PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE LEAVES OF *COMMIPHORA KERSTINGII* Engl (*Burseraceae*)

BY

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OCTOBER, 2009

DECLARATION

I declare that the work in this dissertation entitled PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE LEAVES OF *COMMIPHORA KERSTINGII* Engl . ***(Burseraceae)*** has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry, under the supervision of Dr. M.I. Sule, Professor Ilyasu Muhammad and Dr Ibrahim Ilya. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Sallau Muhammad Sani Karaye _____

(Signature/Date)

CERTIFICATION

THIS DISSERTATION ENTITLED "PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE LEAVES OF *COMMIPHORA KERSTINGII Engl.* **(*Burseraceae)***" meets the regulations governing the award of Doctor of Philosophy degree in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This piece of work is whole-heartedly dedicated to my beloved parents Abu-Sufyan (Sallau) and Maimunat and for the ever-lasting memory of my maternal grandmother, Hauwa'u.

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ABSTRACT

Chemical and pharmacological investigations were carried out on the leaves of *Commiphora kerstingii* (Engl). The plant belongs to the family of *Bursaraceae,* which consists of about 17 genera and over 600 species of tropical shrubs and trees. It is of extensive ethnomedical usage. In particular it is used as a stomachic, in the treatment of inflammation of the eyes, scorpion bites, diarrhea and dysentery. Preliminary phytochemical studies carried out on the leaves revealed the presence of alkaloids, carbohydrates, flavonoids, saponins, steroids and tannins in the crude acetone extract and generally to a lesser extent in the methanol extract. This result is in good agreement with earlier reports on the stem bark of the plant. The acute toxicity studies have suggested that the acetone extract is slightly more toxic *(LD_{50} of 2154.10mg/kg* in mice) than the methanol extract (LD_{50} > 5000mg/kg). The pharmacological investigations indicate that both extracts of the plant leaves particularly that of acetone, exhibited effective anti-nociceptive (peripheral), anti-inflammatory and anti-oxidant properties. The acetone extract also exhibited a broad spectrum of activity against both gram-negative and gram-positive bacteria. The results also show that even some bacteria that have a higher intrinsic multi-drug resistance to most anti-microbial agents were found to be susceptible to particularly the acetone extract. Extensive phytochemical studies resulted in the isolation and characterization of two isomeric pentacyclic triterpenoids as Glutinol and Rhoiptelenol in addition to 11-methoxy-β-Boswellic acid from the acetone fraction of the leaves of the plant. This is the first time in history that these compounds are being reported in *Commiphora* species. Structure elucidation of the compounds were carried out with the help of MS, and GC/MS Spectrometry as well as IR, ^{1}HNMR ^{13}CNMR, 2DNMR; ^{1}H-^{1}HCOSY, ^{1}H-^{1}H long range COSY, HMQC and HMBC Spectroscopy. The anti-nociceptive and anti-inflammatory properties of the acetone extract are attributable to the presence of these triterpenoids. Generally, results from these investigations show that the plant leaves of *Commiphora kerstingii* are rich with bioactive compounds and could therefore be potential sources of compounds that are effective analgesics, anti-inflammatory agents and or with higher potency against most gram-positive and gram-negative bacteria.

CHAPTER ONE

1.0 Native Medicines from Plant Sources

From the very early ages of history, plants and plant products have been the primary sources of food, shelter and transport materials, clothing, fragrances, flavours and as ingredients of medicinal substances for humankind. In this context myrrh, gums, oils and resins which are the main plant products of *Commiphora* species played an important role. They have been used as adhesives, as ingredients of cosmetic preparations, for their fragrance in daily rituals, in religious ceremonies, for coating materials and for their different medicinal effects especially wound healing (Basar, 2005).

Another extensive consumption of natural resins in the old times was their use as ingredients in cosmetic ointments. These resins were also used for their medicinal properties since antiquity. They served as disinfectants in operations and during the healing period of injuries. In the first pharmacopoeia written by the Greek botanists, there were around 600 remedies, advised, which were prepared from mixtures of natural resins and balsams as well as some herbal preparations. The antiseptic activity of resins was assumed to be known long before, since Egyptians used to burn a special mixture of them during plague and Indians had used gurju balsam (from *Dipterocarpus* species) against leprosy.

It has been also reported that mankind's experience of thousands of years with plants has led him to nurse a notion that herbal remedies, being natural products are inherently safer than the potent synthetic drugs which often produce side effects. This notion conveys a special and popular appeal for research on medicinal plants by scientists (Sofowora, 1993).

Plants historically have served as models in drug development for some major reasons. First, being that each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. It has also been established by the World Health Organisation (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs. The second reason involves biologically active substances, derived from plants, have served as templates for the synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles. While the reason concerns the fact that highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1994).

Man has employed the use of traditional medicine civilization after civilization. Recently evidences that prove the use of plants as drugs were excavated from caves in Texas and dated to about 7000BC. In lowland South America, an artifact from coastal Ecuador indicates that coca (*Erythroxylum* specie) was in use in 2100BC. The Sinu culture of Northwestern Columbia produced numerous gold pectorals with mushroom like representation which was thought to be the representation of hallucinogenic mushroom of the genus *Psilocybe*.

The major setback often encountered with drugs of plant sources is that most of the active compounds occur in minute quantity which require tedious extraction procedures. In an effort to overcome this limitation the synthetic chemist takes the approach of determining the structure – activity relationship of the isolated, biologically active constituents to synthesize simpler analogues, some of which might be more active and less toxic than the natural products (Trease and Evans, 1985). A good example is the anti malarial drug – quinine, obtained from cinchona bark and its synthetic analogue, chloroquine. Indeed structural modification of pure bioactive

molecules of plant origin as with Naloxone (narcotic analgesic antagonist of morphine) isolated from *Papavera somniferum* has presented a breakthrough.

Plants have undoubtedly played a major role in the treatment of human ailments. Natural products and their derivatives represent more than 50% of the drugs in clinical use in the World (Sofowora, 1993). The use of herbal medicines in India which dates back over 5000 years and has become codified in the Ayurveda, which contains over 8000 herbal remedies (Huxley 1984). Surprisingly this same ancient system of treatment is still used in over 14,000 dispensaries. Another ancient record has indicated the use of chaulmoogra oil obtained from species of *Hydrocarpus gaertn* for the treatment of leprosy (Le Strange, 1977). This treatment was recorded in the pharmacopoeia of the Emperor Shen Nung of Chinese between 2730 – 3000 BC. In addition, *Ricinus communis* L (Castor oil seeds) and *Papaver somniferum* L(opium poppy seeds) were excavated from some ancient Egyptian tombs. This indicates their use in that part of Africa as far back as 1500BC (Sofowora, 1993). The use of wild ginseng, a Chinese herbal drug for vitality and male potency dates back to more than 2000BC.

In much the same way as almost no portion of *Commiphora kerstingii* has not been reported to be of ethno medical significance (Dalziel, 1956, Keay, 1964, Burkill, 1985, Kubmarawa, *et al*, 2007 and Musa, 2008) so also no portion of *Commiphora* species has not been indicated to contain one chemical constituent of medicinal importance or the other (Enrico and Carlo, 1972; Hanus, 2005 and Basar 2005). It is therefore necessary to review the active chemical constituents of the medicinal plant *Commiphora kerstingii* used in this study with the aim of discovering the prospects for the present and future sources of useful drugs as well as providing alternative modern medicines. Inspite of the tremendous advances, certain human ailments have not reached

complete remission with these drugs, but evidently some defiant diseases have found complete treatment with bioactive substances of plant sources. For example, Vincristine and Vinblastin; two important alkaloids from *Catharanthus roseus* have superseded the synthetic antitumour agents in the palliative treatment of certain carcinomas, while reserpine obtained from *Rauwolfia serpentina* still remains an indispensable drug in the management of malignant hypertension (Sofowora, 1993).

Plants have traditionally served as man's most important weapon against pathogens. In fact it seems that even Neanderthals knew and made use of medicinal plants (Solecki, 1971). As early as 2000BC, the Chinese were using moulds to treat ulcers and the ancient Egyptians were known to have applied moldy bread to open wounds (Oldfield, 1984).

1.1 GENERAL BOTANICAL DESCRIPTION OF *COMMIPHORA* SPECIES

Commiphora species are small trees or shrubs with short thorny branches. The genus *Commiphora* comprises over 150 species most of which are confined to Eastern Africa, with few species also occurring in Arabia and India and other Asian countries. In particular *Commiphora opobalsamum* was abundant in Judea. It is native to Southern Arabia and Abyssinia, was cultivated on the plains of Jericho and the Jews believed it was planted by King Solomon. According to history the Queen of Sheba brought balm to Solomon on the occasion of her visit (Hanus, *et al*, 2005).

The name *Commiphora* is coined from Greek word meaning "gum bearing". This refers to the resin which exudes from the bark of some of the species. The species of *commiphora* yield myrrh, gums, oils and resins. In particular, the bark and leaves are fragrant. It yields a gum-resin

which gives an arguable myrrh-like odour when burning and is used to fumigate clothing (Dalziel, 1956).

This large genus is abundantly represented in arid regions in Africa and Asia by shrubs or small trees with a short trunk and tangled branches, the twig often being spiny though they bear leaves. Occasionally the leaves are simple, but in the tree species in Nigeria there are 1-5 pairs of leaflets plus an odd terminal one. The small bisexual or unisexual flowers are arranged in clusters at the ends of the branches of a narrow panicle, or in stalked head; with 4-lobed cup-like calyx, 4-valvate petals, 8 free stamens (with rudimentary anthers in the females) and a short style. The trees are deciduous, sometimes for a long period and the flowers often appear before the new leaves. The fruits are drupe-like and usually contain two leathers or woody stones (Keay *et al*, 1964).

Generally, *Commiphora* species have been described as those with leaves that are imparipinnate; flowers in pedunculate inflorescences. Leaflets 5-6 pairs, very acutely acuminate, oblong-ovate; unequal-sided at base, 6-12cm long, 2.5-4.5cm broad, thin, obscurely ovenulate, glabrous; petiole 1cm, long; panicles as long as or longer than the leaves, the flowers crowded at the ends of the lateral branches (Hutchinson and Dalziel ,1966).

The most reknown *Commiphoras* are *Commiphora myrrha* (Nees) Engl, *Commiphora molmol* (Eng), *Commiphora abyssinica* (Berg) Engler, *commiphora madagascarensis* (Jacq), *Commiphora africana* (A. Rich) Engl, *Commiphora guidottii* (choir), *Commiphora mukul* (Hook ex stocks) Engl, *Commiphora wightii* (Arnott) Bhanol, *Commiphora apobalsamum* (*C.*

gileadensis), *Commiphora erythraea* (Ehrenb) Engl and *Commiphora kataf* (Forssk) Engl (Honus, *et al*, 2005).

1.2 ETHNOMEDICAL APPLICATION OF THE *GENUS COMMIPHORA*

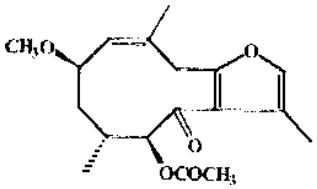
The genus *Commiphora* comprises more than 200 species, all native to Africa, Arabia, Madagaskar and India. Myrrh is the gun-resin obtained from several species of *Commiphora*, notably C *abyssinica* (Berg) Engler, *C. Schimperi* (Berg) Engler, and *C. myrrha* (Nees) Engler (Guenther, 1950). In order to collect gum, the natives make incisions into the bark, causing the exudation of a yellowish oleoresin. When exposed to air, this dries, hardens and turns reddish-brown.

Commiphora myrrha is the chief source of myrrh today but *Commiphora erythrea* was the principal source of ancient and classical time. A number of aloe-gum-resins called bdelliums are produced in Arabia and Somalia from various species of *Commiphora* and resemble myrrh; these were probably counted as myrrh in classical times and are probably used for adulteration today. The "perfumed bdellium" (opopanax or bisabol myrrh) is from *C. erythrea* (Hanus, *et al*, 2005).

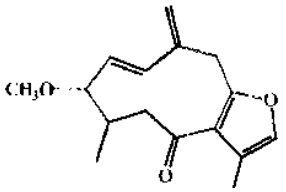
In Arabia the word *murr* means "bitter", it has been used throughout history in incense and as a perfume. It is said that the Greek soldiers would not go into battle without a poultice of myrrh to put on their wounds. It is often used in toothpastes and in the perfume industry. It tends not to dissolve very well in water however (Honus, *et al*., 2005).The gum myrrh has been used as incense for many centuries. In more recent times the gum has found medicinal usage as an antiseptic, the tincture being applied to inflammatory and ulcerated conditions of the throat and mouth.

In ancient times, Hindus, Babylonians, Assyrians, Persians, Romans, Chinese and Greeks as well as the people of old American civilizations like Incas, Mayers and Aztecs used natural resins primarily for embalming and for its incense in cultural ceremonies. Those people believed that when these materials were in contact with fire, the smoke and the fragrance they produce would not only soothe their souls but also please their gods. Burning of these natural resins had become an important action of their cultural life. They burned these resins during sacrification ceremonies or in their daily rituals to prevent the influence of bad spirits on their souls or to honour the dead or living people. The Egyptians used for these purposes Olibanum (from *Boswellia* spp) myrrh (from *Commiphora* spp), bdellium (*Commiphora wightii*), mastic (*Pistacia lentiscus*), styrax (*Liquidamber orientales)* as well as santal (*Santalium album*) (Hanus, 2005).Nevertheless, the resin of *Commiphora erlangeriana* is known to be poisonous to humans and animals and has traditionally been used as an arrow poison.(Dalziel,1956).

Even though the resins from the African *Burseraceae* are important items of commerce, as glues, tick repellent, medicinals and perfumes, the content compounds of these resins are not yet well identified. Three known – (1E) – 3-methoxy-8, 12-epoxygermacra-1,7.10,11-tetraen-6-one, (**I**) *rel*-2R-methyl-5S-acetoxy-4R-furanogerma-cr-1 (10)*Z*-en-6-one (**II**) and (1(10)E,2R,4R)-2-methoxy–8,12-epoxy-germacra-1(10),7,11-triene-6-one(**III**) and one novel furanogermacrene have been isolated and identified from the ethanolic extract of a resinous exudates (a commercial sample) of *Commiphora holtziana* from Kenya (Cavanagh. *et al*, 1993).

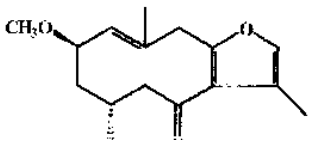


I



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MITAT



III

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A number of studies in recent years have demonstrated the increasing importance of plants in support of, or as alternative to orthodox or western medicine. More than 200,000 plants out of about 300,000 species identified in the whole planet are in Africa and other tropical countries. Plants have great potential uses especially as traditional medicines and pharmacopoeial drugs. Eighty percent of the population of Africans living in rural areas relies on traditional medicine. Many of the plants have a variety of phyto pharmaceuticals which are very important in the fields of agriculture, human and veterinary medicine (Sofowora, 1993).

The plant kingdom represents an extraordinary reservoir of novel molecules. Of the estimated plant species around the world only a small percentage has been investigated phytochemically and the fraction submitted for biological or pharmacological screening is even lower. Thus there is currently a resurgence of interest in the plant kingdom as a possible source of new lead compounds for introduction into therapeutic screening programmes. The rapid disappearance of forests and other important areas of vegetation has meant that it is essential to have access to methods which lead to the rapid isolation and identification of bioactive natural products. The approach adopted to obtain exploitable pure plant constituents involves interdisciplinary work in botany, pharmacognosy, chemistry and toxicology.

The coming of this new millennium has brought with it a very good prospect for traditional herbal remedies. This is particularly so when one considers the rate at which people from both the developed and the developing nations are switching over to the mother, nature, for the treatment of their ailments. The reason for this titillating switch over towards the traditional herbal drugs might be different for the developed and the developing nations; however the extent of recognition of the herbal remedies by orthodox practitioners and respective governments is

remarkable. In fact it is based on this that World Health Assembly acknowledged the potential value of herbal remedies and passed a number of resolutions (Pateh, 2006).

It is however, worthy to note that the greater surge of public interest in the use of plants in developed nations as medicine has been based on the fact that herbal remedies have either no untoward effects or that the side effects are minimal when compared to the synthetic remedies. In fact the common believe is that plant drugs are completely harmless. Another reason is that many of these remedies are very effective. In a similar reasoning it has been reported that opposing herbal practitioner in developing nations is making more people curious. Those who oppose them do not know that they are actually opposing what is right, natural and native to us. It cannot be discarded because that is the main thing we are gifted with for health care (Kafaru, 1999).

The cost of drugs is a sizable proportion of the total health expenditure in most developing countries. In some of these countries, drug related expenses account for up to 30-50% of the total cost of health care. This situation is becoming increasingly unbearable to many nations including Nigeria. The World Heath Organisation (WHO) have observed that up to 80% of the rural populace in the developing countries depend on herbal or alternative medicine and requested member countries to explore safe indigenous medicines for their national health care (Sofowora,1993).

However, the major set back in the recognition and utilization of herbal traditional medicine is lack of standardization. Herbal drugs are generally complex and multi componential. It is therefore important to scientifically establish the biological activity of a plant, its toxicity and its

dosage regimen. It is also pertinent, for consistency in content and activity, to standardize the whole process involved in the manufacture of the herbal remedies from the stages of cultivation, collection, drying, storage, extraction procedures of the right plant, at the right time to the final formulation of the dosage form using modern scientific and technological facilities. This does not in any way preclude the pharcognostic standardization of the plant material.

Scientific strategies for the study of natural products from plants have changed substantially in the past few years, especially in the plant selection and collection, isolation techniques, structure elucidation, biological evaluation, semisynthesis, dereplication and biosynthesis. This is as a result of advances in technology and heightened awareness of the chemical and biological potentials of tropical plants (Cordell, 1995).

The practice currently is that in which extracts are ultra purified to one major active component. However, in most instances if not all, this single compound exhibits higher side effects. In some cases, better activity and less undesirable side effects are noticed in semi pure preparations than in ultra pure compounds. In fact, it has been verified that the activities of some drugs are potentiated by the presence of other compounds. For such drugs getting them ultra pure will strip them of their ultimate potency. In addition, if there happens to be another compound in the mixture, which militates against the unwanted side effects, this useful property would be lost on purification (Adesogan, 1999).

The situation in semi-purified extracts is that in which the active component is surrounded by substances which may marginally influence its shape through any of the following: hydrophobic or hydrophilic bonds; hydrogen bonds thereby making the surrounding more or less ionic; giving steric hindrance or steric compression and or forcing conformational or other type of changes.

Any of these or combination of them or indeed any other changes may influence the binding of the molecule on to the receptor site and cause the needed biological response. Therefore the presence of other compounds near the molecule of interest may significantly affect its activity. If these compounds are not there, we may have diminished response (Adesogan, 1999).

The development and encouragement of research in this field in Africa is to ensure

(i) the scientific exploitation of Africa's flora and fauna for our benefits

(ii) a diversification in the sources of medicaments and to reduce dependence on synthetic drugs

(iii) to provide a source of lead compounds into our therapeutics and compounds for introduction into combinatorial chemistry

(iv) the codification of oral tradition which at present in Africa and other developing nations is being confused with religious rites.

These natural resources have to be developed for us to achieve the needed interdependence. Africa has considerable human and natural resources crying out for development (Sofowora, 1993).

1.4 STATEMENT OF RESEARCH PROGRAMME AND OBJECTIVES

Commiphora kerstingii has so far been scarcely investigated in spite of its ethno-medical importance and also the very close relationship with other *Commiphora* species that have been so exhaustively studied.

Phytochemistry or plant chemistry has developed in recent years as a discipline, somewhere between natural products chemistry and plant biochemistry and is related to both. It is concerned

with the variety of organic compounds that are accumulated by plants and deals with the chemical structure of these compounds, their biosynthesis, turnover and metabolism, natural distribution and biological functions (Harbone, 1973). In Africa in general and Nigeria in particular, indigenes have used certain parts of plants in traditional medicine or for other empirical uses. The role of scientists such as phytochemists is to determine the structures of the compounds used in these practices and to elucidate the mechanism of action. Their phytochemistry is then correlated with their physiological and pharmacological activities so that the use of such plant materials can be explained on a scientific basis. A plant whose use has been ascertained is included in the World Health Organisation's list of medicinal plants.

The range and number of discrete molecular structures produced by plants is huge and such is the present rate of advance of our knowledge of them that a major problem in phytochemical research is the collation of existing data on each particular class of compound. It has been estimated, for example, that there are now over 5500 known plant alkaloids and such is the pharmacological interest in novel alkaloids that new ones are being discovered and described, possibly at the rate of one a day (Harbone, 1973).

Almost every natural product is found in more than one species, although there is a taxonomic pattern of restricting a compound or a class of compounds to a certain genus, family or order. In other cases such restriction is statistical rather than absolute or quantitative rather than qualitative. Accordingly, the vast number of chemical constituents reported in literature from *Commiphora* species is a positive indication that every specie of the genus is a potential source of these and possibly other valuables. In particular the best known products of the family are myrrh, frankincense, American elimi and java almond which are all also gum resins. Most of these

resins possess antiseptic, anti arthritic and anti-inflammatory effects in addition to being used in religious ceremonies, as important fixative in perfumes, soaps, creams, lotions and detergents (Basar, 2005).

The need to screen plants for new pharmaceuticals is particularly urgent in the light of rapid deforestation and the concurrent loss of biodiversity throughout the world. It is against this background that *Commiphora kerstingii*, which is one of the herbal plants used extensively in some parts of Africa and Nigeria in particular was chosen for study to ascertain or isolate the medicinally active principles and characterize them using spectroscopic analysis.

Resistance to anti microbial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one-half of all deaths in countries of tropical regions. In industrialized nations, despite the progress made in the understanding of micro-organisms and their control incidents of epidemics due to resistant micro-organisms and the emergence of hitherto unknown disease-causing microbes pose enormous public health concern (Iwu, *et al*, 1999).

Therefore, in this study, the aim was to carry out the preliminary phytochemical screening, extraction, isolation, characterization, structural elucidation, determination of some pharmacological properties, the antimicrobial and antioxidant properties of the isolates from the leaves of *Commiphora*, *kerstingii*. The pharmacological properties intended for analysis include the determination of median lethal dose (LD_{50}), the analgesic and anti-inflammatory studies while the anti-microbial studies involves an assay of the anti-bacterial properties of the extracts of the leaves of *Commiphora kerstingii*. It is expected that as with other *Commiphora* species very

useful pharmaceuticals of immense medicinal and or commercial significance will be harnessed in the course of the research. The methods used for the fulfillment of these targets were primarily chromatographic techniques using silica gel and sephadex LH-20 to isolate and purity the compounds. The structure elucidation was done by spectroscopic methods such as IR, GC-MS and NMR (^{1}HNMR, ^{13}CNMR, ^{1}H-^{1}HCOSY, NOESY HMQC and HMBC). The biological activities and microbial assay of the extracts were also determined.

1.5 JUSTIFICATION

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. Subsequently developments on vacuum distillation techniques provided the possibility to determine the volatile components of these extracts.

The therapeutic revolution produced by the sulphonamides and later by the penicillin's and cognate antibacterial agents has had very wide consequences with unimagined developments in medicinal chemistry. This has been through the design of drug via pure synthesis of organic compounds to the isolation, structure determination and synthesis of medicinal agents of natural origin or structures that are closely related to those of naturally occurring compounds (Olaniyi, 2005). Many classes of medicinal agents such as the sulphonyl ureas as hypoglycaemic, the thiazides as diuretic, the benzodiazepines, as anti-anxiety agents, sildenafil as an effective drug for

erectile dysfunction have been uncovered quite serendipitously during chemical and clinical studies.

