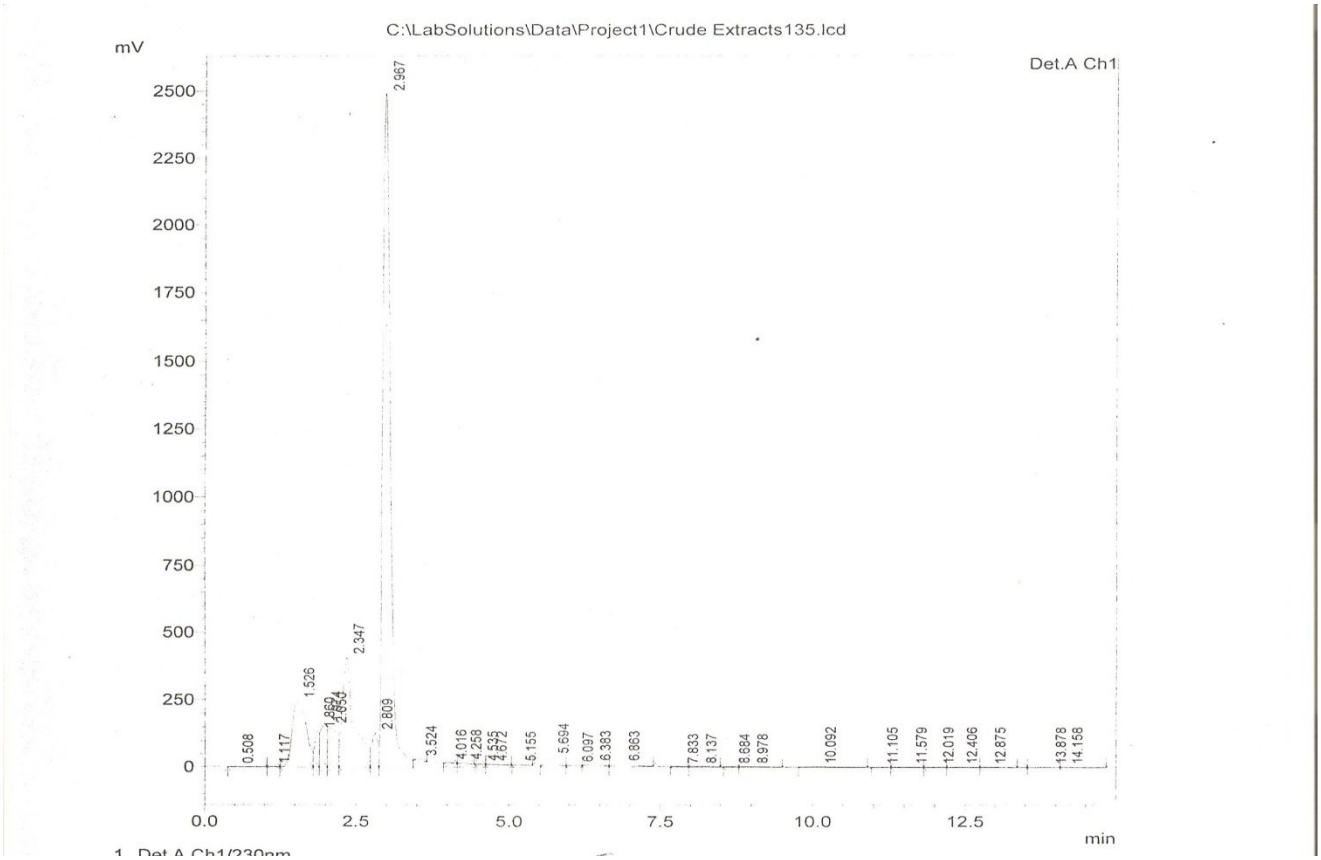


Fig 20: HPLC spectrum ethylacetate fraction using a flow rate of 1.0ml/min

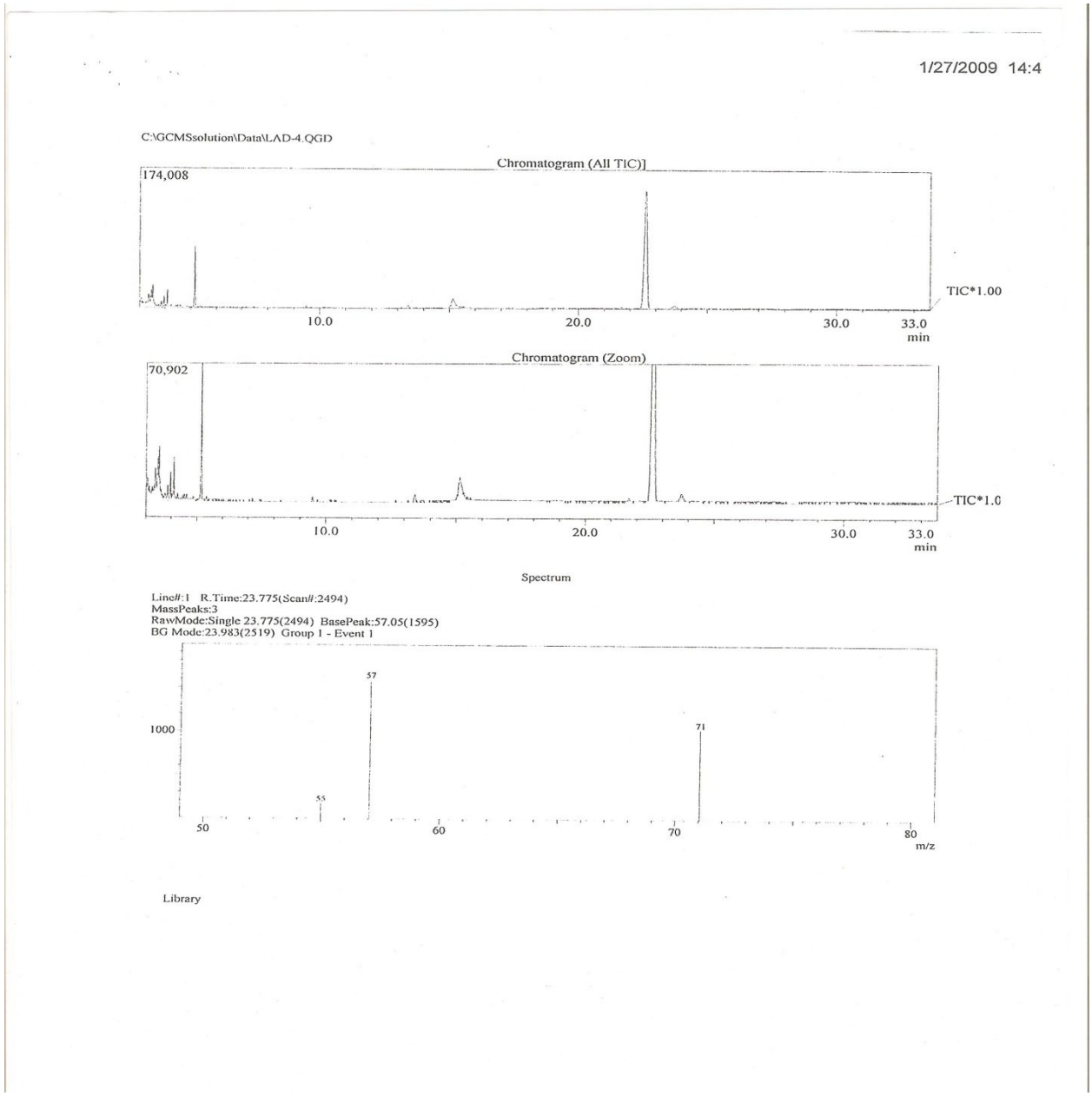


Fig.21: GC spectrum of