The screening of plant components and standard tests of the extracts for the presence of flavonoids, alkaloids, saponins, glycosides, tannins and other phenolic compounds in itself may not lead to discovery of new biologically active compounds but if consistent and exhaustive evaluation is carried out, it could provide useful information from which plants with potential biological activity could be identified for further research.

The traditions of collecting, processing and applying plant and plant-based modifications have been handed down from generation to generation. In many African countries, traditional medicines with medicinal plants as their most important components are sold in market places or prescribed by traditional healers. Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994).

In Nigeria there are a number of unexplored plants with historical ethno medical applications. Unless such species are thoroughly studied, the alleged medicinal potentials remain hoax and continuous application and usage could therefore culminate into serious health hazards. Some of these plants however will most certainly contain biologically active compounds as such it is only in the course of these studies that the various valuable pharmaceuticals could be explored. It was in similar ways that a number of alkaloids such as vincristine which has since 1963 been clinically used for cancer chemotherapy (Sofowora, 1993) and Maytansine an anti leukemia agent (Kupchan, *et al*, 1972) have been discovered from some African medicinal plants.

Commiphora kerstingii has been in use for a number of medical purposes. The gum-resin is boiled in West Africa for the treatment of inflammation of the eyes by holding the face over the steaming pot (Burkill, 1985). The leaves are pounded with bulrush millet and milk and taken as a stomachic (Hutchinson and Dalziel, 1966). For scorpion-bite the bark is applied after chewing it along with natron. The present research studies would be a basis for establishing the identity of the chemical compounds present in the plant, the pharmacological basis for the observed action and to determine the toxicity of the plant if any.

The use sometimes of the bark of the plant as an antidote to arrow poison suggests that the plant could be a potential source of certain compounds that would be used in the treatment of certain illness. Furthermore even the anti-oxidant properties of the extracts of the plant are worth evaluating.

The use of synthetic analgesics, anti-inflammatory and antimicrobial agents are but with an attendant fear for their oncogenic and or terratogenic effects and their tendency to cause ulcer. As such naturally occurring treatments for these disease conditions will go along way in eliminating unwanted side effects.

The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine, because the search for new potential medicinal plants is frequently based on an ethno-medicinal bases. In the ethno-pharmacological approach, local knowledge about the potential uses of the plant is very useful as compared to the random approach where indigenous knowledge is not taken into consideration. Compounds inhibiting microorganisms, such as benzoin and emetine have been isolated from plants (Cox, 1994).

It is possible that anti-microbial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains. For this reason, it is therefore important to investigate plants as alternative sources of anti-microbial compounds. Nevertheless the active principles of many drugs found in plants are secondary metabolites (Ghani, 1990). Hence, basic phytochemical investigations of these extracts for their major phytoconstituents are also vital.

Finally, the synthetic drugs used either as antimicrobial, anti-inflammatory or analgesic agents are usually beyond the reach of the common man. Unfortunately this class of people constitutes the majority of African population. Therefore the abundance of well explored plant products of relevant medicinal values will serve as better substitutes.

CHAPTER TWO

LITERATURE REVIEW

2.0 LITERATURE

2.1 THE PLANT *COMMIPHORA KERSTINGII*

The botanical origin of Commiphora species has been characterized as follows

Division:	*Spermatophyta*
Subdivision:	*Angiospermae*
Tribe:	*Rosopsida*
Sub-tribe:	*Rosidae s. lat*
Over-class:	*Rutanae*
Class:	*Anacardiales*
Family:	*Burseraceae*
Genus:	*Commiphora*

Commiphora kerstingii (Engl) belongs to the family of *Burseraceae*. This is a large family comprising of about 17 genera and over 600 species of tropical shrubs and trees. The major genera include *Commiphora* (185 species), *Bursera* (80 species), *Canarium* (75 species) and *Boswellia* (24 species).

Commiphora. *kerstingii* is a tree 20-30ft high with smooth green bark eventually peeling in brownish papery strips; panicles crowded at the ends of the branches which are brownish tomentellous and marked by leaf-scars; often planted (Huchinson and Dalziel, 1966).

Fig. 2.1: The plant *Commiphora kerstingii* (a, c and d) in its natural habitat and (b) a branch sample.



(a)



(b)



(c)



(d)

This specie with leaves in tufts, at the ends of stout branchlets and large leaflets, resembles a *Lannea*. It is called "*Ararrabi*" or "*hana gobara*" (Hausa) and "*Kabiwal*" (Fulani). The people say it has seven lives (Hausa nickname "*dashi mai yawan rai*") because it is cut and not killed and they therefore plant it to form a hedge in arid districts. It grows well from cuttings and it is venerated by the Tuaregs who regard it as a symbol of immortality and place it on graves (Dalziel, 1956). It spreads from Northern Nigerian states of Sokoto, Plateau, Bauchi and parts of Kaduna State, to the Eastern and North Southern of Nigeria. Its habitat is savannah, but often in towns and villages planted as a fence. The tree is up to 30 feet high with ascending branches. The bark is smooth green, as with *Boswellia* species, eventually peeling off in papery strips and rolling up; slash creamy-brown, exuding a white gum. Young twigs covered with fine brownish hairs (Keay *et al*, 1964).

The leaves are with a glabrous common stalk 6-12 in long; 5-6 pairs of leaflets 3-4.5 in. long by 1-1.5 in broad, ovate to broadly lanceolate, sharply acuminate, often unsymmetrical at the base with one side rounded and the other cuneate; glabrous, papery, entire or very vaguely toothed; with 8-12 pairs of regular up curving lateral nerves running out parallel with the margin leaflet – stalk about 0.25in. long (Dalziel, 1956).

The flowers (Mar-Apr) are in clusters at the ends of the branches of a narrow panicle as long as or longer than the leaves; panicles finely hairy, crowded at the ends of the shoots, appearing when the tree is leafless; individual flowers shortly stalked, about 0.25 in. long including the stalk, calyx top-shaped. The fruits are subglobose, 0.5 to nearly 1 in. in diameter and the wood is soft.

2.1.1 Medicinal Uses of *Commiphora* Species

The resinous exudates obtained from the trees of certain Commiphora species of the *Burseraceae* family are mainly Myrrh and Opopanax oils. The People's Republic of China is the largest market for all two resins mainly for use in traditional medicines. (Hanus *et al*, 2005). Guggul, the exudes of *C. wightii* are used in *Ayurvedic* literature as medicine. Guggul lipid (a mixture of lipid steroids isolated from the resin) is a potent hypolipidemic agent. It was found that the ethyl acetate extract of C. wightii showed significant *in vitro* cytotoxicity.

The gum myrrh, an exudate of *Commiphora myrrha*, has found medicinal usage as an antiseptic, the tincture being applied to inflammatory and ulcerated conditions of the throat and mouth (Hanus *et al*, 2005), as food additive, fragrances and in traditional medicine in African and Asian countries, as toothpastes and tinctures for the treatment of gingivitis, as anti inflammatory agent on rat paw edema produced by carrageen as anti-inflammatory agent on edema and an adjuvant-induced arthritis (Duwiejua *et al*, 1993) as hypolipidemic agent in India (Satyavati, 1988), and as hypocholestrolemic tablets and drugs (Mesrob *et al*, 1998) and numerous other medicinal applications.

Myrrh is used today in Chinese medicine to treat wounds, relieve painful swelling and to treat menstrual pain due to blood stagnation. Myrrh is called *mo yao* in China. It has long been used in the Ayurvedic system of medicine.

Various parts of C*ommiphora* species have also been reported to be of great medicinal values. The ethanolic extract of air-dried trunk of *Commiphora wightii* was separated on column packed silica gel to give a new antifungal flavone named *muscanone* and already known *naringenin* (Fatope, 2003). Muscanone was active against *Candida albicans* in microbial sensitivity assay.

The stem bark of *Commiphora kua* was also exhaustively analysed to obtain a number of triterpenes from the petroleum ether extract. The essential oils obtained from the leaves of *Commiphora africana* and *Xylopia aethiopica* from Benin were analysed by GC, GC/MS and C-NMR. The leaf essential oil of *C. africana* contained fairly high amounts of *sesquiterpenes* (Ayedoun, *et al*, 1998). Gas chromatography of the oil from the stem bark of *Commiphora rostrata* grown in Kenya, Somalia and Ethiopia revealed at least 30 components. All the aliphatic ketones and aldehydes showed comparable or greater activity than the synthetic commercial insect repellent N, N-di-ethyl-toluamide (DEET). The bark of *Commiphora kerstingii* is however sometimes used as an antidote to arrow poison (Dalziel, 1956 and Burkill, 1985). It is also used traditionally in Northern Nigeria to treat fever, cancer, measles, asthma, rheumatism and venereal diseases (Musa, 2008) and for the treatment of diarrhea and dysentery. Incidentally the inhibition of E. coli by the ethanolic extract of the stem of the plant has vindicated its traditional usage (Kubmarawa, *et al*, 2007). The leaves of *Commiphora africana* are pounded with bulrush millet and milk and taken as stomachic.

2.1.2 Other Uses of *Commiphora* Species

Since antiquity, myrrh the main exudates of *Commiphora myrrha* and many other C*ommiphora* species was used by the Egyptians for embalming and by the Jews as anointing oil. In St. Marks Gospel, "Vinum Murratum", wine with myrrh was offered to Jesus before crucifixion (Hanus *et al*, 2005). Olibanum, the exudates of *Boswellia* species has been used in religious ceremonies, as an important fixative in perfumes, soaps, creams, lotions, and detergents with an oriental note in its scent in the leading products of the perfume and cosmetic industry.

The stems of *Commiphora africana* are used as a chew stick and the wood for making rosary beads. The people say it has seven lives (Hausa nickname it *dashi mai yawan rai*) because it is cut and not killed and they therefore plant it to form a hedge in arid districts. The tree is planted as a live-fence in towns for its aesthetic value and as a shade plant (Burkill, 1985). It is venerated by the Tuaregs who regard it as a symbol of immortality and place it on graves. C. *kerstingii* has also been indicated (Dalziel, 1956) to be a very common live fence in towns. The *Hausa* name *hana gobara* is suggested by the ever green bark which seems to have engendered an idea that the tree is little likely to burn, so that it has acquired vernacular names suggestive of protection against fire and survival of property and therefore of inheritance (Burkill, 1985).The Fulani herdsmen feed the leaves to goats and in a superstitious token, lay sticks of the plant across graves perhaps in the sense of conferring protection.

Commiphora kua a 3-5m tall tree is found mainly in Kenya, Somalia, Ethiopia and Arabia. It produces wood that is used to make household utensils, furniture and tools. During rainy season, its trunk is cut and sucked to quench thirst. In dry season, the tree produces a brown resin which is used as incense (Hanus, 2005).

2.1.3 Micro-organisms as Causative Agents of some Diseases

Most infectious diseases are caused by microorganisms. *Pseudomonas aeruginosa*, a gram – negative bacteria causes otitis external, urinary track infections, infected bedsores and eye infections etc. *Escherichia coli*, *Klebsiella* species and *Proleous* species are also responsible for urinary tract infections. In spite of the fact that all persons have a rich flora of *Escherichia coli* in the lower ileum and in the colon, it however, incriminates as a pathogen outside the gut. It causes

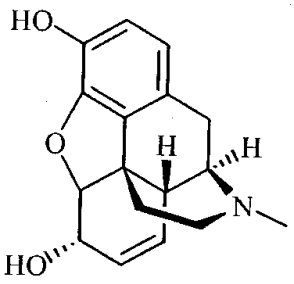
infections of the urinary tract particularly in married women. It could be responsible for appendicular abcess, pesitonitis, cholecystis and wound infections (Malik, 1992).

Salmonella typhi and *Salmonella paratyphi* are responsible for typhoid and para-typhoid fevers respectively. These pathogens are also responsible for gastro-enteritis in swine and abortion in sheep. The most important diseases caused by *Staphylococcus aureus* in animal is mastitis. The organism is also responsible for causing abscesses, carbuncles, wound infections, endocarditis, arthritis and food infections in man and other animals. Generally, *Staphylococcus aureus* causes infections most commonly in tissues and sites with lowered host resistance such as boils, wound infection, whit low of fingers or palm of hand, breasts, abscess, osteomyelitis, bronchopneumonia and lymphadenitis etc.

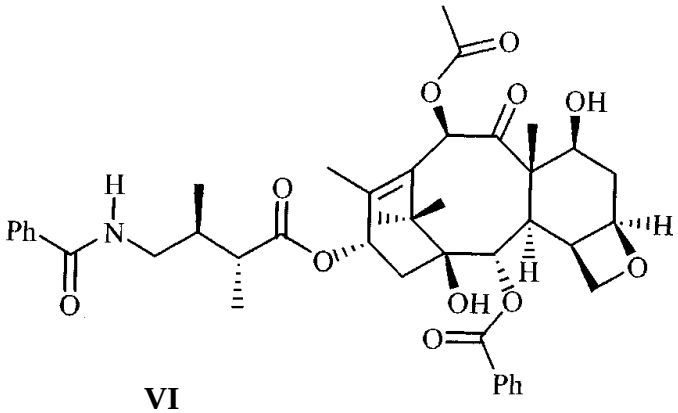
Streptococcus pyogenes is also known to cause scarlet fever, sore throat called transillitis, acute rheumatic fever, and pneumonia in calves, suppurative lesions in cattle, buffalos, sheep, goats and pigs (Thomas, 1979: Malik, 1992).

2.2 PLANT METABOLITES

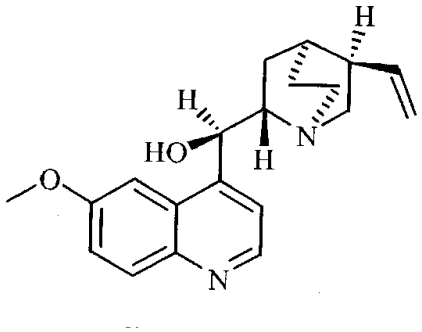
Although plant extracts and herbal products were widely used throughout history, it was not possible to successfully isolate their active constituents prior to the 19th century.



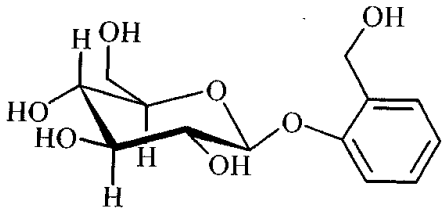
IV



VI



V



VII

The complexity and diversity of these products and their specific pharmacological effects stimulated researchers to find explanations for the biosynthetic pathway for plant constituents starting with photosynthesis and resulting in most complex structures: Morphine (**IV**), isolated from opium (*Papaver somniferum and P. setigrum*) that has powerful analgesic and narcotic effects; quinine (**V**), isolated from the bark of the *Cinchona* tree showing antimalarial activity; taxol (**VI**), isolated from the stem bark of the western yew, *Taxus brevifolia*, having antitumor, antileukemic effects and today being used in the treatment of refractory ovarian cancer, metastatic

breast and lung cancers, and Kaposi's sarcoma; and salicin (**VII**), a glycoside, first isolated from the bark of the willow tree in 1828 which led to the development of acetylsalicylic acid, the world's most widespread anti-inflammatory and painkilling drug, aspirin (Basar, 2005).

Within a short period the discovery of new bioactive natural compounds has increased and it has become crucial to understand the biosynthetic metabolism of plants in detail. Since these natural compounds generally do not belong to the group of compounds which are essential for the continuity of the lifecycle of a plant, it is common to distinguish between the primary and secondary metabolic pathways while studying these routes.

Primary metabolism, which actually proceeds in all living species, refers to the processes for synthesis and consumption of nucleic acids, α-amino acids, proteins, fats and carbohydrates that are essential for the survival and well-being of the organism (Basar, 2005).In contrast, secondary metabolism, predominantly found in plants, microorganisms, fungi, marine organisms, and, to a lower extent, in animals, was proved to include processes nonessential for the continuity of the lifecycle or for growth and development. In plants, this metabolic pathway ends up with products like essential oils, resins, balsams, saponins and glycosides. Secondary metabolites are produced in separate biosynthetical routes as compared to primary metabolites although primary metabolism provides some precursor molecules for the secondary metabolic pathway, such as acetylcoenzyme A which would complete its metabolic pathway with the formation of isoprenoids, the largest group of secondary natural compounds. Therefore, it is impossible to exactly distinguish between the two metabolic pathways (Bratt, 2000).

Structures, distributions and levels of occurrence of secondary metabolites in general are valuable tools in defining evolutionary pathways and providing taxonomic markers. Quite a number of these metabolites are common in many species but some of them are characteristic to a particular family, genus or only to a single species. In fact, the specific constituents of certain species have

been used for systematic determination. Groups of secondary metabolites were used as markers for chemo-taxonomical classification. Such chemotaxonomy is based on the assumption that systematically related plants will show similar chemical characteristics. For such purposes, simply constructed compounds of widespread distribution are less valuable than more complex compounds formed in long reaction chains by the mediation of many enzymes and specified by many different genes (Banthorpe *et al*, 1972).

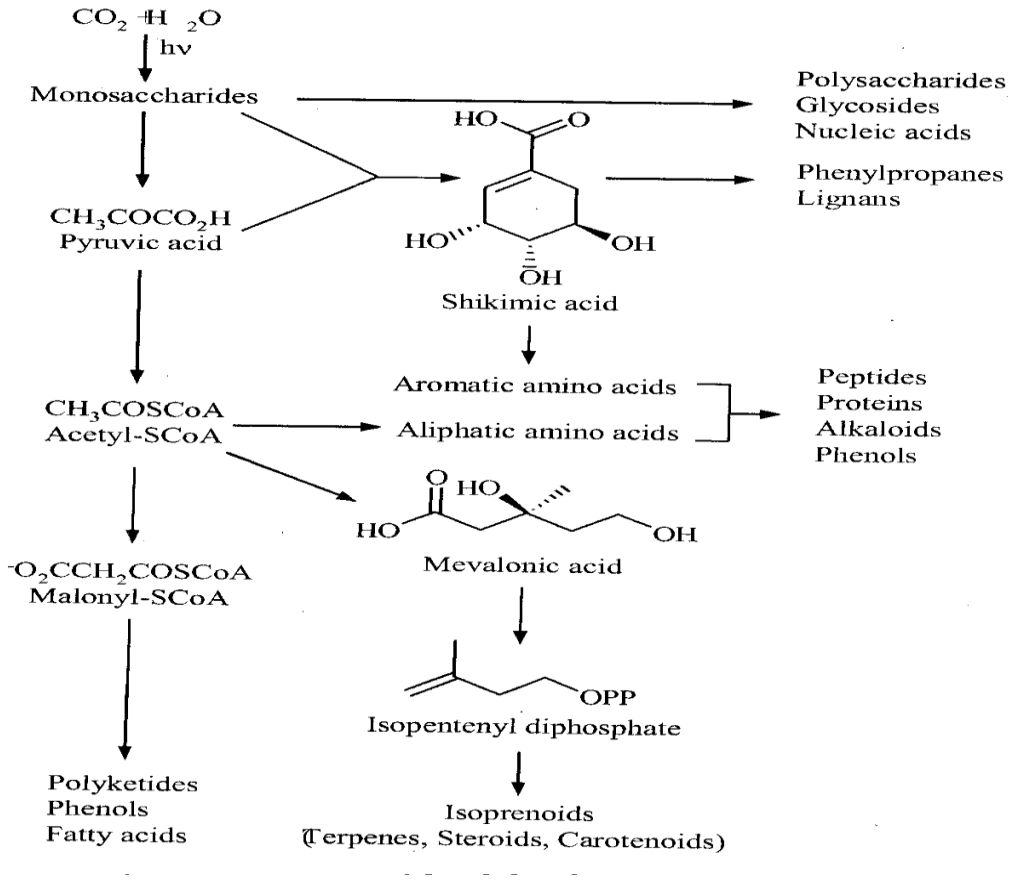


Fig. 2.2.: An overview of primary metabolites and their links to secondary metabolism

2.2.1 Essential Oils

Essential oils are defined as mixtures of volatile compounds excreted by glandular hairs or deposited in the plant body in cell organelles, idioblasts (oil cells or bodies), schizogenous or lysigenous cavities or ducts. The essential oils occur in various parts of the plant, sometimes in all organs and sometimes in certain tissues only. They are the end products of secondary metabolism, and most of their components are terpenoids, generally monoterpenes and sesquiterpenes, as well as some diterpenes and aromatic compounds, such as phenylpropane derivatives. Hydrodistillation is the most frequently used technique to isolate these lipophilic mixtures.

In addition to their ecological significance in nature, essential oils are important raw materials for perfume, cosmetic, food and pharmaceutical industries. Some of them have a long history as phyto-pharmaceuticals, especially in Egypt and in India, since 1600 B.C. They have proved to possess antimicrobial, spasmolytic, antiseptic, diuretic, sedative, antiphlogistic and many other activities.

2.2.2 Natural Resins

Besides the essential oils, considerably larger amounts of non-volatile substances are produced in the plant cells as a result of their metabolic routes. They are generally known as "*resins*" and their solutions in essential oils as "*balsams*". Nevertheless, it is difficult to classify the different types and find a common identification for resins.

Since resins and essential oils are mostly composed of terpenoids they would be classified by plant physiologists as secretions, as the end-products of plant metabolism which are collected in

secretory depots and become immobile although research results proved that they may undergo chemical rearrangements.

A. Tschirch was the first scientist who tried to classify them. He thought that common features among resins were not their properties but their origin. He also indicated that resins represent plant excretes, that is substances, which should be eliminated from the plant at the end of the metabolic pathways, in contrast to secretions actually serving the plant metabolism. He divided resins into two groups: "*physiological*" for the usual metabolic end products and "*pathological*" where the production of these substances was observed first when the plant was wounded. Although the chemical route for resin formation was not very clear, for their accumulation in the plant body he assumed that cellulose in many cases played an important role. He called "*lysigenous resin formation*" when the cell walls in the plants were dissolved and instead of a cell a cavity was formed which was then filled with resin.

A second type of formation was the development of the resin in the cell and its diffusion through the cell wall. The plants which had this kind of cell formation had usually bigger intercellular gaps where the resin was collected. These gaps were usually small at the beginning but grew with time either equally in all directions or only in one direction which was mostly parallel to the stem to form the "*resin channels*". This kind of formation of resin channels through restructuring of the cell gaps and accumulation of the resin in these gaps was called "*schizogenous resin formation*".

At the end of his survey about the resins from different plant families, *Tschirch* concluded that mostly these two types of resin formation occur together as "*schizo-lysigenous*" in which first the schizogenous formation occurred then the cell wall disappeared with a chemical regeneration,

and the resin channels were enlarged. His terminology in resin chemistry is still in use although some of his hypothesis was proved to be incorrect such as the disappearance of the cell walls. They only became unrecognizable because of the enlarged excrete channels (Basar, 2005).

Such channels are typically observed in many plant families; *Pinaceae*, *Anacardiaceae*, *Hypericaceae*, *Guttiferae*, *Dipterocarpaceae*, *Burseraceae*. These channels are surrounded with epithel, thin walled and very tight placed cells, like the excrete pockets. This kind of cellular organization has a double function for the plant; first it isolates excrete in a separate space and secondly it closes the excrete passage hermetically from the intercellular system of the body. These resin channels are generally connected to each other through horizontal gaps to the interior. By this way, the plant succeeds to impede secretions to flow outside.

It is possible to make different classifications for the resins, for example, according to their appearance or their chemical composition. In many cases three classes of resin types were recognized: "*balsam*", viscous but fluent plant products; "*resin*", when this fluent product becomes solid after a short time; "*gumresin*", contains the resin constituents together with the plant gum.

Chemical classification of resins is based on their constituents. It is common to classify them into three groups: "*terpenresins*", "*benzresins*" and "*gumresins.*" *Terpenresins* contain mainly diterpenoic and triterpenoic acids or triterpenoic alcohols. Colophonium, dammar, mastics belong to this class of resins. On the other hand, *benzresins* are composed of mostly phenylpropane derivatives. Besides cinnamic acid and coniferylalcohol they contain lignanes, xanthone and highly condensed coumarines. In most cases they are found in their esterified forms. Peru balsam and guajak resin can be considered as examples of this class (Basar, 2005).

Gumresins flow as a yellow or white latex from the incisions of the plant and hardens there into amorphous, tear shaped products with an aromatic scent. They are composed of 30-60 % resin, 5-10 % essential oil, which is soluble in organic solvents, and the rest is made up of polysaccharides, which are soluble in water. The darkening of the color is a result of autoxidation, polymerization and enzymatic reactions. The most well-known examples are myrrh and olibanum.

Still, the significance of the resin for a plant is not precisely known. But in contrast to *Tschirch* who believed that they do only serve for the healing of wounded parts in the plant organism since some of them exhibit no obvious physiological activities, they, nevertheless, have been used in folk medicine since very old times, as anti-inflammatory and antiseptic materials or to cover wounds like the plasters of today. In daily rituals and religious ceremonies, resins have been burned for their aromatic scents. Olibanum and myrrh are the oldest scents mentioned in history since 5000 B.C. Today the utilisation of resins is still common in pharmaceutical, perfume and cosmetic, aroma, laquer and varnish industries in addition to their cultural and folkloric uses (Hanus *et al*, 2005).

2.2.3 The Chemical History of Olibanum

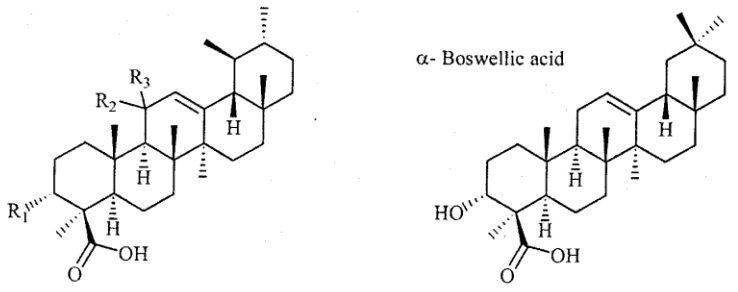
Although the oil of olibanum had occupied the shelves of the 16th century pharmacies as "*oleum thuris*", the first investigation on its chemical composition was performed in 1788 at the University of Erlangen. Following this work, the first elementary analysis was carried out in 1839. The constituents of the essential oil were first investigated in 1840, and identifications depending on the origin of the resin of fourteen monoterpenoic constituents including pinene,

dipentene, phellandrene and cadinene were made. In 1898 a publication for the first time was made that olibanum had an acidic constituent called, boswellic acid, with a molecular formula of $C_{32}H_{52}O_4$ but the actual structure could not be suggested at that time (Basar, 2005).

At the beginning of the 1930's, the olibanum resin was investigated in more detail. The study in 1932 drew the attention to the resin acids, the pentacyclic triterpenoic α- and β-amyrin like skeletons with different functional groups, which were attempted to be isolated and identified with the analytical methods possible for that time. Nevertheless, by the 1960´s several of these acids

such as α- and β-boswellic acids, 11α- hydroxy-β-boswellic acid(**LXIII**) and 3-*O*-acetyl-11-hydroxy-β-boswellic acid(**LXIV**) were identified by various derivatisation methods (Basar, 2005).

Fig. 2.3: some of the triterpenoic acids that were identified from olibanum.



In 1967, the structures of acetyl-11-keto-β-boswellic acid(**LXVIII)** as well as *epi*-α- and *epi*-β-amyrin and their acetates, α- and β-amyrenone and viridiflorol(**LXV**) were elucidated from the neutral fraction of olibanum, adding that it is composed of 5-9 % essential oil, 15-16 % resin acids, 25-30 % of material insoluble in ether containing the polysaccharides and 45-55 % ether soluble compounds (Basar, 2005).

In 1978 identification was made of tirucallic acids as well as β-boswellic acid(**LXVI**), acetyl-β-boswellic acid(**LXVII**), 11-keto-β-boswellic acid(**LXVIII**), acetyl-11-keto-β-boswellic acid(**LXIX**) from *B. serrata* Roxb (Pardhy and Bhattacharyya, 1978) and a diterpenoic cembrene(**XXV**) derived alcohol, "serratol". Studies on the isolation and identification of the boswellic acids with modern analytical techniques and on their pharmacological effects are still going on.

The first important and comparative study on the essential oil of olibanum of different origins was performed in 1977. It was carried out on two different commercial brands of olibanum, "*Eritrea*" and "*Aden*" by GC-MS, which corresponded to *B. carterii* and *B. serrata* resins, respectively. As a result of this investigation it was reported that not only the fragrance of these two qualities but also the composition of the constituents in the oils were different. (Basar, 2005)

The "*Eritrea*" oil was reported to have octylacetate as the major constituent (52%) as well as α-pinene(**LXX**), camphene(**LXXI**), p-methoxytoluo(**LXXII**), hexyl acetate(**LXXIII**), limonene(**XIX**), 1,8-cineole(**LXXIV**), octanol, linalool(**LXXV**), bornyl acetate(**LXXVI**), cembrene A(**LXXVII**), incensole, incensyl acetate(**LXXIX**) and an unknown diterpenoic constituent. In contrast, "*Aden*" oil was found to contain α-pinene(**LXX**) as the major constituent

(43%), camphene(71), β-pinene(**LXXIX**), sabinene**(LXXX**), o-cymol, limonene(**XIX**), 1,8-cineole(**LXXIV**), p-cymol, campholenaldehyde(**LXXXI**), verbenone, octyl acetate(**LXXXII**) and cembrenol(**LXXXIII**), a diterpene alcohol with cembrene skeleton which was identified later by the same group, which was expected not be different than "serratol" described before.

In 1985 a detailed review was published on the "*Aden*" brand of olibanum. It cited 47 new constituents identified in the resinoid and in the oil of olibanum in addition to 169 formerly identified substances including the pyrolysis products. Recent studies on *B. serrata* oil and by Humprey *et al* (1993) comparing *B. carterii* oil with cumin, ginger, rosemary oil, were reinvestigations of known facts. These studies pointed to the difficulties in the identification of the origin of olibanum resin as well as in the determination of standard olibanum oil. A complete list of major constituents of olibanum resin hitherto described are as given in **Fig 2.4,2.4 and 2.6.**

Basar (2005), carried out a number of phytochemical investigations on *Boswellia* species. In the report, the essential oils, acid fractions, and pyrolysates of the olibanum resins from *Boswellia carterii*, *Boswellia serrata*, *Boswellia frereana*, *Boswellia neglecta* and *Boswellia rivae* were investigated. In addition antibacterial and antioxidative activities of the samples were tested. During this investigation, verticilla-4(20),7,11-triene(**XI**) and a dimer of α-phellandrene(**XII**) were isolated and identified for the first time from natural source from *B. carterii* and *B frereana* respectively. In addition 5,5-dimethyl-1-vinylbicyclo-(2,1,1) hexane(**XIII**), m-camphorene(**XIV**) and p-camphorene(**XV**) were isolated from *B. serrata*. Moreover a nortriterpenoic constituent, 24-norursa-3,12-diene(**XVI**) was isolated for the first time from natural sources from *B. carterii* and *B. serrata*.

Fig. 2.4: Some of the monoterpenoic constituents identified in olibanum resin.

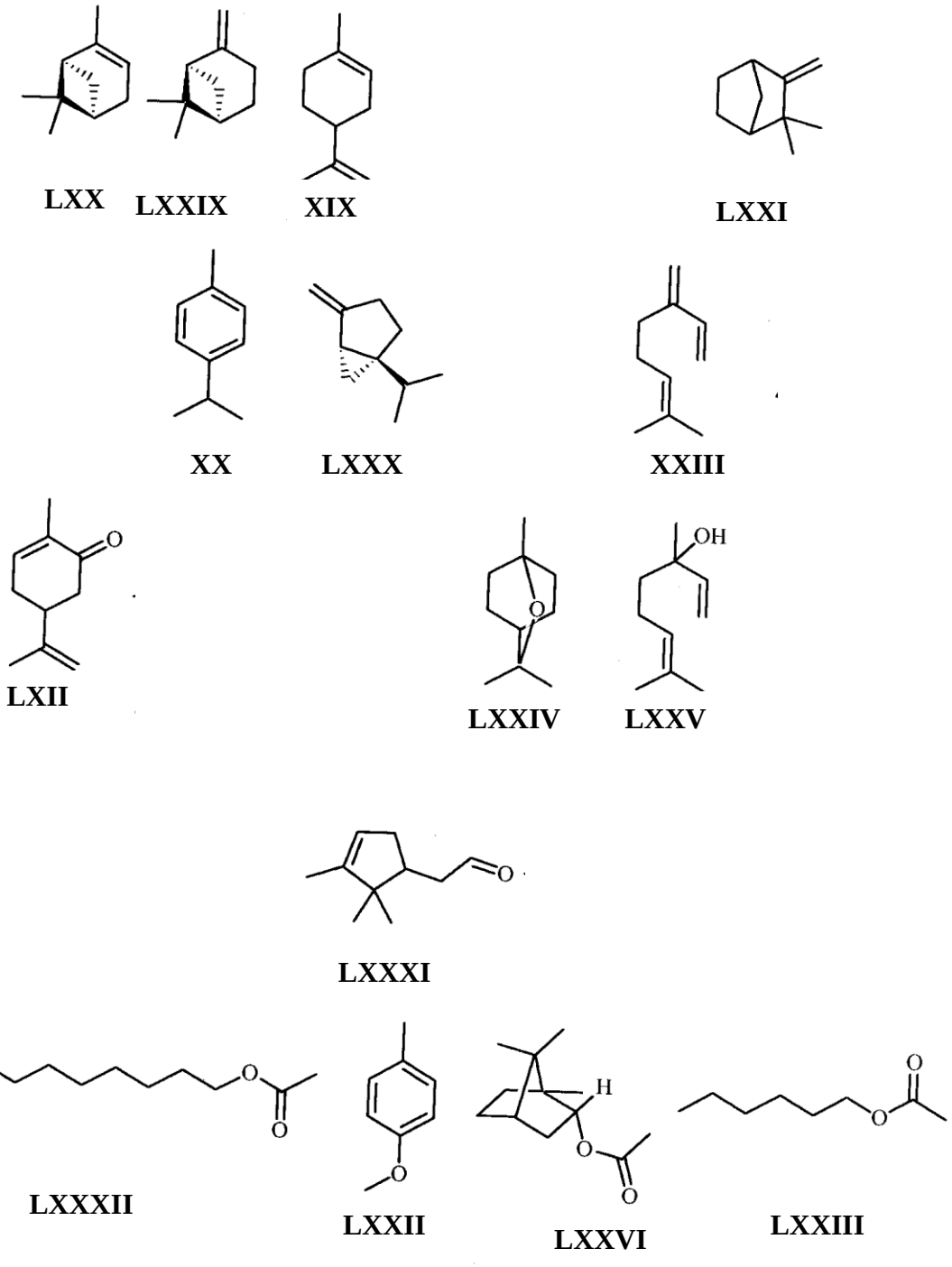
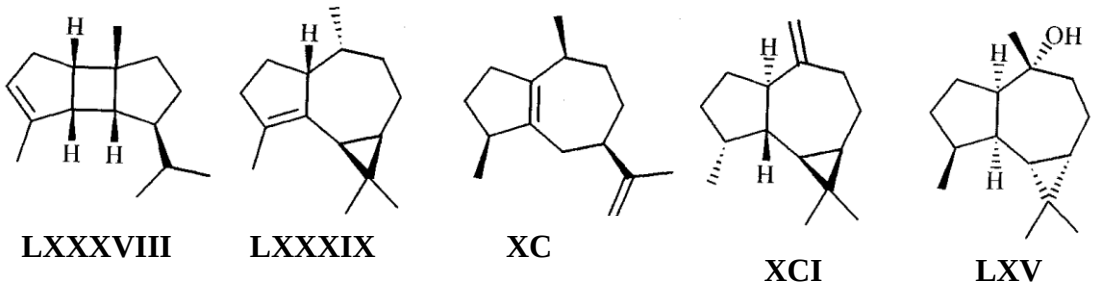
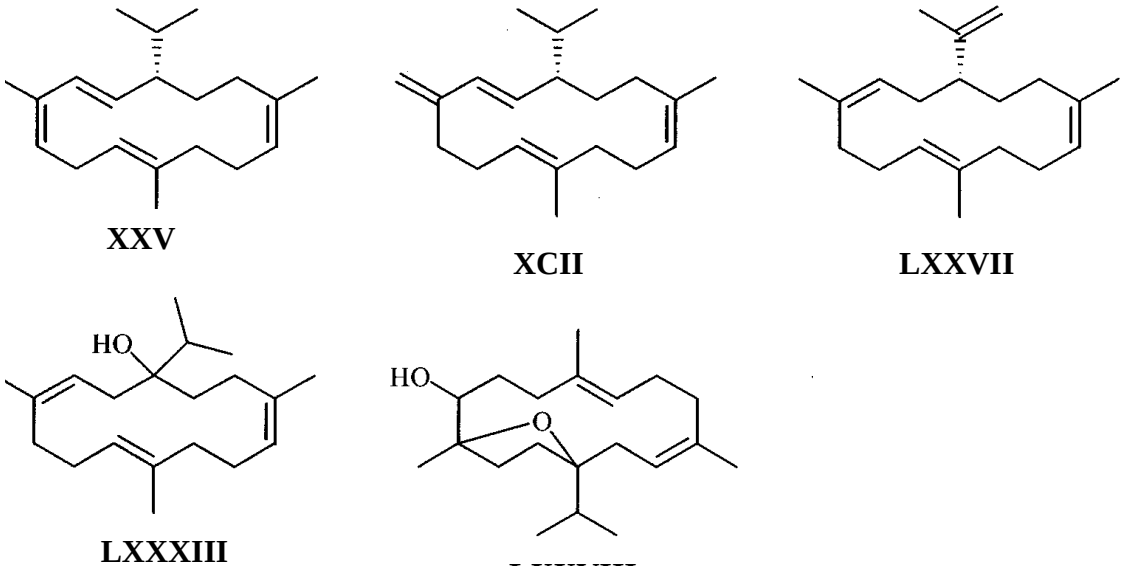


Fig. 2.5: Some of the sesquiterpenoic constituents identified in olibanum resin.



LXXXVIII **LXXXIX** **XC** **XCI** **LXV**

Fig. 2.6: Some of the diterpenoic constituents identified in olibanum resin.

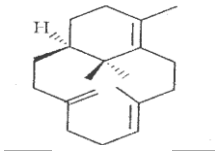


XXV **XCII** **LXXVII**

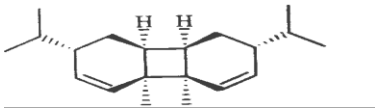
LXXXIII **LXXVIII**

2.3 Chemical Constituents of *Commiphora* Species

Myrrh and similar but lower quality opopanax are hardened resinous exudates obtained from trees of certain *Commiphora* species of the *Burseraceae* family. Tree resins are composed of terpene and terpenoid compounds, diterpenoids and triterperoids are the most common. Diterpenoids and triterpenoids have not been found together in a resin, thus resins from each group have different properties (Hanus, 2005).



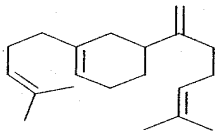
XI



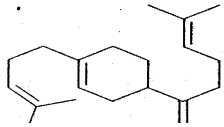
XII



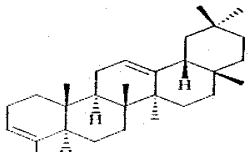
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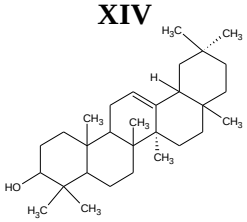
XIV



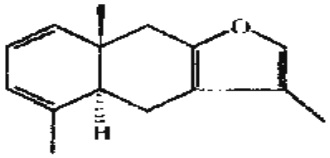
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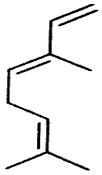
XVI



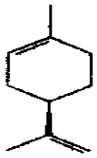
XXXVIII



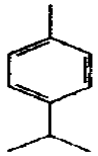
XVII



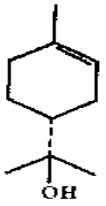
XVIII



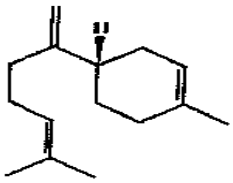
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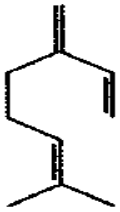
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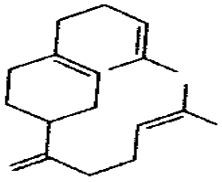
XXI



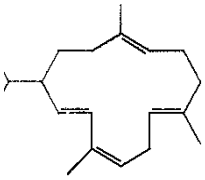
XXII



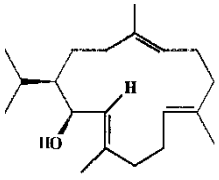
XXIII



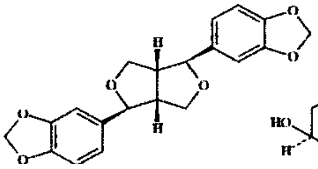
XXIV



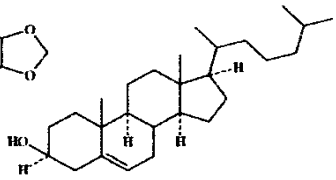
XXV



XXVI



XXVII

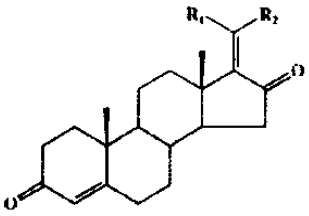


XXVIII

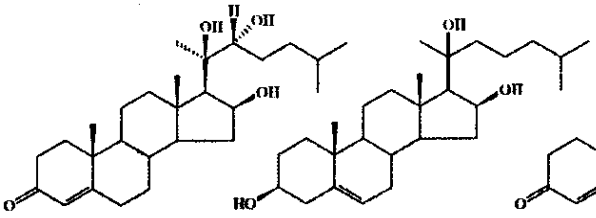
The constituents of essential oil in myrrh and gum opopanax were analysed by GC-MS. Fifteen compounds in Myrrh and 33 compounds in gum opopanax were identified and their percent content was detected. The main constituents myrrh was furanoeudesma-1,3 diene (**XVII** and the main constituent of gum opopanax was *trans*-β-ocimene (**XVIII**).

Demissew (1993) also reported the chemical investigations carried out on some *Commiphora* species from Kenya and Ethiopia. The main volatile components include monoterpenes (limonene **XIX**, p-cymene **XX**, α-teripineol **XXI** and a sesquiterpene; β-bisabolene **XXII**).

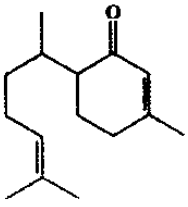
With the help of column chromatography of petroleum ether extract of *Commiphora mukul* (Hooks, ex stocks) Engl some crystalline needles were isolated. An isolated compound (steroid) showed significant anti-inflammatory activity on rat paw edema produced by carrageenin. The activity is dose dependent and much more potent than the resin fraction present in *C. mukul* (Arora *et al*, 1971).



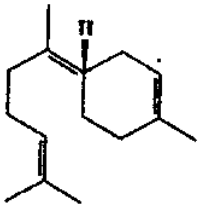
XXIX R_1-H,R_2-CH_3
XXX, R_1-CH_3,R_2-H



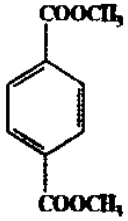
XXXI **XXXII** **XXXIII**



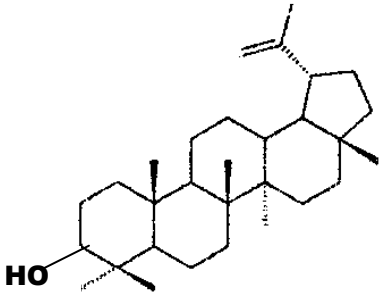
XXXIV



XXXV



XXXVI



XXXVIII

The compound was reported to be three times more potent than the resin fraction isolated from the same drug in inhibiting carrageenin in edema in rats. It looks like this drug is likely to be therapeutically effective as anti-inflammatory or anti-rheumatic drug (Rucker, 1972). Furthermore, myrcene (**XXIII**), the monocyclic diterprenoids α-camphorene (**XXIV**) and cembrene (**XXV**) were isolated from the gum resin of *Commiphora mukul* (Indian gum gugul). For the diterpene alcohol allycembrol (**XXVI**), isolated from the overgrown parts of *C. mukul*, the structure 2-hydroxy-4,8,12 trimethyl-1-isopropyl-3,7,11–cyclodecatriene was proposed. The allylcembrol structure was established by mild dehydration which yielded cembrene and by spectral analysis. Allylcembrol(**XXVI**) and its C2 epimer were synthesized from isocembrol (Raldugin *et al*, 1976).

Guggulu (Sanskrit) is the gum resin exudates from the tree *Commiphora mukul* and is an article of commerce in India. Chromatography of petroleum ether soluble fraction gave a diterpene hydrocarbon ($C_{20}H_{32}$), a diterpene alcohol ($C_{20}H_{34}O$), (+)–sesamin(**XXVII**) cholesterol (**XXVIII**) and two other new isomeric $C_{21}H_{28}02$ steroids which were identified as 4,17(20)-(*trans*)–pregnadiene-3,16-dione(**XXIX**) (guggulsterone, Z-isomer) and 4,17 (20)-(*cis*)-pregnadiene-3,16-dione (**XXX**) (gugulsterons, E-isomer). These two steroids were assigned trivial names, Z-and E-guggulsterone respectively. The ethyl acetate fraction gave additional three new sterols and a long-chain aliphatic triol. The three new sterols have been designated guggulsterol–I(**XXXI**), guggulsterol–II(**XXXII**) and guggulsterol-III (**XXXIII**) (Patil *et al*, 1972).

Moreover, the leaf oil of *Commiphora africana* obtained by hydrodistillation, has been analysed by GC, GC/MS coupling and ^{13}C –NMR spectroscopy. The two major compounds identified in

the oil were α - oxobisabolene(**XXXIV**) (61.6%) and ϒ-bisabolene (**XXXV**) (10.0%) (Ayedoun *et al*, 1998). The methanolic extract of the bark of the plant *Commiphora africana* gave a homogenous product through chromatographic separation which after crystallization from methanol furnished needles characterized as dimethylterephthalene (benzene-1-dicarboxylic acid dimethylester) (**XXXVI**) on the basis of spectral analysis (Choudhury *et al*, 2000).

Extraction of the stem bark of *Commiphora dalzielli* (Hutch), (a shrub or small tree indigenous to Ghana) with petroleum ether gave seven dammarane triterpenes. Two compounds were identified as common lupeol(**XXXVII**) and β-amyrin (**XXXVIII**). Five others were separated by column chromatography with silica gel and circular preparative thin-layer chromatography. After purification, a number of new compounds were discovered (Waterman and Ampoto, 1985).

Similarly, in the essential oil of *Commiphora quadricincta*, a large number of compounds were identified by GC-MS. A significant number of the compounds identified were terpenoids (Assad *et al*,1997).