ethylacetate fraction from HPLC

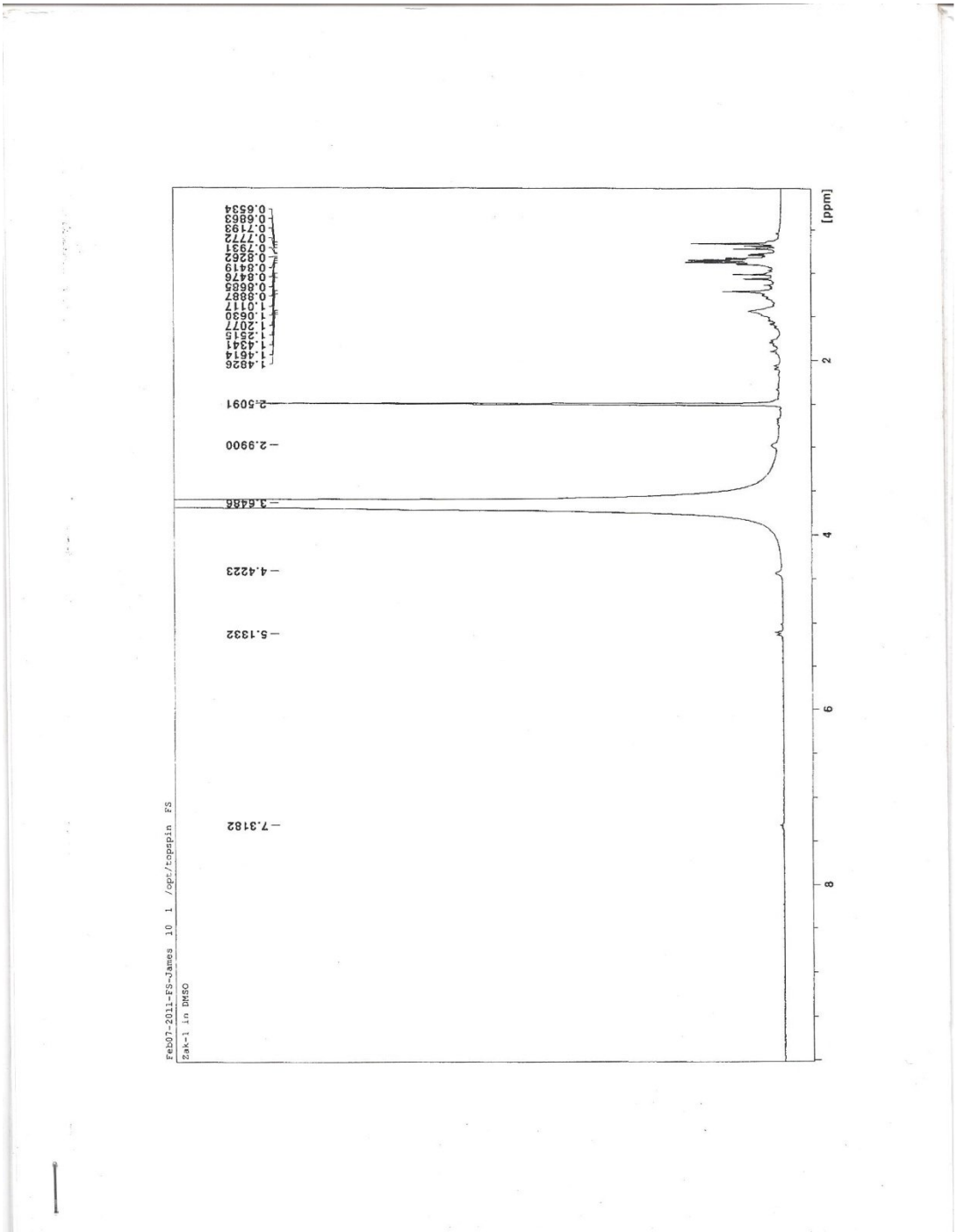


Fig 22: ^{1}HNMR