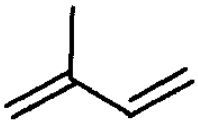
2.3.2 Isoprenoids: Terpenes, Terpenoids and Steroids

Isoprenoids are universal metabolites present in all living organisms. They include essential metabolites such as sterols, acting as membrane stabilizers in eukaryotes or as precursors for steroid hormones; the acyclic polyprenols, found in the side chain of the prenylquinones or in phytol from chlorophylls, which, via their phosphodiesters, act as sugar carriers for polysaccharide biosynthesis; the carotenoids in photosynthesizing organisms as well as a large variety of compounds with a less evident physiological role (Conolly and Hill, 1992).The term "terpenoid" is preferred for reference to all compounds built of isoprene (**XXXIX**) units, regardless of the functional groups present, while "terpene" refers specifically to hydrocarbons.

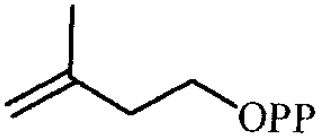
Terpenes, or terpenoids, are represented with more than 8000 compounds in this group of secondary metabolites comprising more than 17,000 structures. They show extraordinarily diverse structures and exhibit a large variety of physical and physiological properties. Their number increases every year with the addition of new structures, most of which have different biological effects (Basar, 2005).

In plants, the production of terpenoids is much more common than in animals or microorganisms. The production of large quantities of these natural products, their accumulation in cells and other storage compartments, their secretion in the plant body or emission from the plant to its surrounding is a challenging subject for biochemists, phytochemists and biologists in terms of finding out not only the biosynthetic pathway of these compounds in such specialized organisms, but also identifying their physiological properties in the association with other living organisms.

All isoprenoids, consequently terpenoids, are synthesized from one precursor "*isopentenyl diphosphate*", IDP,(**X**), which is the biological equivalent of isoprene (2-methyl-1,3-butadiene). It was formulated hypothetically as the "*isoprene rule*" first by *Otto Wallach* in 1887 that, terpenoids are the sequential combinations of isoprene (C5) units. The proposal of the "*biogenetic isoprene Rule*" emphasized that this biosynthetical route has to be accomplished through a sequence of oligomerization reactions along with methylation, hydroxylation, oxidation, decarboxylation and with other cyclization and rearrangement reactions, which provide an enormous structural diversity. This hypothesis worked empirically for terpenoid biosynthesis without considering the involvement of the other possible precursors, assuming that isoprene is the only and the key precursor for this biological route (Basar, 2005).



XXXIX

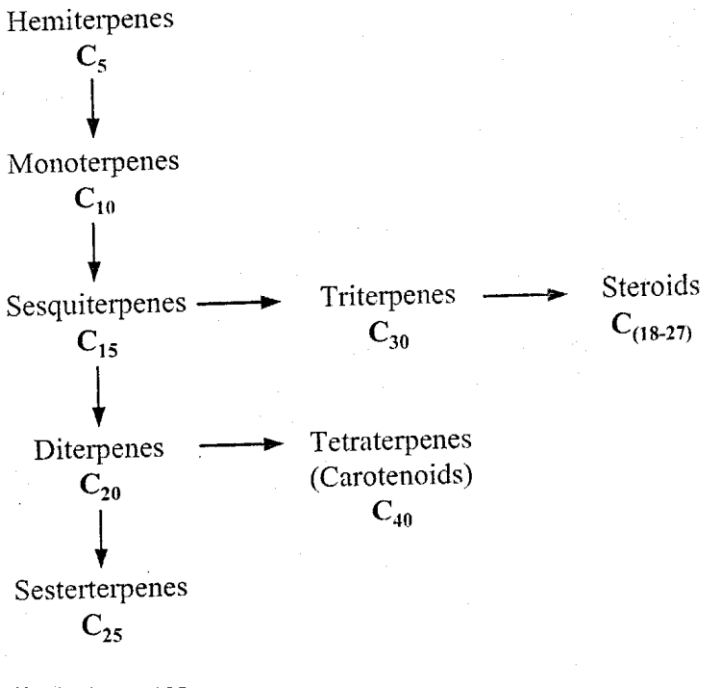


X

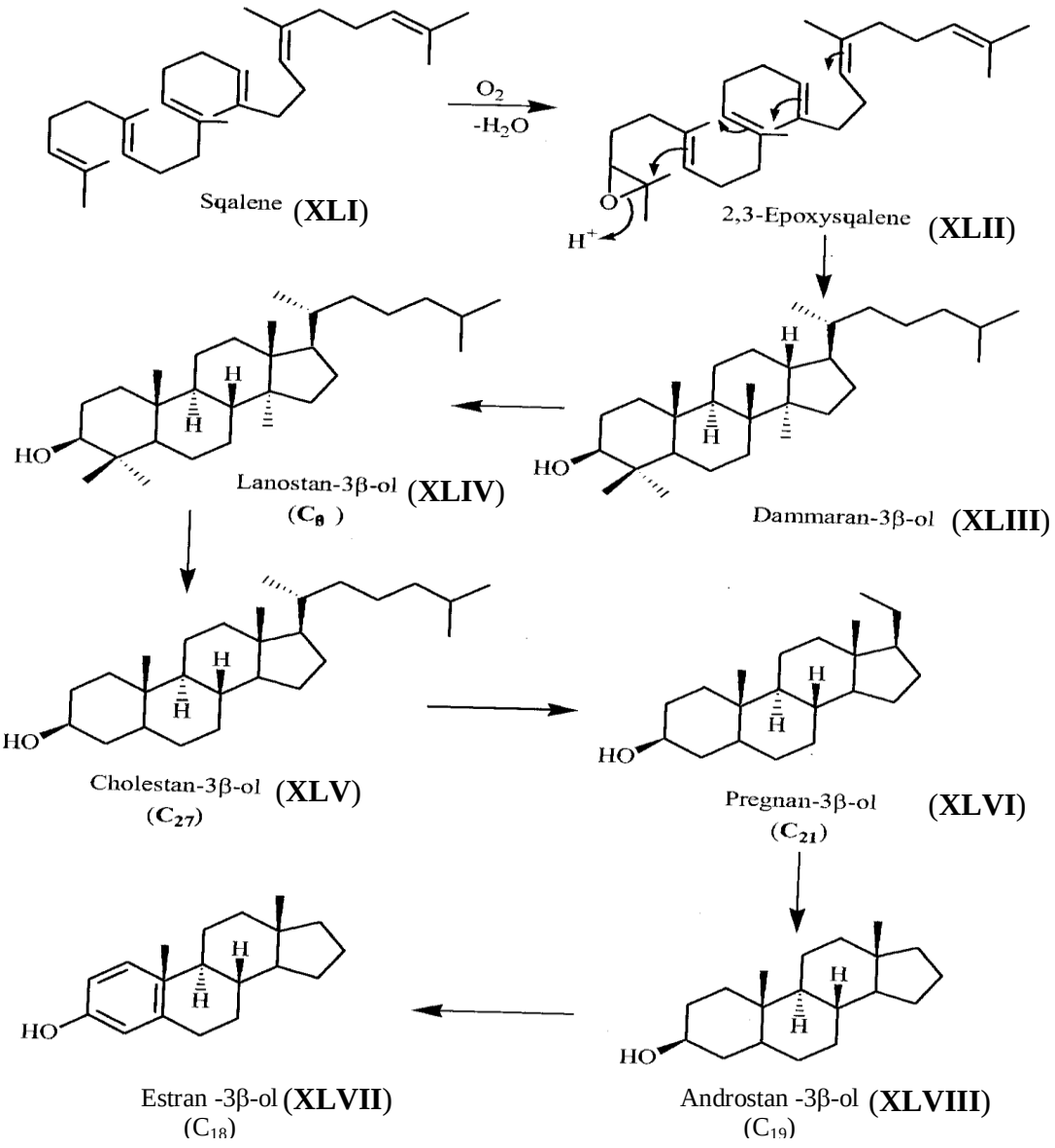
An isoprene unit and its biological equivalent, isopentenyl diphosphate.

The classification of terpenoids depends on the number of isoprene units present in the skeleton of the product. Compounds, which are composed of one isoprene unit, are called *hemiterpenes* (C5), in the case of dimerization of two isoprene units they are called *monoterpenes* (C10). *Sesquiterpenes* (C15), *diterpenes* (C20), *sesterterpenes* (C25), *triterpenes* (C30) and *tetraterpenes* (C40) follow this sequence, each having an isoprene unit more than the preceeding one. However, the biosynthetic routes to these terpenes do show some differences. The polymerization of an indefinite number of isoprene units results in *polyisoprenes* or *polyprenes* as in the case of natural rubber (Thiele and Verleg, 1979).

Steroids (C18-27) belong to the group of isoprenoids as they originate from triterpenes. Labeling experiments showed that the formation of tetracyclic triterpenoids from 2,3- epoxysqualene is an introductory step in the formation of steroids. The loss of carbon atoms and rearrangement reactions during their biosynthesis from triterpenes makes the isoprene units of steroids unrecognizable. Therefore, they are classified separately from terpenes (Thiele and Verleg, 1979).



Steroids occur throughout the plant kingdom as free sterols and their estes in many lipids and as more complex derivatives. In plants they have no known function although they have profound importance in animal metabolism as hormones, coenzymes, bile acids and pro-vitamin D. Certain animal steroids have been, shown to influence plant growth strongly, but whether presently unknown plant steroids may act similarly is an open question. The steroid alkaloid tomatine may be somehow related to flowering since its concentration may be photoperiedically controlled in the same way as the flowering responses. Some mammalian estrogens stimulate flowering of *Lemna minor* (Czygan, 1962) and inhibitors of steroid synthesis inhibit flowering of *Pharbitis nil* (Sachs, 1966).



The biosynthesis of some steroids from 2,3–epoxysqualene

Such observations hint a hormone role for steroids in plants. Typical "animal" steroids such as cholesterol and estrons have been found in plants, but if they are wide spread, it is only at very low concentrations.

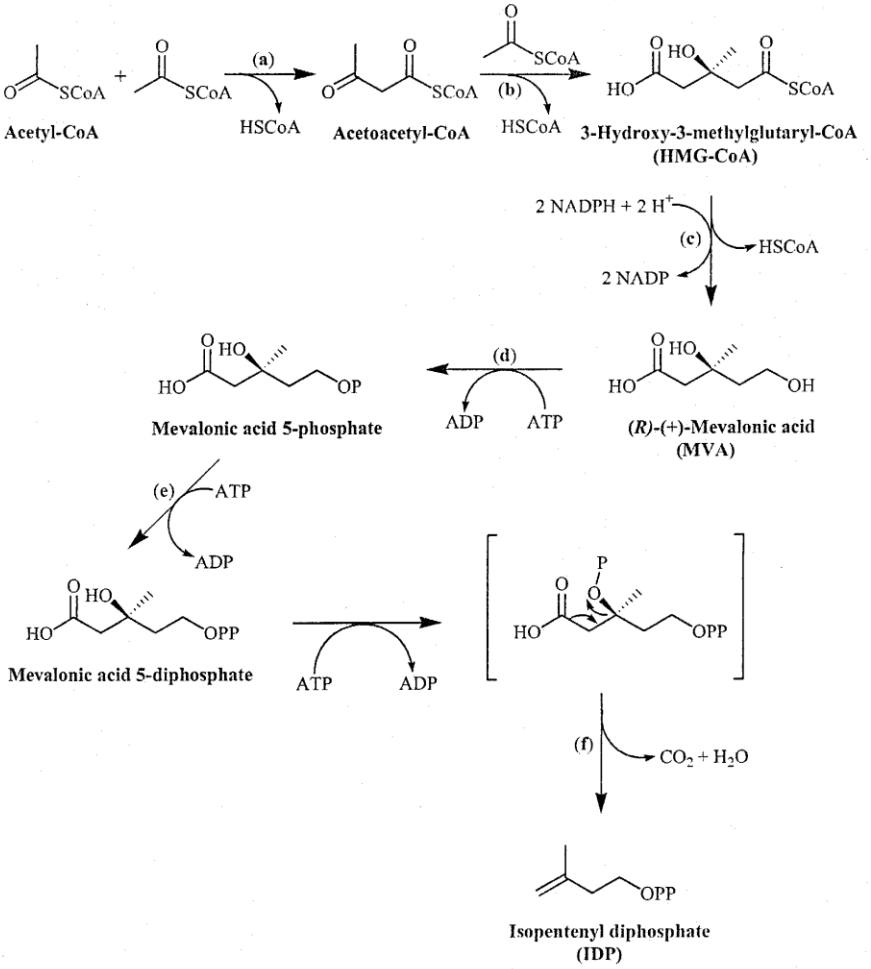
2.3.2 Biosynthesis of Isoprenoids

Isopentenyl diphosphate, IDP,(**X**) has proved to be the building block of isoprenoid biosynthesis. Until today several individual metabolic pathways were proposed for its biosynthesis. Except the classical "*acetate-mevalonate pathway*" and the recently proposed "*mevalonateindependent pathway*"-in other words "*triose-pyruvate pathway*" or "*deoxyxylulose phosphate pathway*"-there are also studies that concentrate on the incorporation of amino acids in the isoprenoid biosynthesis especially where leucine degradation in higher plants took place through a "*mevalonic acid shunt pathway*". However, this pathway has not been accepted completely as the former ones, because of the contradictory results on the formation of intermediates making use of free acids rather than coenzyme esters, as well as the insufficient information on the intermediates, their regulation and their metabolic fate in plants (Batch, 1995).

2.3.3 Biosynthesis of Isopentenyl diphosphate via Acetate-Mevalonate Pathway

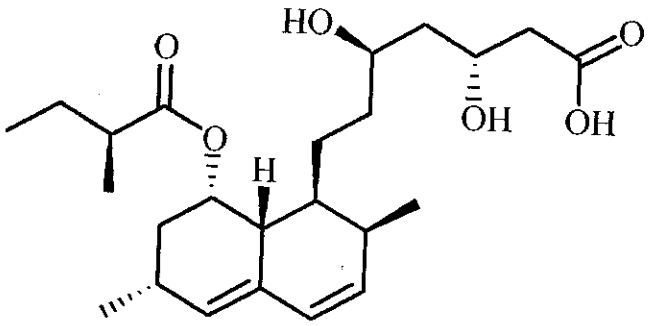
Since the 1950's feeding experiments with isotopically labeled precursors were performed in the field of isoprenoid biosynthesis. Mevalonic acid was first recognized as a precursor of cholesterol (Tavormina *et al* , 1956 and Cornforth *et al, 1966)*. Following research proved that it serves as an effective precursor of the acyclic isoprenoids squalene and rubber as well as carotenoids. As a result of these studies on the incorporation of mevalonic acid into isoprenoids, the acetate-mevalonate pathway was established. The initial step in the acetate-mevalonate pathway is the reaction of two acetylcoenzyme A molecules via Claisen condensation by acetyl-coenzyme A acetyltransferase (**a**). The resultant acetoacetylcoenzyme A reacts with a third molecule of acetylcoenzyme A. This Aldol addition is catalyzed by 3-hydroxymethylglutarylsynthase (**b**) and (*S*)-3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is formed which is reduced to *(R)*-mevalonic acid in the next step by HMG-CoA reductase28 (**c**) with the use of (NADPH + H^+).

The stepwise phosphorylation of mevalonic acid by mevalonate kinase (**d**) and consequently phosphomevalonate kinase (**e**) utilizing adenosine triphosphate (ATP) converts it into mevalonic acid 5-diphosphate(Bloch, *et al,1959)*. The subsequent phosphorylation-assisted decarboxylation by mevalonate 5-diphosphate decarboxylase (**f**) yields isopentinyl diphosphate (IDP) (Dewick, 2002).



Biosynthesis of isopentenyl diphosphate via acetate-mevalonate pathway. The enzymes involved in this pathway are: a: Acetyl-CoA acetyltransferase; b: 3-Hydroxymethylglutaryl synthase; c: 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase; d: Mevalonate kinase; e: Phosphomevalonate kinase; f: Mevalonate 5-diphosphate decarboxylase.

The acetate-mevalonate pathway has been accepted as the universal biosynthetic pathway until the end of 1980's. Nevertheless, some contradictory results have been observed. Isotopically labeled mevalonic acid and acetate were usually not, or very poorly, incorporated into monoterpenes, diterpenes and carotenoids in higher plants although they incorporated into sterols, triterpenoids and sesquiterpenes quite efficiently. In addition to this, it is proved that mevinolin(**XLIX**) which is an inhibitor of the HMG-CoA reductase, strongly inhibited the sterol synthesis in plants, but did not affect the production of carotenoids and chlorophyll, containing the diterpenic phytyl side-chain. These results were interpreted as the impermeability of the chloroplast membrane towards the precursor or the inhibitor. However, the role of IDP was obviously accepted as isoprenoid precursor in chloroplasts via the mevalonate-dependent pathway although the possibility of the existence of another route could not be ruled out.

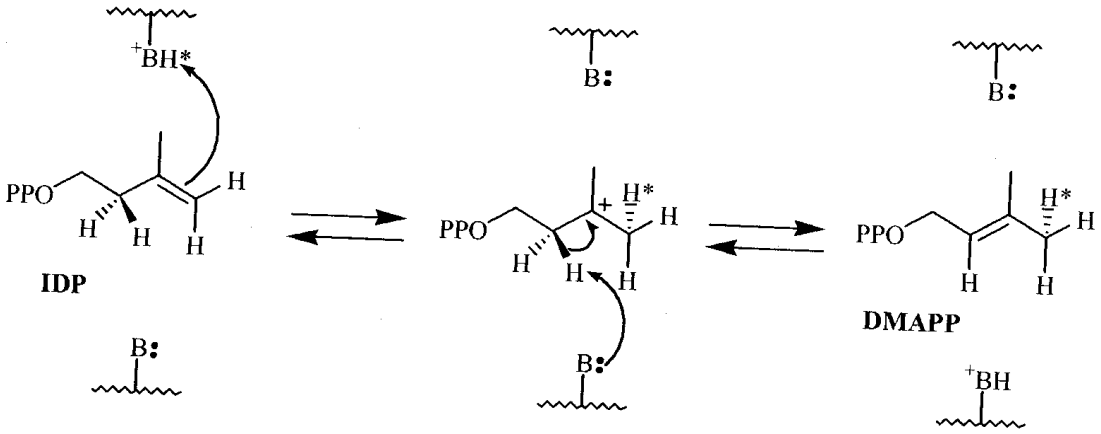


XLIX

2.3.4 Biosynthesis of Isoprenoids from Isopentenyl diphosphate

After the biosynthesis of isopentenyl diphosphate (IDP), the next step in the biosynthesis of isoprenoids is its isomerisation into dimethylallyl diphosphate (DMAPP). The isomerisation of

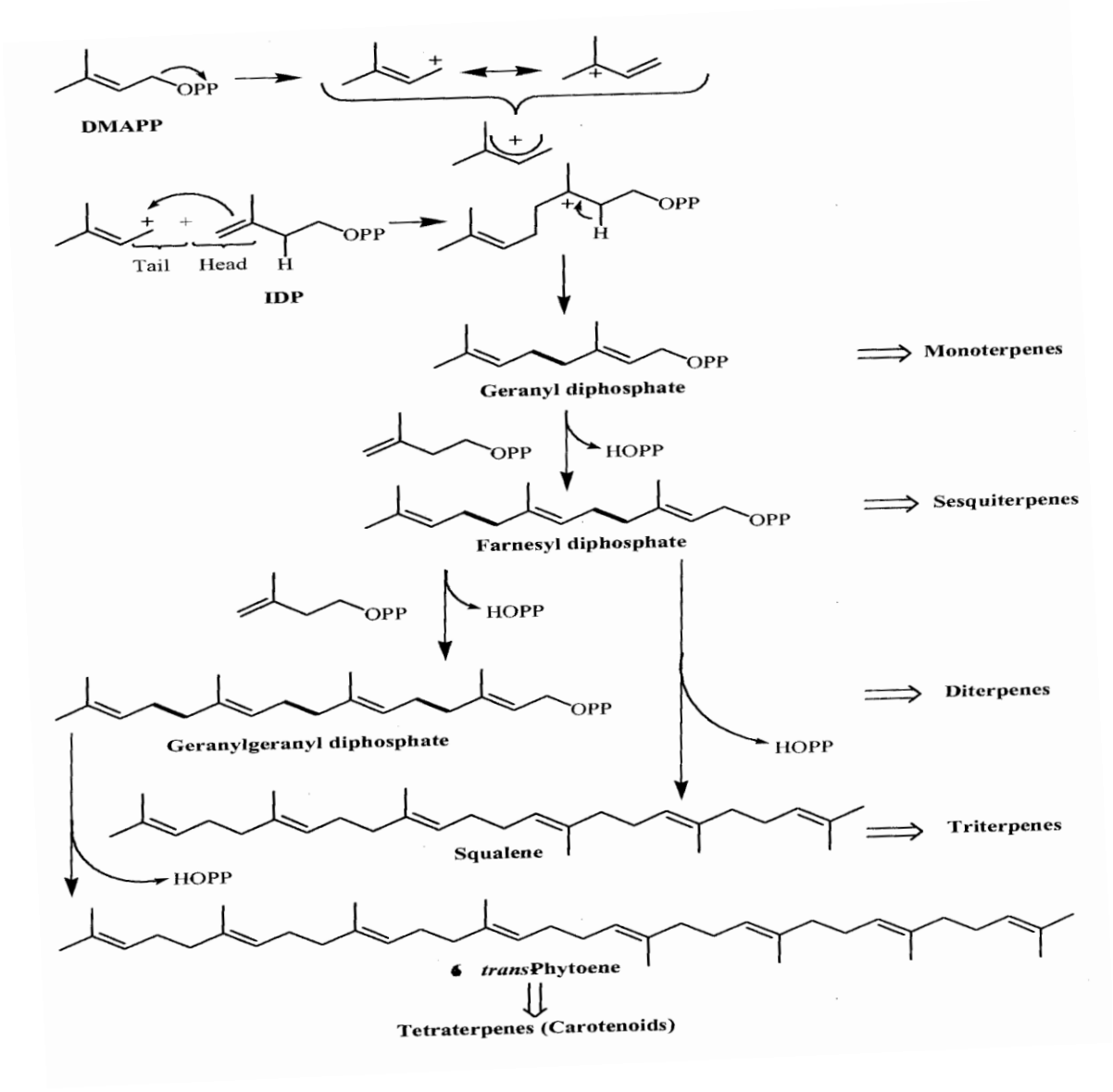
IDP into DMAPP is postulated as a 1,3-allylic rearrangement reaction via a two-base cationic mechanism. This interconversion is catalysed by isopentenyl diphosphate isomerase (IDP isomerase) (Dewick,2002).



Isomerisation of IDP into DMAPP

The isomerisation of IDP to DMAPP is followed by the condensation of IDP and DMAPP or other prenyl diphosphates to give higher homologues which are the precursors of all isoprenoids. The reaction sequence is catalyzed by prenyltransferases. It is assumed that the first step in this sequence is the formation of the allylic cation which will condense with a second molecule of IDP to allow the formation of geranyl diphosphate or the monoterpenes in general. The condensation occurs in a "*head-to-tail*" fashion where the nucleophilic site of IDP is named "head" and the electrophilic site "tail". Analogous condensation reactions may proceed either by addition of IDP in each step or the condensation of the synthesized prenyl diphosphates in "*tail-to-tail*" fashion, to biosynthesize the precursor molecules of other terpene classes; i.e., the addition of an IDP to geranyl diphosphate yields farnesyl diphosphate, which will form geranylgeranyl diphosphate after the addition of another IDP molecule. The latter two diphosphates are the starting substances in the biosynthesis of sesquiterpenes and diterpenes, respectively. The condensation of two farnesyl diphosphate molecules produces squalene for

further biosynthesis of triterpenes. Similarly, the condensation of two geranylgeranyl diphosphates ends up with the formation of 16-*trans*-phytoene the precursor of the biosynthesis of tetraterpenes, the carotenoids, (Basar, 2005).