spectrum of sample EL-2

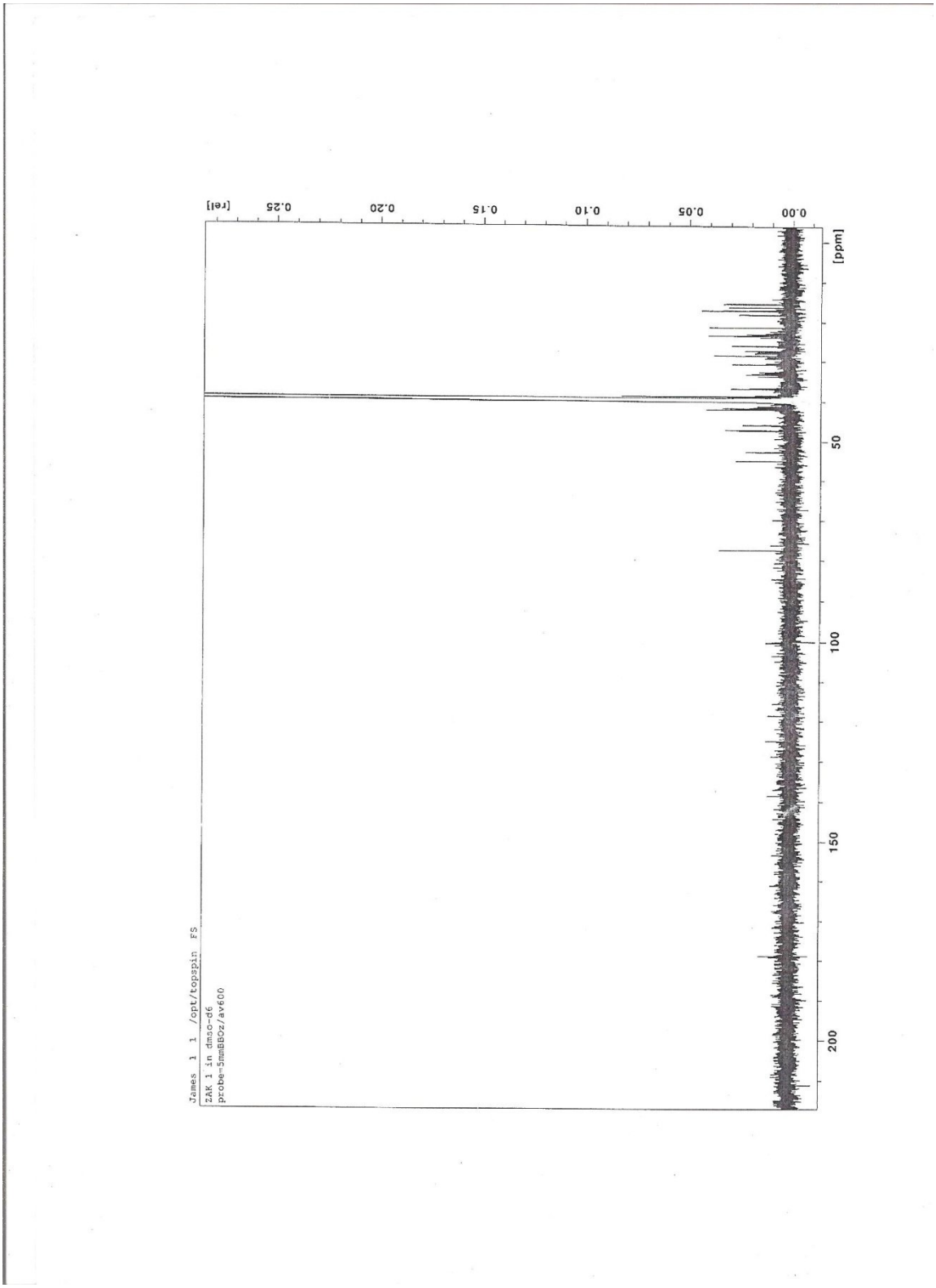


Fig 23: ^{13}CNMR spectrum