Schematic expression of the biosynthesis of different terpene classes

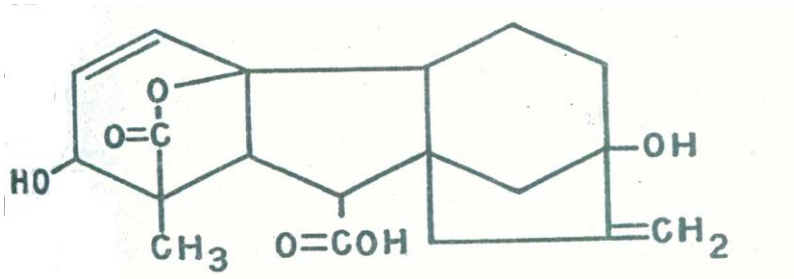
2.3.5 Biological Significance of Terpenoids and Other Secondary Metabolites

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 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 (Harborne, 1993).

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. Many of the cycle diterpenoids may be regarded as derived from phytol by ring closures, but others do not show the head-to-tail type of linkage. This group of diterpenoids includes some compounds of considerable physiological interest such as the group of plant hormones known as gibberellins. The best known of these is gibberellic acid (**LXXXVII**).



(LXXXVII)

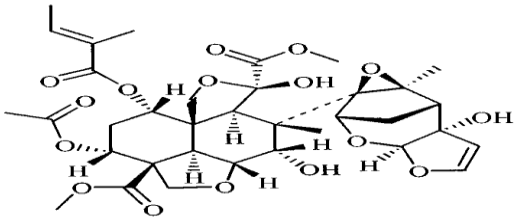
Higher plants apparently have nine or more gibberellins with somewhat similar structures. They act to stimulate *dl novo* synthesis of certain enzymes. Gossypol of cotton and gummiferin of *Atraclylis gummifera* are powerful inhibitors of oxidative phosphorylation *in vitro* and may function similarly *in vivo* (Myers and Throneberry, 1966). The insecticide ryanodine of *Ryania speciosa* is also a diterpenoid as are the toxins of *Ericaceae* species.

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.

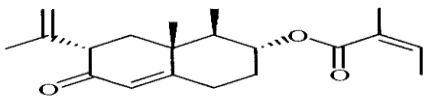
It should however be noted that most investigators are of the opinion that the function of most lower terpenoids in plants may be described as ecological rather than physiological. Many of them inhibit the growth of competing plants and may also be insecticidal. The possible ecological significance of terpenoids (and other secondary plant constituents) is well demonstrated (Frenkel, 1959). The great specificity possible in this area is illustrated by the

finding that the Douglas fir beetle *(Dendroctonus pseudotsugae)* is attracted to α - pinene but repelled by β-pinene. Both are present in the host tree but vary in relative concentration from tree to tree.

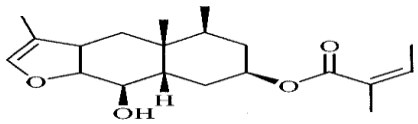
Most sesquiterpene lactones and some diterpenes appear to have repellent effects towards insect herbivores. The triterpenoid azadirachtin(**L**) is a well known natural insecticidal agent. Slugs and snails are also known to feed on a wide variety of plant species. But two sesquiterpenes, petasin(**LI**) and furanopetasin(**LII**) which occur in *Petasites hybridus* repel snails feeding on this plant (Harborne, 2001).



L



LI



LII

It should be noted that the volatile isoprenoids also control insect behaviour and development. The volatile semiochemicals which generally cover isoprenoids in the range C5-C20 , are used by insects for signalling over some distance(Hick *et al*, 1999).

The various biological effects of secondary metabolites, which are recognized through traditional medicine, affect the drug discovery and development nowadays. Many sesquiterpenes showing a variety of pharmacological effects, like cytotoxic, antibiotic, antifungal, antiviral, even sedative effects, (Rucker, 1972) as well as many biologically active diterpenes and triterpenes, are utilized

or modified as precursors of active substances to discover new antimalarial, antiviral, anticancer, and many other drugs.

Physiologically one of the most interesting of the sesquiterpenoids is abscisin II, a hormone which antagonizes the effects of gibberellins. It has been isolated from cotton and found identical to the growth inhibitor, dormin, of dormant sycamore buds (Comforth *et al*, 1965). Some of these cannot be conveniently constructed from isoprene residues. Presumably, rearrangements and oxidation play a part in their formation from isoprenoid precursors.

The most important and widely distributed triterpenoids are the pentacyclic compounds. They have been found in plants as primitive as *Sphagnum* but are most common among seed plants, both free and as glycosides. The nonglycosidic triterpenoids are frequently as excretions and in cuticle where they may have a protective or water proofing function. Some scientists have proposed the interesting generalization that monohydroxy triterpene alcohols are not accompanied by pigments in plants whereas triterpene diols occur along with carotenoids and triterpene acids with flavonoids.

2.3.6 Spectral Properties of Terpenes

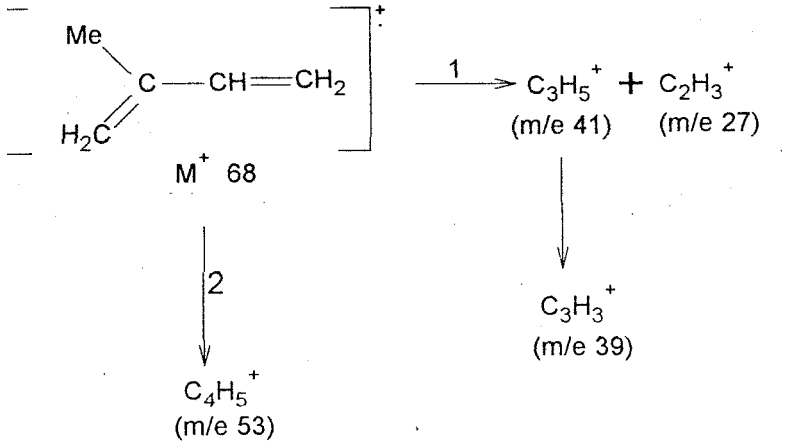
Gas chromatography was the method of choice for characterisation of a terpenoid. It was applied to essential oils, oils of oranges and black currants and has made possible the separation of several dozens of components (Anderson and Von Sydow, 1966). With advancements in scientific research however, more sophisticated spectroscopic techniques are now employed.

Infra red spectroscopy (IR) is valuable in terpenoidal chemistry as it is employed to detect the presence of hydroxyl group (~ 3400cm^{-1}), an oxo group at 1750 – 1700cm^{-1} for saturated and 1700-1660cm^{-1} for the α-β-unsaturated. I.R. spectroscopy is particularly useful for detecting the presence of isopropenyl group in terpenes occurring usually between 895-885cm^{-1}. This is exemplified by myrcene(**XXIII**) with λmax 224nm but no band at 890cm^{-1} because of the absence of isopropenyl group. The λmax at 890cm^{-1}.

Ultraviolet (U.V.) spectroscopy has been employed in the study of terpenes to detect isolated double bonds as well as conjugation. The λ max for simple dienes is 217-228nm but if the diene is homoannular the λ max is 256-265nm but there is a weak band at 315-336nm. The λmax is 230-240mm when the diene is heteroannular. In any case the presence of substituent affect the maximum value for the diene system and the modified wood ward empirical rule is then employed to calculate the λmax.

Proton NMR or more specifically *Nuclear Magnetic Resonance Spectroscopy* has been used to detect and identify double bonds, to determine the nature of the end groups present and the number of rings present. NMR can also ascertain the orientation of methyl group in the molecule. For instance the NMR spectrum of carvone (**LXII**) shows a multiple signal at δ3.25 for proton at C^6 which is characteristic of a β-proton in α, β - unsaturated carbonyl compounds. On the other hand a multiple signal for C-8 methylene group has δ4.78 which falls within the normal range for olefinic protons.

Generally speaking, terpenoids being built by isoprene units give the following peaks 68 ($C_5H_8^+$), 67 ($C5H_7^-$), 53 ($C_4H_5^+$), 41($C_3H_5^+$), 39($C_3H_3^+$), 29($C_2H_5^+$) 27(C_2H_3+). The fragmentation pattern that accounts for some of such reactions is as shown:



2.3.7 Spectral Properties of Steroids

Mass spectrometry. This is the only instrumental technique (of those used for the structural elucidation of an organic compound) that does not employ electromagnetic radiation. It allows us to determine the molecular mass, molecular formula of a compound as well as some of its structural features. Steroids usually give an abundant molecular ion from which it becomes quit easy to determine the molecular formulae. There are four common peaks usually observable for steroids which are $[M-R]^+$, where R is the side chain; $M-[R+42]^+$ where 42 is the molecular mass of C_3H_6; $[M-15]^+$, due to the loss of an angular methyl group and $(M-[R+42+15])^+$. Steroids

usually show the presence of a peak at M/Z $(M-18)^+$ as a result of loss of water and a peak at $[M-(18+15)]^+$ due to the loss of water and angular methyl proton group (Finar, 1991).

The *Ultraviolet/Visible* (UV/Vis) Spectroscopy provides information about organic compounds with conjugated double bonds. The UV absorption spectra of steroids are characteristics of the functional groups present and the observed maxima are in agreement with those calculated using Wood Ward's rule (Finar, 1991). The value of the UV and visible spectra in identifying unknown constituents is obviously related to complexity of the spectrum and to the general position of wavelength maxima. If a substance for instance shows three distinct peaks in the range of 400-500mm region, with little absorption elsewhere, it is almost certainly a carotenoid. Further spectral measurements in two or three other solvents and comparison with literature data might even indicate which particular carotenoid it is (Harbone, 1973).

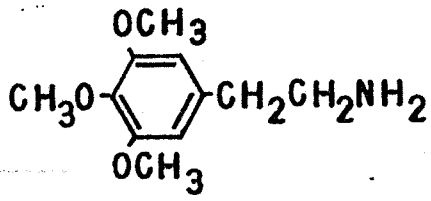
Infrared Spectroscopy (IR) is another valuable instrumentation technique in structural elucidation of unknown steroids. The fact that many functional groups can be identified by their characteristic vibration frequencies makes the IR spectrum the simplest and often the most reliable method of assigning a compound to its class. Inspite of this, IR spectroscopy is most frequently used in phytochemical studies as "fingerprinting" device for comparing a natural with a synthetic sample. IR spectroscopy can also usefully contribute to structural elucidation when new compounds are encountered in plants. Measurements of carbonyl frequencies between 1800 and 1650cm^{-1} in quinones can show at once whether the carbonyl group is chelated with an adjacent hydroxyl or not. With Anthraquinones, for example, non-chelated quinones have a band between 1678 and 1653cm^{-1}, a quinone with one α-OH shows two bands at 1675-1647cm^{-1} and at 1637-1621 cm^{-1}; and a quinone with two α-OH groups has bands at 1675-166 cm^{-1} and at 1645-1608 cm^{-1} (Harbone, 1973). The most important groups identified by IR are C=O, OH, C=C, C=C-C=O, COOH and COOR. For saturated ketones (C=O strs cm^{-1}): 3-CO (5α and 5β);

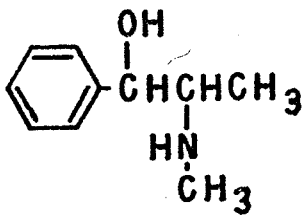
1719-1712; 4-CO (5α):1712; 4-CO (5β) 1713; 6-CO (5α) 1714-1712; 6-CO (5β) 1708-1706 and 11-CO (5β) at 1710-1704cm^{-1}, Devlin and Cooney, 1972).

Nuclear Magnetic Resonance Spectroscopy (NMR) essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. It cannot however give any direct information on the nature of the carbon skeleton of the molecule: this must be obtained in the first instance by application of other spectral techniques. Steroids appear to have a finger print region which is characteristic of the CH and CH_2 protons in the nucleus. Also the proton in the group = CH – has a definite value. These groups can be detected and their number estimated from the area of the proton peak. The protons of the angular methyl groups C_{10} and C_{13} have their own characteristic chemical shifts and the actual shift depends on the presence of various functional groups such as C=C, C=O. One major advantage of NMR spectroscopy over MS spectrometry is that the sample can at least be recovered unchanged after the operation and used for other determinations.

2.3.8 Alkaloids

Alkaloids of which over 5500 are known, comprise the largest single class of secondary plant metabolites. There is hardly any one definition of the term "alkaloid" which is completely satisfactory. Generally they are compounds which include basic organic substances with one or more nitrogen atom, usually in combination as part of a cyclic system (Harbone, 1973). As the name indicates, they are generally basic and contain nitrogen, frequently in a heterocyclic ring, such as mescaline (**XCIII**) and ephedrine (**XCIV**).





XCIII **XCIV**

Many alkaloids are terpenoidal in nature and some (e.g. solanine, the steroidal alkaloid of the potato, *Salanum tuberosum*) are best considered from the biosynthetic point of view as modified terpenoids. Others are mainly aromatic compounds e.g. colchicine the tropolone alkaloid (Harbone, 1973).

The functions of alkaloids in plants are still largely obscure, although individual alkaloids have been reported to be involved as growth regulators or as insect repellents or attractants. In man however, quinine, cocaine and strychnine which are sourced from *Cinchoma* species, *Coca* species and *Strychnos nux-vomica* are known with the physiological action of cardiac depressants, local anesthetics and nervous stimulants respectively.

The functions of alkaloids in plants include:

i. as nitrogen waste products like urea and uric acid

ii. some alkaloids may serve as nitrogen storage reservoirs, although many seem to accumulate and are not further metabolized even in severe nitrogen starvation

iii. in some cases alkaloids may protect the plant against attack by parasite or harbivoros.

iv. alkaloids may serve as growth regulators since structures of some of them resemble known of both regulators and that

v. alkaloids being mostly basic, might serve in the plant to replace mineral bases in maintaining some balance.

2.3.9 Carbohydrates

These are the most abundant class of compounds, in the biological world, making up more than 50% of the dry weight of the Earth's biomass. Carbohydrates are important constituents of all living organisms and have a variety of different functions. Carbohydrates can be defined as polyhydroxy aldehydes, polyhydroxy ketones and compounds formed by linking polyhydroxy aldehydes and ketones together. Some are important structural components of cells, some act as recognition sites on cells while others serve as major sources of metabolic energy (leaves, fruits, seeds, stems and roots of plants contain carbohydrates that plants use for their own metabolic needs but that can also serve the metabolic needs of the animals that eat the plants).

As early products of photosynthesis, carbohydrates are key compounds in the biochemistry of green plants. Ultimately, all other constituents can be derived from them. Aside from this role as precursors, the different varieties of carbohydrates themselves serve quite different functions. Starch and the simple sugars are generally involved with the storage and utilization of the energy required for the process of growth, ion transport, water uptake etc. As cellulose and the hemicellulose carbohydrates contribute to structural strength and binding cells together.

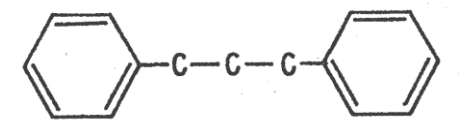
Carbohydrates and derivatives have been reported in the entire plant kingdom. Both simple and complex sugar molecules have been reported in most *Commiphora* species. Hydrolysis of methylated *Commiphora mukul* gum furnished 2,3,4,6–tetra-O-methyl-D-galactose(**LIII**), 2,3-di–O-methyl–L-arabinose (**LIV**), 2,3,4-tri-O-methyl-D-galactose(**LV**), 2, 4-di-O-methyl-D-galactose(**LVI**) and 2,3, 4-tri-O-methyl-D-glucuronic acid(**LVII**) in the ratios of 1:1:1:2:1. Methanolysis and subsequent acid hydrolysis of the methylated polysaccharide from the

degraded gum of *Commiphora mukul* furnished neutral and acidic sugar fractions. Paper chromatographic separation of the neutral fraction furnished three sugars; namely, 2, 4, 6-tri-O-methyl-D-galactose,(**LVIII**) 2,3,4-tri-O-methyl- D-galactose (**LV**) and 2,4-di-O-methyl-D-galactose (**LVI**) and an acidic sugar fraction gave 2,3,4-tri-O-methyl-D-glucoronic acid (**LVII**) (in the ratio of 1:6:2:3) (Bose and Gupta, 1964).

As with all other *Commiphora* species, *Commiphora kerstingii* also remains a potential source of most of these metabolites.

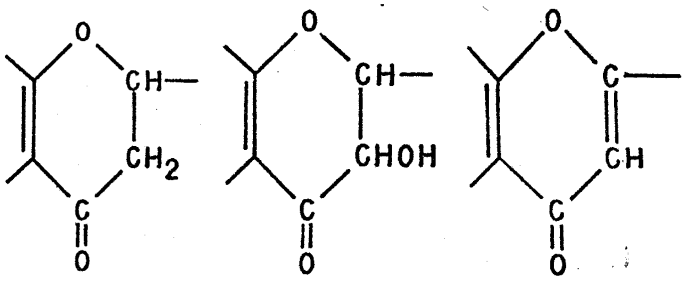
2.3.10 Flavonoids

They are a group of polyphenolic compounds which are widely distributed throughout the plant kingdom. They comprise of a series of C_6-C_3-C_6 compounds. The skeleton (**XCV**) comprises of two substituted benzene rings connected to a three-carbon system (aliphatic or alicyclic or even aromatic). The different classes within the group are distinguished by additional oxygen-heterocyclic ring and by substituted groups in different patterns.

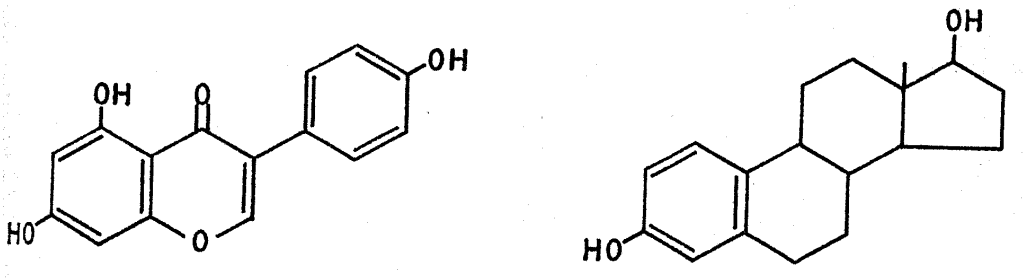


XCV

The various flavonoids (of which genistein, **XCIX** and estradiol, **C** are members) are distinguishable by oxidation state of the C chain. Flavonoids (flavonones , **XCVI**, Flavononols, **XCVII** and flavonones, **XCVIII)** occur as a glycones, glycosides and methylated derivatives. From the C_3 ring, the following classification can be made.



XCVI **XCVII** **XCVIII**



XCIX **C**

Flavonoids include many of the most common pigments and occur throughout the entire plant kingdom from the fungi to the angiosperms. One of the main functions is, as flowering pigments in attracting pollinating buds and insects. They also serve as growth inhibitors so that plant growth could be controlled by the balance between inhibiting and activating flavonoids (Furuya, *et al*, 1962).

Flavonoids commonly occur in foliage, bark, sapword and heartwood in trees. Approximately 9000 different flavonoids have already been reported from plant sources. They are beneficial to man as powerful antioxidants, stress modifiers, anti-allergic agents, antiviral compounds and anti carcinogens. Some are able to stimulate protein synthesis and some are known anti-inflammatory agents and some others have demonstrated vaso-protective activity. Amongst flavonoids are also diuretics, antispasmodic, antibacterial and antifungal agents (Harbone and Baxter, 1993).

Reports have indicated that the major flavonoid components of the flowers of *Commiphora mukul* as quercetin (**LIX**), quercetin-3-O-α-L-arabinoside(**LX**) and quercetin -3-0-β-D-galactoside (**LXI**) amongst others (Kakrani and Dev, 1981).

CHAPTER THREE

MATERIALS AND METHODS

3.0 METHODOLOGY

3.1 SYMBOLS AND ABBREVIATIONS

ABBREVIATIONS

A	Amperes	AET	Acetone extract
Amu	Daltons	Bp	Boiling point CC
CC	Column Chromatography	CD	Circular dichroism
$CHCl_3$	Chloroform	Cm	Centimeter
CT	Constant temperature	Cm^3	Centimeter cube
EI	Electron ionization	cm^{-1}	Wave number
EtOAc	Ethyl acetate	Expt	Experiment
FID	Flame Ionisation Detector	Ev	Electron volts
GC	Gas Chromatography	Fig	Figure
GIT	Gastro intestinal tract	g	Grams
IR	Infra-red spectroscopy	Hz	Hertz
LD_{50}	Median lethal dose	L	Litre
MET	Methanolic extract	mg	Milligrams
Ma	Milli amperes	mbar	Millibers
UV	Ultraviolet spectroscopy	MeOH	Methanol
MS	Mass spectrometry	mmol	Millimoles
NMR	Nuclear Magnetic Resonance	M	Meter
ORD	Optical Rotatory Dispersion	M	Molar
Pet ether	Petroleum ether (60 – 80^oC)	V	Volts
UV	Ultraviolet spectroscopy	Wt	Weight
Ppm	Parts per million	µl	Microliter
S.d.t.q	Singlet, doublet, triplet, quartet	δ	Chemical shift
C.	*Commiphora*	RT	Retention time
TLC	Thin layer chromatography	M^+	Molecular ion
UV	Ultraviolet spectroscopy	ppt	Precipitate
GC-MS	Gas Chromatography/Mass Spectrometry	TMS	Tertramethylsilane
PTLC	Preparative thin layer chromatography		
HPLC	High performance liquid chromatography		
Sh.str.w.vw	Sharp, strong, weak and very weak		

ATCC American Type of Culture Collection

3.2 Apparatus, Reagents, Instrumentation and General Techniques

3.2.1 Apparatus and Equipment Used

Glass aspirators

Distillation kits

Water bath

Quick fit flask

Rotary evaporator

Churchil (Coolant)

Glass column with sintered disc at the bottom

Adaptors

Conical flasks

Precoated glass TLC Plates (size 5 x 20cm, layer thickness 250µ,catalog No. 4855-621)

Soxhlet extractor

Thermometers

Analytical balance (mettler, H31)

Vernier calipers

Centrifuge

Chromatographic tanks

Capillary tubes

Dessicator

Separatory funnels

Buckner funnels and flasks

Beakers, test tubes and funnels

Volumetric flasks

Pestle and Mortar Petri dishes

Heating mantle

Reagent Sprayer

3.9.2 Instrumentation and General Techniques

i. Thin Layer Chromatography

This was carried out using LK5D Linear-K Silica gel glass TLC precoated plates with (size 5 x 20cm, layer thickness 250µ, catalog No. 4855-621. The plates Lot No was 002849 from Whatman Chemical Separation Inc. U.S.A. The components were observed under ultraviolet light or after derivertisation with appropriate reagent (anisaldehyde, prepared by mixing 0.5ml anisaldehyde with 10ml glacial acetic acid, 85ml methanol and 5ml

concentrated H_2SO_4 in this order) and heated at day light to 105^0C for 10 minutes for colour development. The derivatisation reagents were sprayed with glass reagent sprayer. The ascending TLC technique was used.

ii. Preparative Thin Layer Chromatography

Glass precoated silica gel TLC plates, LKD5D (size 20x20cm, layer thickness 250μ) from Whatman Chemical Separation Inc, USA. The derivatisation reagents were sprayed with glass reagent sprayer were used. The components were observed under Ultraviolet light before scrapping the bands of interest. The scrapped portions were grinded to small particle size before extracting with suitable solvents. The ascending TLC technique was used.

iii. Column Chromatography

a. Column dimensions: 100cm length 5.0cm width and 30cm length x 1.5cm width both with sintered disc at the bottom.

b. Technique: Gradient elution, using appropriate solvents

c. Absorbent: Silica gel 100 – 200 mesh size

d. Column packing: Wet slurry method

e. Sample application: Dry load method

iv. Determination of optimum solvent system for column chromatography various solvent systems were used for TLC of the fraction in question on a trial and error basis and the one that gives a good resolution of the sample (i.e. one in which there was a good degree of separation) is used for the column chromatography. The polarity of the solvent system was gradually increased as at when necessary.

v. Melting point (MP) determination

The Gallenkamp capillary melting point apparatus, equipped with a thermometer, was used.

vi. Infra-red Spectroscopy.