of sample EL-2

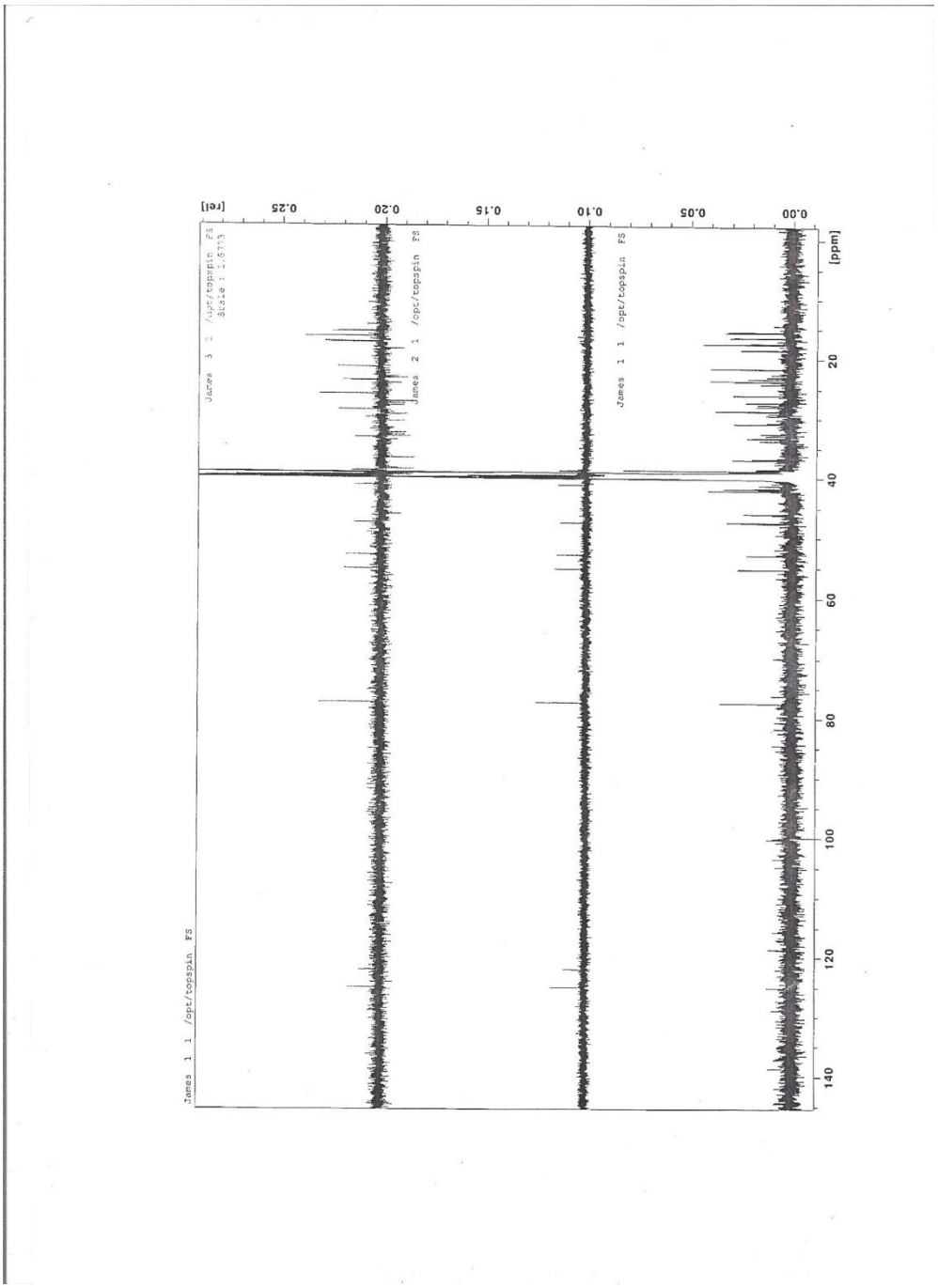


Fig 24: DEPT spectrum of sample EL-2