Measurements were carried out with Shimadzu FT-IR 8400S Model at a resolution of 2[1/cm]. Apodization was Happ-Genzel and the solvent used in both cases was $CHCl_3$.

vii. Mass Spectrometer

Mass spectra and accurate mass spectra were obtained on a fisons VG platform II mass spectrometer at University of Virginia, USA

viii. Gas Chromatography/Mass Spectrometry (GC/MS)

The electron impact (fVeV) GC-MS measurements were performed with a Hewlett-Packard HP5890 gas chromatograph that was equipped with a 25m fused silica capillary column with poldimethy/siloxane CPsil 5CB, coupled to a VG Analytical 70-250S mass spectrometer (ion source 250^0C). A temperature programme with an initial temperature of 80^0C increased 10^0C/min up to 270^0C was used with two minutes solvent delay by injection. The injector temperature was adjusted to 200^0C for these analyses.

ix. NMR – Spectrometry

The measurement of ^{1}H, ^{13}C-, HMBC, HNQC, COSY, NOESY–spectra were performed with instruments JNM-ECA 600MHz (^{1}H: 600.1MH_Z, ^{13}C:150.6MH_Z) Joel spectrometer. Tetramethyl saline (TMS-d_{12}) was used as an internal standard (δ = 0.00). TMS and the deutoriated solvents (C_6D_6, $CDCl_3$) were used. The chemical shifts were shown as δ values. Peak multiplicities were recorded in MHz.

3.10 Collection, Identification and Preparation of Plant Materials

Several collections of the plant were made between 10th to 20th May, 2006 from the bushes around Samaru to Basawa village of Sabon Gari Local Government Area of Kaduna State. The plant was identified at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria where a voucher specimen (006) were deposited.

The aerial part of the plant (leaves) were separated from the remaining parts of the plant, air-dried in the glass house, powdered, sieved, weighed and stored in screw-capped glass containers before further analysis.

A summary of the experimental techniques employed in the present research work is presented in a schematic format (**fig. 3.1**).

Isolation diagram

3.4 Method of Extraction

The powdered plant material (750g) was macerated exhaustively in a 10L aspirator bottle with acetone and the eluates combined, filtered through glass wool and concentrated using a rotary evaporator. The dark green oil extract was completely dried under vacuum weighed labeled AET and stored in refrigerator. The resulting marc was air dried at room temperature and then exhaustively macerated with methanol. The eluates were combined and the solvent recovered under reduced pressure to yield a dark residue. This residue was then completely dried under vacuum, weighed labelled, Met and stored in a refrigerator for further analysis. The weights and percentage of each extract are as given in table 4.1.

3.5 Preliminary Phytochemical Analysis of the Extracts

The phytochemical screening of the powdered leaves of *Commiphora* kerstingii was carried out on the acetone and methanol extracts using standard methods of Trease and Evans (1989). In all cases small portions of either powdered leaves or extracts were used to test for the presence of the following constituents.

3.5.8 Test for alkaloids

A sample of the extract of the leaves (1g) was taken in a small beaker and moistened with concentrated ammonia solution and left to stand for 10 minutes to enable the ammonia solution penetrate the drug. A mixture of chloroform and ethanol (1:1) was added to soak the leaves and allowed to stand for 20 minutes with occasional stirring. The mixture was filtered through a cotton wool plug. The marc was washed with chloroform twice ($5cm^3$) and the washings were combined with the filtrate.

After evaporating the filtrate to dryness to the residue was cooled and dissolved in chloroform ($5cm^3$). The chloroform solution was transferred into a separating funnel and shaken with of 2M H_2SO_4 ($3\ cm^3$). The chloroform layer was discarded. The acid layer was neutralized with 10% ammonia solution ($5cm^3$). This alkaline mixture was shaken with chloroform to extract the alkaloid. The extract was passed through a cotton wool plug and collected. The filtrate was evaporated to dryness to give a mixture of compounds. The residue was dissolved in chloroform ($5cm^3$) and neutralized with 2 M H_2SO_4. This was divided into two portions ($1\ cm^3$ into each test-tube).

General Test: To the first aliquot ($1cm^3$), two drops of freshly prepared Dragendorff's reagent was added. The second aliquot ($1cm^3$), Mayer's reagent was added.

3.5.9 Test for Carbohydrates (Brain and Turner, 1975; Trease and Evans, 1989)

i. General test for carbohydrates (Molisch Test)

A sample of each extract (0.5g) was transferred into a test-tube and water was added. The filtrate was treated with a few drops of Molisch's reagent. Concentrated H_2SO_4 ($1.0cm^3$) was allowed to flow gently down the side of the test-tube in an inclined position so that the acid formed a layer beneath the aqueous solution without mixing. The solution was then shaken, and the mixture was allowed to stand for two minutes and then diluted with $5\ cm^3$ of distilled water. The formation of a red colour at the interface of the two liquid layers when the concentrated H_2SO_4 was added, is a positive indication for the presence of carbohydrates.

ii. Test for sugar (Barfoerd's Test)

A sample of each extract (0.1g) was moistened with water ($2.0cm^3$) and allowed to stand for 30 minutes. The mixture was filtered and used for the test. $1\ cm^3$ of the filtrate in a test tube was

mixed with 1cm^3 of Barfored's reagent and heated in boiling water for 5 minutes. The formation of a red precipitate of cooper (1) oxide was a confirmatory test for the presence of sugars.

iii. Reduction of Fehling's Solution (Standard Test for Reducing Sugar)

A sample of each extract (0.1g) was treated with 2cm^3 of water and allowed to extract for 10 minutes and then the mixture was filtered. The filtrate was treated with 5 cm^3 of equal volumes of Fehling's Solution A and B and boiled for 5 minutes. Formation of a red precipitate of Cu_2O indicated the presence of reducing sugars.

iv. Standard Test for Combined Reducing Sugars

Aqueous extract (2cm^3) of the leaves was hydrolyzed by boiling with dilute (2M) hydrochloric acid (3 cm^3) and cooled. The acidic mixture was neutralized with 2 M sodium hydroxide and the above Fehling's test was repeated. Formation of a brick red precipitate indicates obtained, which showed that reduction has taken place due to the releasing of reducing sugars on hydrolysis.

v. Test for Starch – Iodine Tests

The aqueous extract (extract heated with 3ml of distilled water) was cooled and treated with a few drops of iodine solution. A blue-black colouration indicates the presence of starch.

vi. Test for Soluble Starch

To the extract (0.1g) in a test tube, 5% KOH solution (5cm^3) was added and heated gently. A brown colour obtained on warming on warming indicates the presence of soluble starch.

vii. Mucilage Test

A small amount of the extract was moistened with water and heated to form a mucilage. The mucilage formed was treated with Iodine solution and heated gently. Formation of a blue-black

colour, when cold, which disappeared when heated and reappeared on cooling is a positive indication for the presence of starch.

3.5.10 Test for Flavonoids

i. *Shinoda Test:* To 2ml of the extract, magnesium chips (1-2) and 5-6 drops of concentrated HCl were added and the colour change observed (Harborne, 1973).

ii. *Sulphuric acid test:* The sample was dissolved in concentrated sulphuric acid and the colour change was observed (Silva et al 1998).

iii. *Ferric Chloride Test:* To the dissolved extract in ethanol, it was boiled with a few drops of 10% $FeCl_3$ solution. A violet colouration was a positive indication for the presence of phenolic hydroxyl groups (Trease and Evans, 1983).

3.5.11 Test for Glycosides

a. *General Test*:

The extract (0.1g) was transferred to a test-tube, and 2M H_2SO_4 ($5cm^3$) was added. The mixture was boiled in a water bath for 15 minutes. The resulting mixture was cooled and neutralized with 20% KOH solution. A mixture of Fehling's solution A & B ($5cm^3$) was added and boiled. The reducing sugars were released as a result of the hydrosis and it was shown by a brick –red precipitate (Trease and Evans, 1983).

b. Test for Digitalis and Cardiac Glycosides

(i). Identification of steroidal aglycone.

Powdered drug (0.5g) was taken in a small beaker and EtOH (50%, $10cm^3$) and lead acetate solution (15%, $5cm^3$) were added. The beaker was placed on a boiling water bath for 2 minutes and allowed to cool. The mixture was filtered into a separatory funnel and shaken with $CHCl_3$ (2

x 5 cm^3). The $CHCl_3$ extracts were combined evaporated to dryness, the residue was treated with 3,5 – dinitrobenzoic acid in EtOH (1%, 2 cm^3) and then NaOH solution added (1M, 1cm^3). The colour change observed within 5 minutes was noted.

(ii). Test for Cardiac Glycoside.

The extract (1g) was boiled with EtOH (95% v/v, 10 cm^3) for 2 minutes, cooled and then filtered. The filtrate was diluted with equal volume of water and 3 drops of strong solution of lead sub-acetate solution was added. The mixture was stirred thoroughly and then filtered. The filtrate was divided into two portions. One portion of the above filtrate was extracted with $CHCl_3$ (5 cm^3) and the extract was evaporated to dryness on a water bath. The residue which is a mixture of glycosides was used for the following tests.

-*Keller – Killiani* test for Digitalis Glycoside

[This reaction depends on the sugar component (Digitoxose) of the glycoside] The residue from (ii) above was dissolved in glacial acetic acid (concentrated, 2 cm^3) containing traces of $FeCl_3$ and the solution was carefully poured on the surface of H_2SO_4 (concentrated 1cm^3) contained in another test tube so that it formed a separate layer. The colours of the two layers were noted. A reddish – brown ring obtained at the interphase indicated the presence of a digitoxose sugar component.

-Additional Test for Digitalis Glycosides – Kedde Test

The reserved filtrate of the extract above for cardiac glycosides was treated with 2% solution of 3, 5 dinitrobenzoic acid (1 cm^3) on 95% ethanol. The solution was then made alkaline with 5% NaOH (1cm^3).

(iii) *Legal* Test for Cardenolide (Aglycone)

A little of the extract was dissolved in pyridine and to it a few drops of sodium nitroprusside together with a few drops of 20% sodium hydroxide solution were added. A deep red colour which faded to brownish yellow was an indication for the presence of cardenolide (Sofowora, 1993).

3.5.12 Test for Saponins

i. *Frothing or Form Test:* A 2 ml aliquot of the diluted solution (1:1) was taken in a test tube and shaken (Brain and Turner, 1975).

ii. *Haemolytic Test:* To 2 test tubes, 2ml of 1.8% sodium chloride was added. To one of the tubes 2ml distilled water was added and to the other, 2ml of the extract was added. 5 drop of fresh blood was added to each of the tubes and the tubes – inverted to gently mix (Brain and Turner, 1975).

3.5.13 Test For Steroids and Terpenoids

i. *The Libermann Buchard Test:* Mixture of chloroform ($1cm^3$) and anhydrous ethanoic acid ($1cm^3$) was cooled to 0^0C and added to a sample of extract (0.1g). One drop of conc. H_2SO_4 was added down the side of the test tube. The formation of a violet ring was observed at the junction, which changed gradually to bluish green is an indication for the presence of steroidal nucleous and or terpenoids. (Silva *et al*, 1998).

ii. *Test for Sterols (Salkawski Reaction):* Chloroform ($2cm^3$) was added to the sample of the extract (0.1g), followed by concentrated sulphuric acid ($2cm^3$). When two phases with a

yellow colour are formed, it is a positive indication for the presence of sterols (Silva et al, 1998)

3.5.14 Test for Tannins

The extract (1g) was boiled with water (15 cm^3) cooled and filtered. The filtrate was used for the tests given below.

i. Filtrate was treated with a few drops of $FeCl_3$ solution. Formation of a deep purple colour confirms the presence of tannins.

ii. Filtrate was treated with lead sub-acetate solution. Formation of a brown precipitate is an indication for the presence of tannins..

iii. Filtrate was treated with a 2:1 mixture of acetic acid (10%) and lead acetate (10%). Formation of a brown precipitate is a likely indication for the presence of tannins(Trease and Evans, 1989).

3.6 Chromatographic Separation of the Extracts

The dark greenish oily acetone extract (AET) and the dark non-oily methanolic extract (MET) were subjected to a number of chromatographic separation and purification techniques.

3.6.4 Thin Layer Chromatography of the Extracts

Both extracts were analyzed by the ascending TLC method of separation using pre-coated plates. Silica gel was the stationery phase and the mobile phase was arrived at through a number of trial and errors. In the process the polarity of the solvent was meticulously adjusted so as to get the best separation.

A good separation of the methanolic extract was obtained with chloroform: methanol (9:1) solvent system in which six spots were noticed. The same solvent system was too polar for the acetone extract as some compounds were noticed to have moved along with the solvent front. In some or all cases, the chromatograms were studied in either some or all of the following:

i. viewed under sunlight

ii. viewed under UV light

iii. exposed to iodine vapour

iv. derivatised with vanillin/sulphuric acid reagent

v. derivatised with anisaldehyde/sulphuric acid reagent

The derivatised chromatograms were heated to 105^0C for 10 minutes before visualization under day light.

3.6.5 Purification of Acetone Extract Column Chromatography

The 100cm (length) / 5cm (width) column was packed with silica gel (100 – 200 mesh size) using the wet packing method. 34.7g of the acetone extract was appropriately loaded and gradual elution began with n-hexane. The solvent polarity was subsequently increased with chloroform. Over 180 eluates were collected and similar fractions (from pilot TLC plates) pooled together. Some of the pooled fractions include NC2 (1.8763g) and NC14 (1.9433g), NC2 was a milky crystalline powder and NC14 was dark green lumpy powder.

3.6.6 Purification of NC2 by Column Chromatography

About 50.0g of silica gel (100 – 200 mesh size) was packed into a smaller column by the wet parking method and 1.8763g of NC2 was loaded and eluted with n-hexane: chloroform in the

ratio of 3:1. In the process 219.6mg of NCA (which was detected by TLC to contain 5 sports) and 27.5mg of C_1S_1 (which appears to have 3 sports of non-polar fractions) were obtained.

3.6.3.4 Purification of NCA by Preparative TLC

NCA was subjected to preparative TLC technique using ethyl acetate: n-hexane (1:9) as developing solvent. The pre-coated silica gel plate (20cm/20cm) was loaded with 87.4mg of NCA and after development 5 fractions A (10mg); B (16.1mg), C (4.9mg), D (6.10mg) and E (1.7mg) were obtained. Only fraction A was confirmed by TLC technique to contain of a single spot. The others consisted, usually of one major spot and one or more other weaker spots. Both samples were labeled and kept for further analysis. The preparative TLC method was repeated several times until a total of 23.7mg of A, 77.2mg of B, and 5.0mg of X were obtained.

3.6.3.5 Purification of C_1S_1 by Preparative TLC

The 69.0mg fraction of C_1S_1 (obtained from the non-polar fraction of column chromatographic separation of NC2) was subjected to preparative TLC with petroleum ether as the mobile phase. This resulted in the isolation of two non-volatile oils, one yellowish in colouration (F) and the other milky in colouration (G) with slight impurities.

3.6.3.6 Purification of Compounds obtained from NCA

Compounds A, B and X were finally obtained as single spots (from TLC chromatogram) and were purified by recrystallisation. Further confirmatory TLC gave single pure spots in each case. The melting points of each one of the compounds was determined.

3.7 Pharmacological Screening of the Extracts of *Commiphora kerstingii*

Acetone and methanol extracts of the leaves of the plant were tested separately for the median, lethal dose (LD_{50}) analgesic and anti-inflammatory effects.

3.7.1 Determination of Median Lethal Dose (LD_{50}) In Mice and Rats

The determination of Lethal Dose (LD_{50}) in mice and rats using conducted using the method of Larke (1983). In the initial phase, 3 groups of three animals each were treated with the methanol and acetone extract at does of 10, 100 and 1000 mg/kg body weight intra-peritonially. In the second phase, 3 groups of one animal each were injected with the acetone and methanol extract at doses of 1600, 2900 and 5000 mg/kg for mice and rats. The animals are observed using each phase for signs of toxicity including death. The LD_{50} values were determined by calculating the geometric mean of the lowest lethal dose and the highest non lethal dose.

3.7.2 Analgesic Studies

Acetic acid – *induced writhing test in mice*. This method was reported by Koster, *et al*, (1959). Swiss albino mice were divided into 5 groups of 6 mice each. 10ml of normal saline per kg body weight and 10mg per Kg of body weight of ketoprofen were injected to each of the mice in the first and the fifth groups so as to serve as negative and positive controls respectively. The other mice in groups 2, 3 and 4 were injected with 400mg, 200mg and 100mg of acetone per kg body weight while similar groups were administered at doses of 2000mg, 1000mg and 500mg of methanol extract per kg body weight respectively. All the administrations were intra-peritoneally.

After 30 minutes 10ml of acetic acid (0.6% v/v) per kg of body weight was administered to all the mince in all the five groups. Five minutes after the acetic acid injection each of the mice was

placed in an individual cage and the number of abdominal contractions were counted for a period of 10 minutes. Accordingly the percentage of inhibition of the writhing was calculated as follows:

$$\text{Inhibition}(\%) = \frac{\text{Mean No of writhing (control)} - \text{Mean No of writhing (test)}}{\text{Mean No. of writhing control}} \times 100$$

3.7.3 Anti-Inflammatory Studies

The effect of the extracts on acute inflammation was studied in rats as specimens. The animals were divided into eight groups of six rats each. Thirty minutes before the administration of the carrageen as a phlogistic agent (Winter *et al*, 1962) the groups were treated as follows: Group I, normal saline (1.0ml/kg of body weight as negative control). Groups II, III and IV were treated respectively with 2000mg/kg 1000mg/kg and 500mg/kg (body weight) of methanol extract whereas groups V, VI and VII received 400 mg/kg, 200mg.kg and 100mg/kg (body weight) of acetone extract respectively. Group VIII received 10mg/kg ketoprofen as positive control. 30 minutes post treatment of 0.1ml of freshly prepared carrageenan was injected into the sub planter region of the left hind paw of each rat. The paw diameter (cm) was measured using Varnier caliper at 0 hour and every hour up to 4th hour. Percent inhibition of the inflammation was obtained using the formula:

$$\text{Inhibition}(\%) = \frac{\text{Mean paw diameter(control)} - \text{Mean paw diameter(test)}}{\text{Mean paw diameter (control)}} \times 100$$

3.8 Anti-Microbial Assay

Test Organisms: Clinical isolates of *Staphylococcus aureus*, *Streptococcus pygenes*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniac* and *Pseudomonas aeruginesa* were obtained from

the Department of Microbiology, Ahmadu Bello University Teaching Hospital, Zaria. The isolates were purified on nutrient agar (MANUC) plates and characterized using standard microbiological procedure (Cowan, 1999).

3.8.1 Determination of Anti-Microbial Sensitivity of the Extracts

The antimicrobial activity of acetone and methanol extracts from *C. kerstingii* plant leaves was carried out by utilizing the agar well diffusion bioassay procedure. Pure culture of each organism was inoculated on to Mueller-Hilton agar (MERCK) and incubated for 24hrs at 37^0 C to obtain discreet colonies. About 5 discreet colonies were aseptically transferred with a sterile wire loop into a tube containing sterile normal saline (0.85% NaCl) and were adjusted to match a turbidity of 0.5 McFarland standards. The suspension of each isolate was used to streak on the surface of Blood agar plates with sterile swaps. A sterile 6mm diameter cork borer was used to make holes into the blood agar plates inoculated with the culture. The wells were filled with 0.2ml of the Acetone and Methanol extracts of *C. kerstingii* at different concentrations (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml).The plates were incubated for 24hrs at 37^0 C. All the tests were performed in triplicates. The antibacterial activities were recorded as mean diameter of inhibition concentration (mm) produced by the plant extract.

3.8.2 Determination of the Minimum Inhibitory Concentration (MIC)

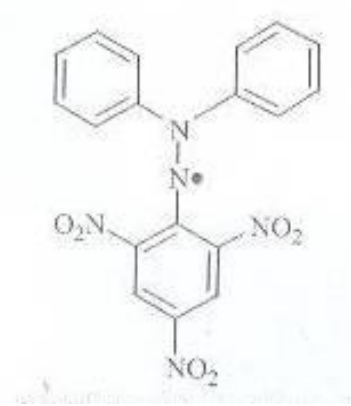
Minimum inhibition concentration was determined using micro broth dilution in accordance with the National Committee for Clinical Laboratory Standards of 2006. Serial dilution of the least concentration of the extract that showed no activity was prepared using test tubes containing 9ml of double strength nutrient broth (OXOID).The test tubes were inoculated with the suspension of the standardized inocula and incubated at 37^0 C for 24 hours. .MICs were recorded as the lowest concentration of extract showing no visible growth in the broth.

3.8.3 Determination of the Minimum Bactericidal Concentration (MBC) of the Extracts

Minimal bactericidal concentration was determined by aseptically inoculating aliquots of culture from MIC tubes that showed no growth on sterile Blood agar plates. The essence was to determine whether the organisms were killed or only inhibited. The plates were incubated at 37^0C for 24 hours after which they were observed for growth. The MBC was recorded as the lowest concentration of the extract showing no bacterial growth.

3.9 Determination of Anti-Oxidant Properties of the Isolates

Anti-oxidative activity of the pure isolates on TLC plates by derivatising the plates with a free radical 2,2-diphenyl-l–picryl thydrazil (DPPH) (**XL**). The reagent was dissolved in methanol (app. 0.2% w/v) and sprayed over TLC plates. The TLC plates had earlier been developed with ethlacetate: n-hexane (1:9) as the mobile phase. The substances which have a potential radical scavenging effect showed white inhibition zones over a violet background.



XL

The isolated compounds from the acetone extract of the leaves were tested for the antioxidant properties.

CHAPTER FOUR

RESULTS

4.0 GENERAL RESULTS

4.2 PHYTOCHEMICAL METHODS

4.2.1 Extraction

Extracts (dry) obtained with acetone from the 750g of the powdered leaves were weighed and the results are presented in table 4.1. Similar extracts using methanol solvent from the mark (left after exhaustive maceration with acetone) were put together, dried and weighed and the results also presented in table 4.1.

The acetone extract was essentially an oily dark green residue while the methanol extract was a dark non-oily residue. The acetone extract, and methanol extract weighed 117.632g and 136.041g respectively. Acetone is expected to have extracted most non-polar as well at some weak polar compounds. Methanol however is expected to have extracted most of the polar compounds.

Solvent	Weight of extract (g)	Percentage Yield of Extract
Acetone	117.6320	15.68%
Methanol	136.5041	18.20%

Table 4.1 Percent recovery of extracts from the leaves of *Commiphora kerstingii*

4.2.2 Phytochemical Screening of Plant Leaves

4.2.2.1 Preliminary Phytochemical screening of the acetone extract (AET)

Test for Alkaloids

General Tests

Dragendorff's reagent test resulted to an orange-red precipitate indicating the presence of alkaloids. The Mayers reagent gave a turbid whitish coloured precipitate confirming the presence of alkaloids.