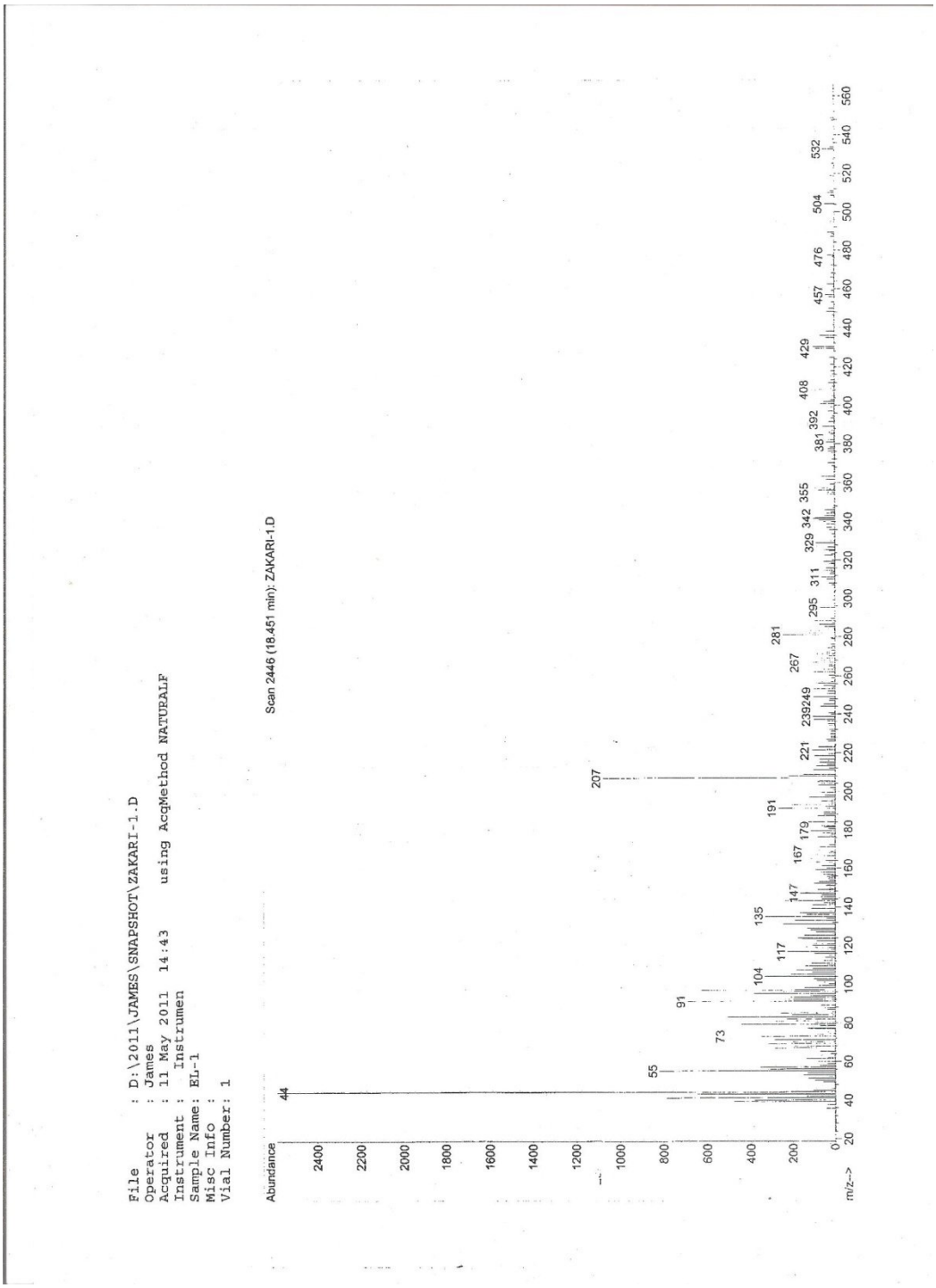
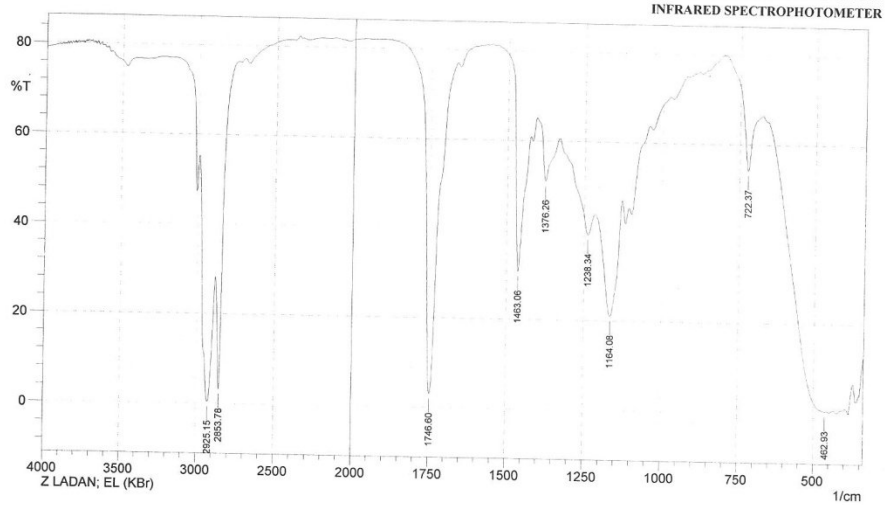