Test for Carbohydrates

General Test for Carbohydrates

i. Molisch's test: A purple ring appeared at the inter-phase of the two layers confirming the presence of carbohydrates.

ii. Test for Sugars (*Barfoerd's* Test): A reddish brown precipitate was observed after heating the mixture of the extract with *Barfoed's* reagent, signifying the presence of sugars.

iii. Test for Reducing sugars (*Fehling Solution's test*): After addition of equal volumes of Fehling solutions A and B to the solution of the extract, a reddish-brown precipitate was formed signifying the presence of reducing sugars.

iv. Standard test for combined reducing sugars: The formation of a brick-red precipitate after addition of the Fehling solutions indicated that reduction has taken place due to the release of sugar. This confirms the presence of combined reducing sugars.

v. Test for Starch (*Iodine Test*): A blue-black colouration resulted, confirming the presence of starch.

vi. Test for soluble starch: The solution turned brown after warming confirming the presence of soluble starch.

vii. *Mucilage test* for starch: The formation of a blue-black colouration when the treated solution was cold which disappeared on warming is a confirmatory test for the present of starch.

Test for Flavonoids

i. *Shinoda test*

A dark green colouration was observed after the addition of concentrated HCl (drop wise), confirming the absence of flavonoids

ii. *Sulphuric acid test*

The addition of concentrated sulphuric acid resulted into a dark-blue colouration of the solution, signifying the absence of flavonoidal aglycones.

Test for Glycosides

(a) *General Test*: A brick-red precipitate resulted as a result of the release of reducing sugars on hydrolysis. This is a general positive test for the presence of glycosides.

(b) Test for *digitalis* and *cardiac glycosides*

i. A pink coloured solution was formed within the 5 minutes period of observation, confirming the presence of steroidal aglycone.

ii. *-Keller-killiant test* for digitalis glycosides

The reddish-brown ring obtained at the interphase confirmed the presence of digitoxose sugar component characteristics of cardenolides.

-*Kedde test*. The formation of purple colouration after the solution was made alkaline with 5% NaOH is a confirmation for the presence of cardiac glycosides.

(iii). *Legal Test*: A deep red colouration which gradually faded to brownish yellow was obtained, confirming the presence of cardinolides.

Test for Saponins

i. *Frothing or Foam Test.* There was frothing which persisted for about 10 minutes confirming the presence of Saponin glycosides.

ii. *Haemolytic test.* The red blood test disappeared as soon as they came into contact with extract solution which is an indication of haemolysis of the red blood cells thus confirming the presence of Saponin glycosides.

Test for Steroids and Tepenoids

i. *Liebermann – Buchard Test*. The colour change from violet to bluish green was a positive indication of steroidal nucleus and or triterpenes.

ii. *Salkowski's Reaction.* The formation of two phases with a yellow colouration indicated the presence of a sterol.

Test for Tannins

i. The formation of a dark green precipitate on addition of the $FeCl_3$ solution confirms the presence of tannins in the extract.

ii. The formation of a brown precipitate on treatment of extract solution with lead sub-acetate solution confirms the presence of tannins.

iii. A brown precipitate was also observed after treatment of extract solution with a mixture of acetic acid (10%) and lead acetate (10%) in the ratio of 2:1. This again confirms the presence of tannins.

4.2.2.2 Preliminary Phytochemical Screening of the Methanol Extract (MET)

Test for Alkaloids

General Tests

Dragendorff's reagent test gave an orange-red precipitate indicating the presence of alkaloids. The Mayers reagent also gave a turbid whitish coloured precipitate confirming the presence of alkaloids.

Test for Carbohydrates

General Test for Carbohydrates

i. *Molisch's test*: A purple ring appeared at the interphase of the two layers confirming the presence of carbohydrates.

ii. Test for Sugars (*Barfoerd's Test*): A reddish brown precipitate was observed after heating the mixture of the extract with Barfoed's reagent, signifying the presence of sugars.

iii. Test for Reducing sugars (*Fehling Solution's test*): After addition of equal volumes of Fehling solutions A and B to the solution of the extract, a reddish-brown precipitate was formed signifying the presence of reducing sugars.

iv. Standard test for combined reducing sugars: No formation of precipitate was observed after addition of the Fehling solutions indicated that no any reduction has taken place. This confirms the absence of combined reducing sugars.

v. Test for Starch (*Iodine Test*): A brown colouration resulted, confirming the absence of starch.

vi. Test for soluble starch: No brown colour was obtained after warning the solution. This indicates the absence soluble starch.

vii. *Mucilige test* for starch: The solution remained brownish before and after heating signifying the absence of starch.

Test for Flavonoids

i. *Shinoda test*

No colour change was observed after the addition of concentrated HCl (drop wise), confirming the absence of flavonoids

ii. *Sulphuric acid test*

The addition of concentrated sulphuric acid resulted into a dark solution, signifying the absence of flavonoidal aglycones.

Test for Glycosides

(a) *General test*. The formation of a brick-red precipitate is a positive test for the presence of glycosides.

(b) *Test for digitalis* and *cardiac glycosides*

i. A pink coloured solution was formed within the 5 minutes period of observation, confirming the presence of steroidal aglycone.

ii. *Keller-killiani test* for digitalis glycosides

-The reddish-brown ring obtained at the interphase confirmed the presence of digitoxose sugar component characteristics of cardenolides.

-*Kedde test*. The formation of purple colouration after the solution was made alkaline with 5% NaOH is a confirmation for the presence of cardiac glycosides

iii *Legal Test* for Cardenolide aglycones

A deep red colouration which gradually faded to brownish yellow was obtained, indicating the presence of cardinolide aglycones.

Test for Saponins

i. *Frothing or Foam Test.* There was frothing which persisted for about 5 minutes confirming the presence of Saponin glycosides.

ii. *Haemolytic test.* The red blood test disappeared as soon as they came into contact with extract solution which is an indication of haemolysis of the red blood cells thus confirming the presence of Saponin glycosides.

Test for Steroids and Tepenoids

i. *Liebermann – Buchard Test.* No colour change was observed indicating the absence a steroidal nucleous or terpenoids.

ii. *Salkowki Reaction.* A clear colouration of the two phases was observed indicating the absences of a sterol.

Test for Tannins

i. The formation of a dark green precipitate on addition of the $FeCl_3$ solution confirms the presence of tannins in the extract.

ii. The formation of a brown precipitate on treatment of extract solution with lead sub-acetate solution confirms the presence of tannins.

iii. A brown precipitate was also observed after treatment of extract solution with a mixture of acetic acid (10%) and lead acetate (10%) in the ratio of 2:1. This again confirms the presence of tannins.

General Test	Group/Specific Test	Sample Inference	
		AET	MET
Alkaloid	i. Dragendorf's reagents	++	++
	ii. Mayer's Reagent	++	++
Carbohydrates	i. Molish's test	++	+
	ii. Barfoerd's test	++	+
	iii. Fehling's test	++	+
	iv. Standard test for combined reducing sugars	++	+
	v. Iodine test	++	+
	vi. Test for soluble starch	++	+
	vii. Mucilage test for starch	++	+
Flavonoids	i. Shinoda's test	-	-
	ii. Sulphuric acid test	-	-
Glycosides	a. General test	++	++
	b. Digitalis Cardiac aglycosides	+	+
	i. Steroidal aglycone	+	+
	ii. Cardiac glycosides	+	+
	- Keller –Killiani Test	+	+
	- Kedde Test	+	++
	iii. Cardinolide aglycone - Legal Test	++	+
Saponins	i. Frothing test	+++	++
	ii. Haemolytic Test	++	+
Steroids and Terpenoids	i. Lieberman –Buchard Test	+++	--
	ii. Salkoski's Test	+++	--
Tannins	i. Ferric Chloride Test	+++	++
	ii. Lead Sub-acetate Test	+++	++
	iii. Acetic Acid and Lead Test	+++	+++

+++: Very High Quantity, ++: Moderate Quantity, +: Low Quantity, _ Absent

Table 4.2: Summary of Preliminary Screening of AET and MET extracts of the leaves of *Commiphora kerstingii*

4.1.3 Thin layer chromatography of the crude ACE

The crude acetone extract was subjected to thin layer chromatography (TLC) analysis with precoated silica gel plates and a number of mobile phases on a trial and error basis.

i. Pet ether: Ethylacetate (7:3). 4 spots observed (after derivatizing with anisaldehyde) were labeled Aw, Ax, Ay and Az with Rf values of 0.50, 0.69, 0.661, and 0.60 respectively. However, a larger proportion of the compounds did not move with the solvent and a small proportion moved along with solvent front.

ii. Chloroform: Methanol (9:1). Six sports observed (after derivatising) with anisadehyde labeled U,V,W, X, Y, and Z with Rf values of 0.18, 0.25, 0.30, 0.62, 0.7 and 0.75 respectively. Some compounds moved with solvent front.

4.1.6 Thin Layer Chromatography of the crude MET

The crude methanol extract was also studied by thin layer chromatography (TLC) using precoated silica get plates. A number of mobile phases were tried and the general purpose spray reagent (Conc. H_2S0_4) was used.

i. Ethylacetate: Chloroform: Methanol: Water (15:8:4:1) 7 spots were observed labeled F,G,H,I,J,K and L with Rf values of 0.22, 0.30, 0.33, 0.45, 0.50, 0.65 and 0.68 respectively. Some compounds moved with solvents front and some not at all.

ii. Chloroform: Methanol: Water (45:15:1), Six spots observed which were not well separated. Some compounds moved with solvent front and some compounds not at all.

4.1.7 Column Chromatography of crude ACE

About 34.70g of ACE was loaded on to a packed column. The column had been previously packed with 200.0g of silica gel (100-200 mesh size). A total of over 180 fractions were collected. Based on TLC studies, 40 similar non-polar fractions were pooled together to give 14 sub-fractions.

Solvent System	Ratio	Fraction	Pooled fractions	Number of Spots
Chloroform: n-Hexane	1:3	C_1S_1		
Chloroform: n-Hexane	1:3	C_2S_1		
Chloroform: n-Hexane	1:3	C_3S_1	C_5C	5
Chloroform: n-Hexane	1:3	C_4S_1		
Chloroform: n-Hexane	1:3	C_5S_1		
Chloroform: n-Hexane	1:3	C_6S_1		
Chloroform: n-Hexane	1:3	C_7S_1	C_7C	4
Chloroform: n-Hexane	1:3	C_8S_1		
Chloroform: n-Hexane	1:3	C_9S_1	$C_{10}C$	3
Chloroform: n-Hexane	1:3	$C_{10}S_1$		
Chloroform: n-Hexane	1:3	$C_{11}S_1$		
Chloroform: n-Hexane	1:3	$C_{12}S_1$	$C_{13}C$	7
Chloroform: n-Hexane	1:3	$C_{13}S_1$		
Chloroform: n-Hexane	1:3	$C_{14}S_1$		
Chloroform: n-Hexane	1:3	$C_{15}S_1$	$C_{15}C$	7
Chloroform: n-Hexane	1:3	$C_{16}S_1$		
Chloroform: n-Hexane	1:3	$C_{17}S_1$	$C_{18}C$	4
Chloroform: n-Hexane	1:3	$C_{18}S_1$		
Chloroform: n-Hexane	1:3	$C_{19}S_1$		
Chloroform: n-Hexane	1:3	$C_{20}S_1$	$C_{20}C$	2
Chloroform: n-Hexane	1:1	C_1S_2		
Chloroform: n-Hexane	1:1	C_2S_2	C_3C_2	2
Chloroform: n-Hexane	1:1	$C3S_2$		
Chloroform: n-Hexane	1:1	C_4S_2		
Chloroform: n-Hexane	1:1	C_5S_2	C_6C_2	2
Chloroform: n-Hexane	1:1	C_6S_2		
Chloroform: n-Hexane	1:1	C_7S_2		
Chloroform: n-Hexane	1:1	C_8S_2	$C_{10}C_2$	4
Chloroform: n-Hexane	1:1	C_9S_2		
Chloroform: n-Hexane	1:1	$C_{10}S_2$		
Chloroform: n-Hexane	1:1	$C_{11}S_2$	$C_{12}C_2$	4
Chloroform: n-Hexane	2:1	$C_{12}S_2$		
Chloroform: n-Hexane	2:1	C_1S_3	C_2C_3	5
Chloroform: n-Hexane	2:1	C_2S_3		
Chloroform: n-Hexane	2:1	C_3S_3	C_5C_3	2
Chloroform: n-Hexane	2:1	C_4S_3		
Chloroform: n-Hexane	2:1	C_5S_3		
Chloroform: n-Hexane	2:1	C_6S_3	C_8C_3	3
Chloroform: n-Hexane	2:1	C_7S_3		
Chloroform: n-Hexane	2:1	C_8S_3		

Table 4.3: Eluates obtained from column chromatography of AET

4.1.6 Some physical properties of isolated compounds.

After purification of samples A, B and X by preparative TLC and recrystallization in suitable solvents, the compounds were subjected to thin layer chromatography using Ethylacetate: n-Hexane (1:9) solvent system. The Rf values determined. The melting point of the dry isolates were recorded. These findings are as presented in table 4.4

Isolated compound	Melting point	Thin Layer chromatography (TLC) Solvent system (ratio) for TLC	R_f values	Solubility
A	202-203^{0}C	Ethylacetate: n-Hexane (1:9)	0.46	$CHCl_3$
B	211-213^{0}C	Ethylacetate: n-Hexane (1:9)	0.42	$CHCl_3$
X	290-292^{0}C	Ethylacetate: n-Hexane (1:9)	0.56	$CHCl_3$

Table 4.4: Some physical properties of isolated compounds

4.1.8 Some chemical properties of isolated compounds

The separated samples on the TLC plates were derivatized with anisaldehyde. Samples A and B gave the characteristic pink colour after heating at 105^{0}C for 10 min. This is an indication that both samples are terpenoidal in nature.

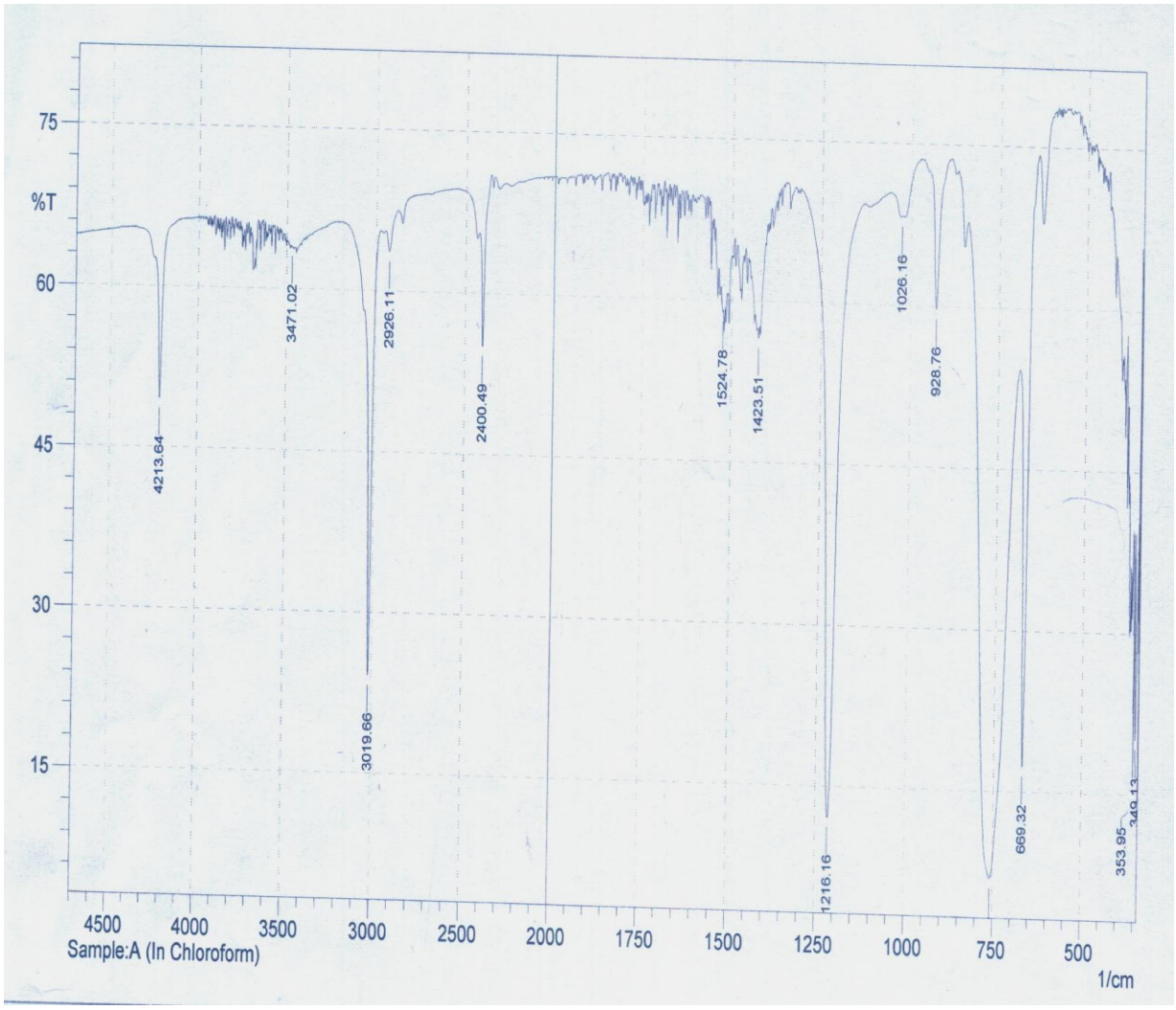
Moreover, when both spots were sprayed with vanillin/sulphuric acid reagent, the purple colouration, characteristics of terpenoids result. However, sample X gave a blue green colouration with anisaldehyde reagent. This colouration of sample X is typical of drugs containing essential oils, balsams and oleo-gum-resins (Wagner and Bladt, 1996).

4.6 SPECTROSCOPIC STUDIES OF ISOLATED COMPOUNDS

4.2.4 spectroscopic identification of compound A

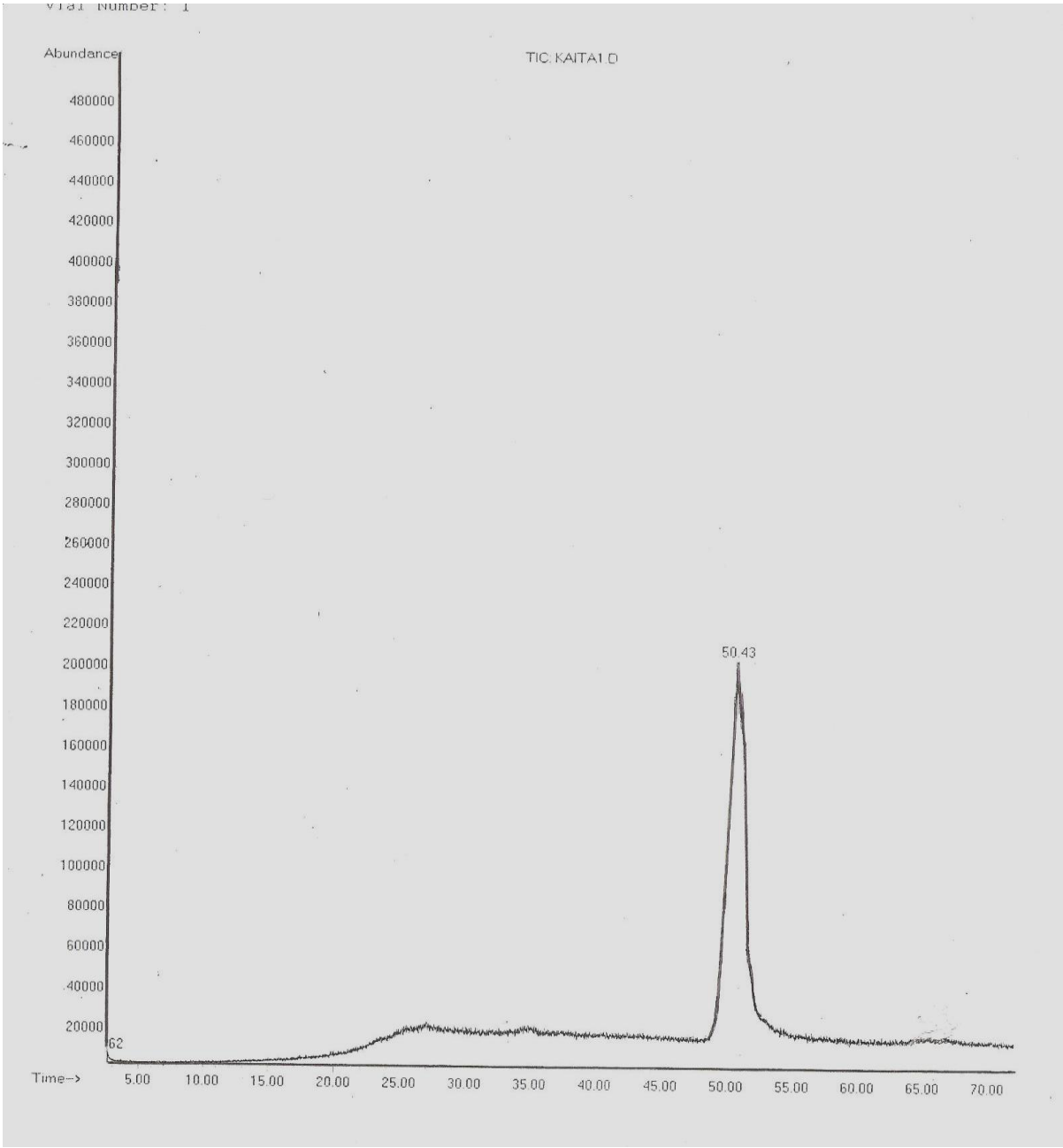
Infra-red spectroscopy. The IR spectrum of compound A in KBr (fig.4.1) showed the absorption peaks at 3471.02cm^{-1} (O-Hstr for alcohols), 3019.66cm^{-1} (SP^2 C-Hstr), 2926.11cm^{-1} (SP^3 C-Hstr) and a weak signal at 1680cm^{-1} (C = C Str) attributable to probably the presence of a single olefinic bond in the molecule.

Figure 4.1 Infra-red spectrum of compound A.