Fig 25: MS Spectrum of sample EL-2

SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM
INFRARED SPECTROPHOTOMETER



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	462.93	0.561	1.272	675.11	459.07	181.224	1.053
2	722.37	54.144	17.379	800.49	676.07	20.352	3.296
3	1164.08	21.035	24.551	1215.19	1129.36	43.233	13.704
4	1238.34	39.243	7.883	1331.89	1215.19	36.157	2.555
5	1376.26	51.061	12.315	1405.19	1331.89	17.517	2.74
6	1463.06	30.748	38.156	1518.99	1424.48	22.219	7.592
7	1746.6	3.042	75.971	1855.58	1665.59	56.665	37.521
8	2853.78	3.159	34.681	2880.78	2745.76	52.157	16.513
9	2925.15	0.428	38.565	2990.73	2881.75	112.529	68.42

Comment;
Z LADAN; EL (KBr)

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User; Administrator
No. of Scans;
Date/Time; 3/14/2011 11:52:01 AM

Fig 26: FT-IR Spectrum of sample EL-2

PUBLICATIONS FROM THIS WORK

Journal Publications:

Z.Ladan, J.O. Amupitan, E.M.Okonkwo, I.A.Aimola and N.Habila (2009).Antimicrobial potency of *Hyptis spicigera* leaf extracts against some pathogenic microorganisms. ***Journal of Medicinal Plant Research.Vol.3 (11), 905-908.***

Z.Ladan, E.M.Okonkwo, J.O. Amupitan, E.O.Ladan and B.Aina (2010).Physicochemical properties and fatty acid profile of *Hyptis spicigera* seed oil. ***Research Journal of Applied Science. 5(2):123-125.***

Z.Ladan**,** J.O.Amupitan,O.A.Oyewale,E.M.Okonkwo,E.O.Ladan,B.Odjobo and N.Habila (2011).Chemical composition and biological activity of the volatile oils of *Hyptis spicigera* against *Trypanosoma brucei brucei,Tbb* Found in Northern Nigeria. ***African Journal of Pure and Applied Chemistry, Vol.5 (4),53-58.***

Conference Paper Presentations:

Z.Ladan,J.O.Amupitan,E.M.Okonkwo (2009).GC-MS and FT-IR of the hexane extract of *Hyptis spicigera* plant.A paper presented at the 32nd Annual International conference of the Chemical Society of Nigeria held at Women Centre,Bauchi on the 5th October,2009.

Z.Ladan, E.M.Okonkwo J.O.Amupitan, E.O Ladan and B.Aina (2010).Physicochemical properties and fatty acid profile of *Hyptis spicigera* seed oil.A paper presented at the 33rd

Annual International conference,Workshop and Exhibition of the Chemical Society of Nigeria held at Ogun State Cultural Centre, Kuto, Abeoukuta on the 20th-24th September,2010.

Z.Ladan, J.O.Amupitan, A.O.Oyewale,E.M.Okonkwo. (2010).Characterisation and biological activity of the volatile oils of *Hyptis spicigera* against *Trypanosoma brucei* brucei.A paper presented at the 33rd Annual International conference,Workshop and Exhibition of the Chemical Society of Nigeria held at Ogun State Cultural Centre, Kuto, Abeoukuta on the 20th-24th September,2010.