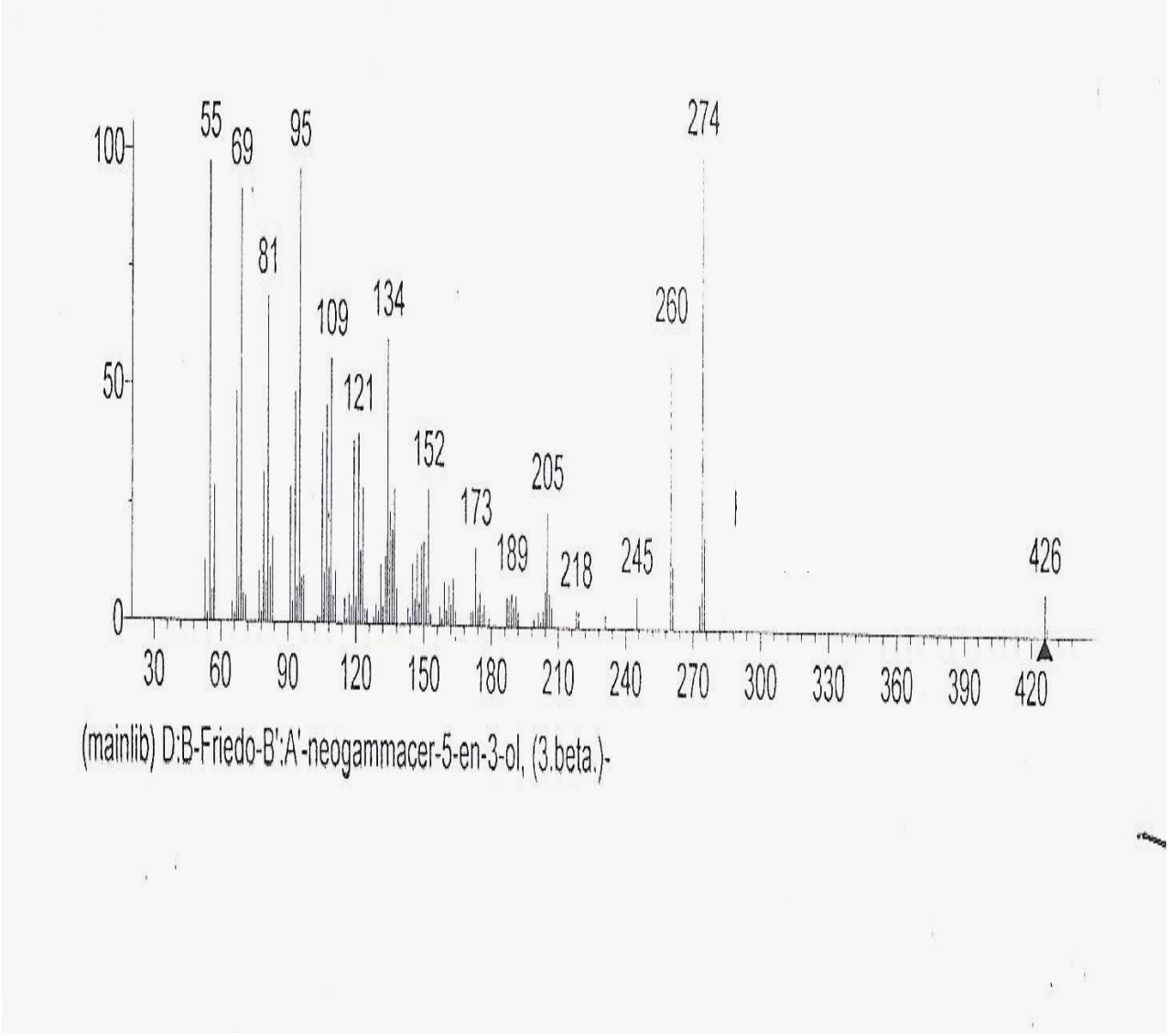
Gas chromatography/Mass spectrometry (GC-MS). The gas chromatogram of compound A is presented in Figure 4.2. The retention time of the compound is 50.43min. The single peak in the chromatogram suggests that the compound is in a state of high purity.

Figure 4.2: Gas Chromatogram of Compound A



The *mass spectrum* (Fig. 4.3) indicated a molecular ion peak of 426 suggestive of a molecular formula of $C_{30}H_{50}O$. From the respective *m/z* values, it could be generalized that all the fragments obtained result from the fragmentation pattern of ursane type of pentacyclic triterpenoids that have a double bond at position 5 (fig. 4.4.) as reported for the Retro-Diels-Alder (RDA) reaction (Budzikiewicz, *et al*., 1963). The fragment peaks obtained for compound A compare very well with all of those reported for Glutinol (Chen *et al*, 2003) as presented in table 4.5.

Figure 4.3: Mass Spectrum of Compound A.



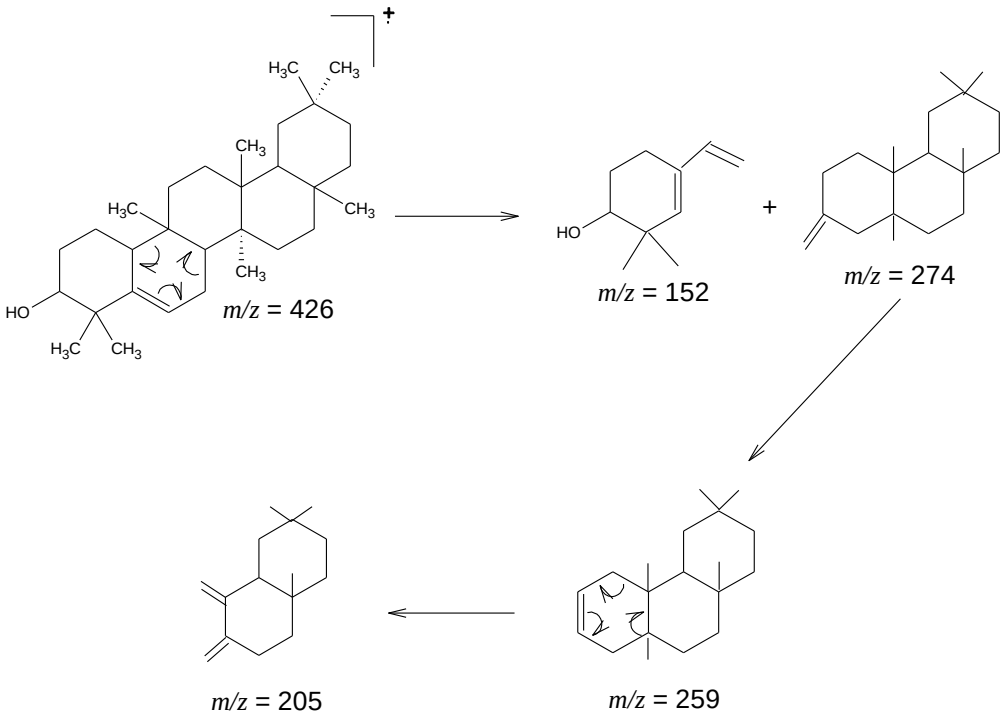
Compound A	**Glutinol ***
426 [M]+ (6.7)	426 [M]+ (3.1)
274 (75)	274 (74.2)
260 (50)	259 (74.8)
245 (3.5)	205 (27.4)
218 (2.0)	152 (21.0)
205 (30.0)	134 (54.0)
173 (20.1)	119 (46.6)
152 (35.3)	109 (56.0)
134 (66.5)	95 (97.9)
121 (48.5)	81 (69.6)
109 (57.5)	69 (100)
95 (100)	
81 (75.8)	

*Chen *et al* (2003)

Table 4.5: Mass Spectrum of Compound A and Glutinol

The possible fragmentation pattern of compound A is given in Figure 4.4.

Fig. 4.4: The possible fragmentation pattern of compound A



The *^{1}H NMR spectra* of compound A are given in figures 4.5, 4.6 and 4.7. The spectra showed eight singlets at δ0.85, (3H, s, H-25), 0.95 (3H,s,4-29), 0.99 (3H, s, H-30), 1.00 (3H, s, H-27) 1.04 (3H,s,H-24), 1.09 (3H,s,H-26), 1.14 (3H,s,H-23) and 1.16 (3H,s,H-28). These accounted for eight methyl groups in the compound. There is also a singlet at δ1.262 (18H) attributable to the presence of 9 methylene protons. In addition, a vinylic proton a triplet and an alcoholic proton have been observed at δ5.6 (1H, br, d, J=5.5Hz, H-6) and δ 3.5 (1H, m, H-3α) respectively.

The ^{13}C-NMR Spectra (fig. 4.8 and 4.9) showed signals for primary carbons at δ25.5, 29.0, 16.2, 19.6, 18.4, 34.0 and 32.4. Similarly, there are eleven secondary carbons at δ18.2, 27.8, 76.4, 23.7, 34.6, 30.4, 32.1, 36.0, 35.1, 33.4 and 39.0 in compound A. Four tertiary carbons also exist at δ49.7, 47.5, 122.1 and 43.1 in addition to seven quaternary carbon atoms at δ 40.8, 141.6, 34.9, 37.8, 39.3, 30.1 and 28.2.

The correlations between ^{1}H and ^{13}C-NMR resonance were derived from ^{1}H-^{13}C COSY (NMQC) Spectrum (fig. 4.12). The connectivity of carbon atoms was deduced from the ^{1}H-^{1}H COSY (figure 4.15 and 4.16) and HMBC Spectrum (fig. 4.13 and 4.14). The HMBC Spectrum (fig. 4.13) has clearly shown the correlation of the oleifinic proton δ5.64 to the carbons at δ23.7 (C-7), 47.5 (C-8) 49.9 (C-10) and 40.8 (C-4). This confirms the position of the vinylic proton to be at C-6.

A double bond was detected in the HMQC spectrum of compound A (fig.4.11) from correlations of the oleifinic protons at δ5.6, to the carbon at δ122.1. The low field shift was related to its proximity to the double bond. The alcoholic proton at δ3.46 correlated to the carbon at δ76.4.

The eight methyl signals at δ 0.85, 0.95, 0.99, 1.00, 1.04, 1.09 and 1.16 were found to be correlated to carbons at δ16.2, 34.0, 32.4, 18.4, 19.6, 16.2, 29.0 and 25.5 respectively in the HMBC Spectrum (fig. 4.14).

The chemical shifts obtainable for the carbon skeleton of compound A (fig. 4.10) are in good agreements with values reported for Glutinol (Chen *et al*, 2003 and Kitajima *et al*, 1994).

Fig. 4.5: Full ^{1}H-NMR spectrum of compound A.

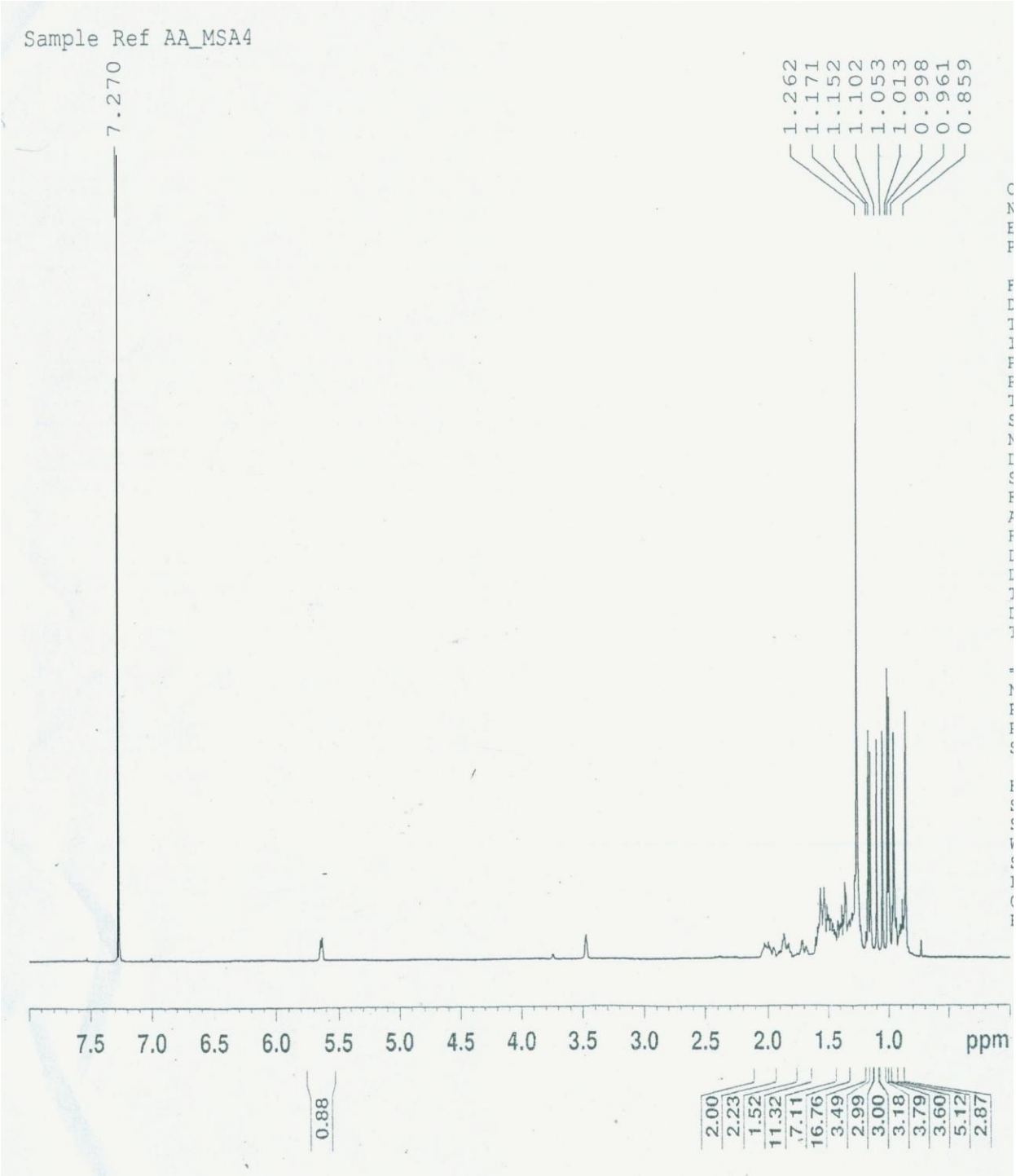


Fig. 4.6: Expanded ^{1}H-NMR spectrum of Compound A.

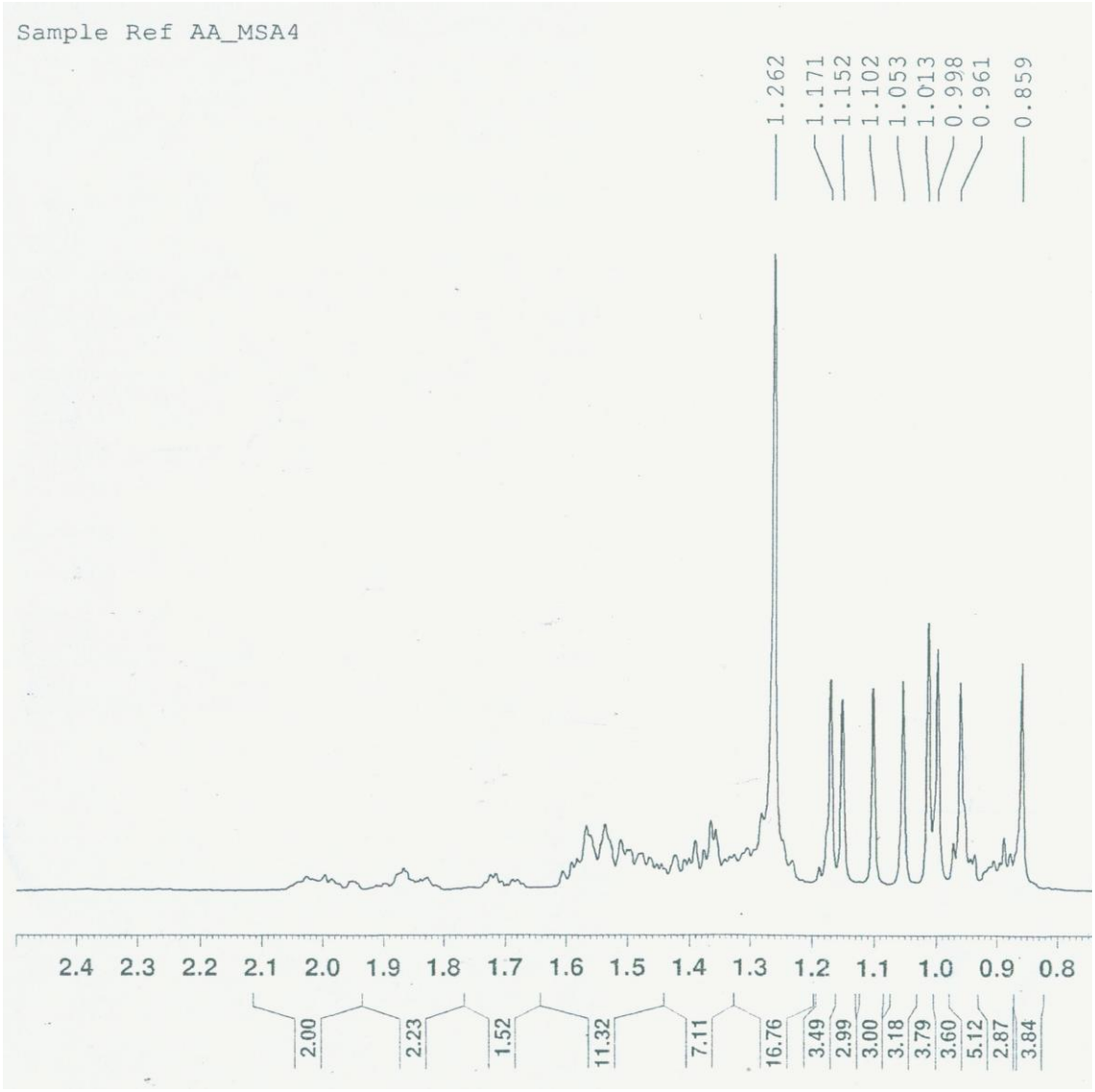


Fig. 4.7 Expanded ^{1}H-NMR spectrum of compound A.

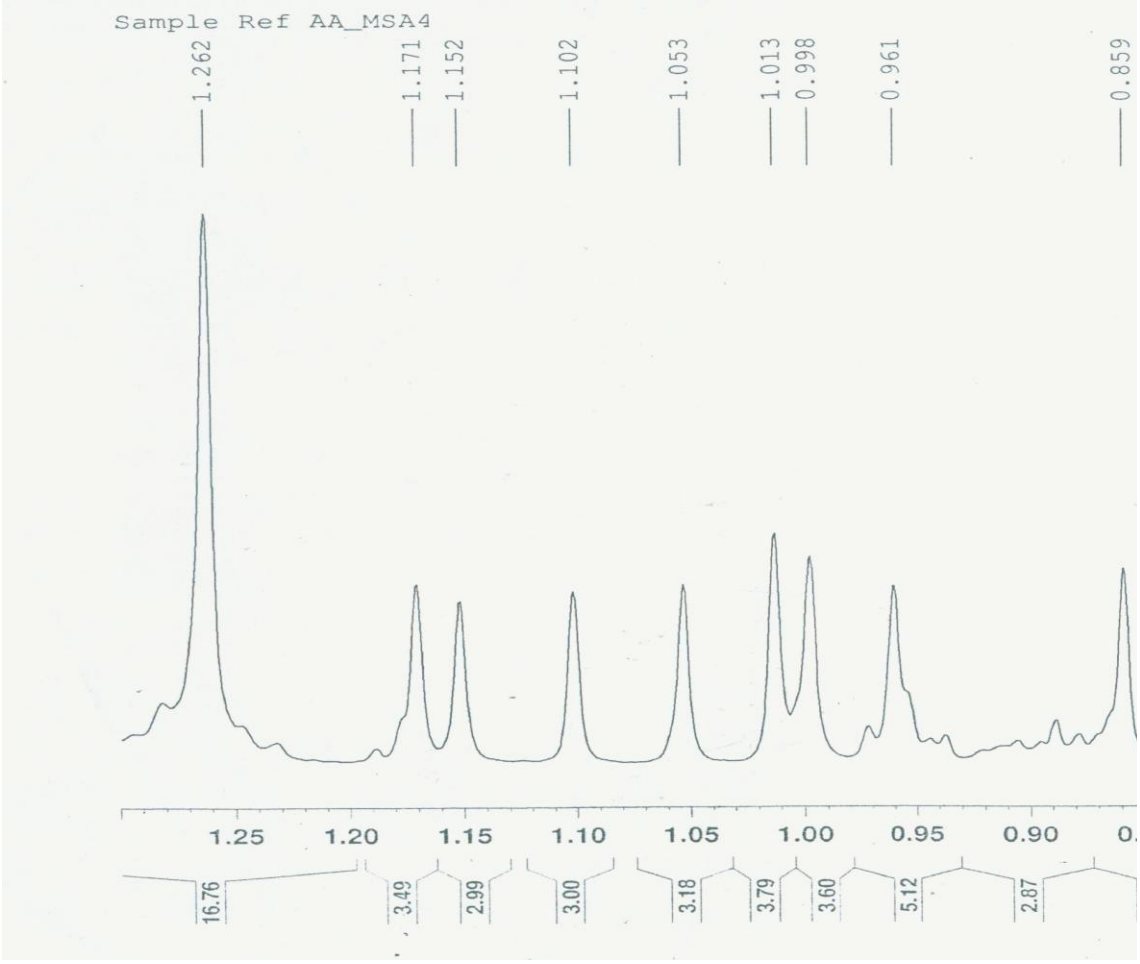


Fig. 4.8: ^{1}H-^{13}CNMR Spectrum of Compound A

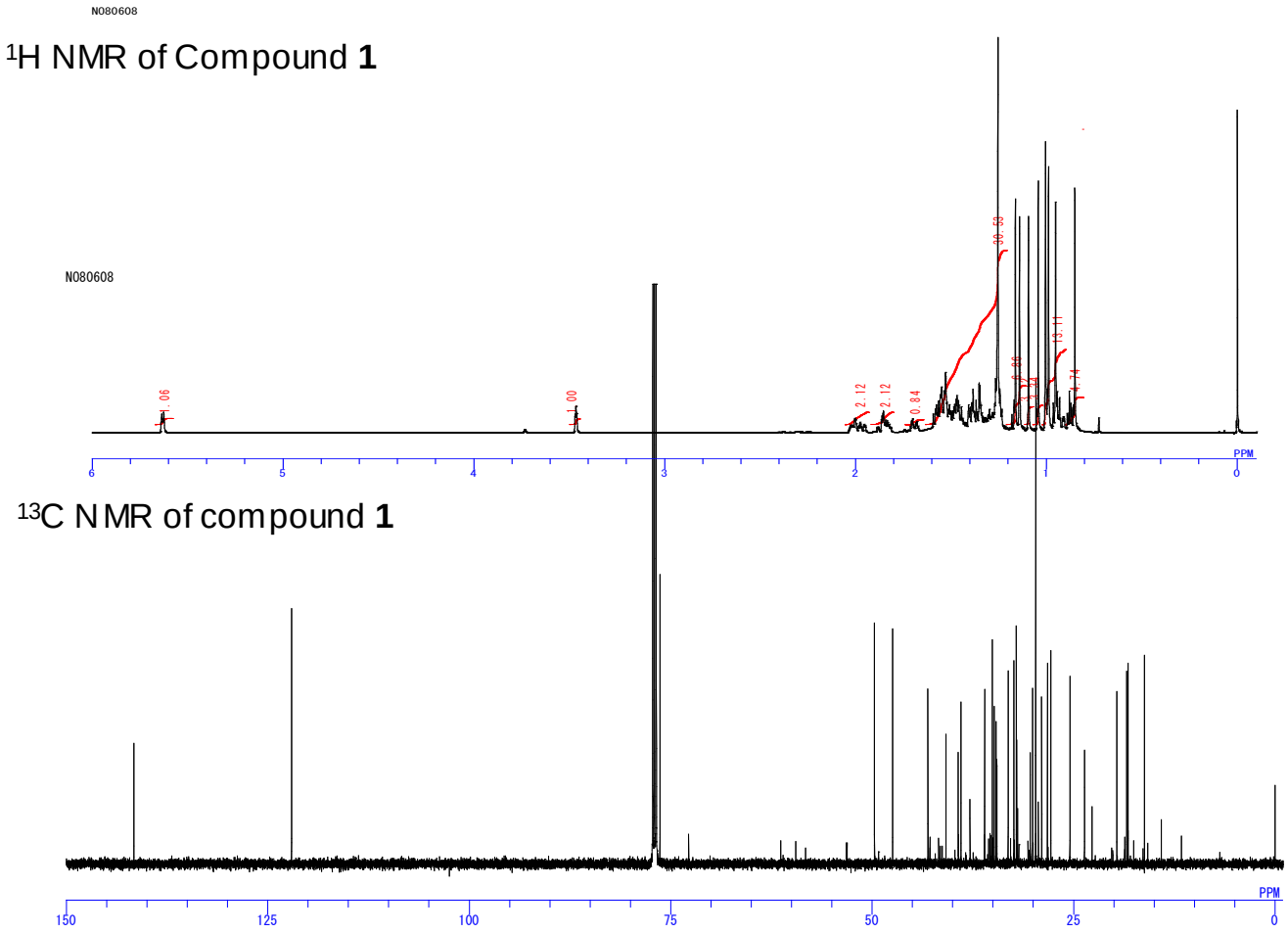


Fig. 4.9: Expanded ^{13}CNMR Spectrum of Compound A.

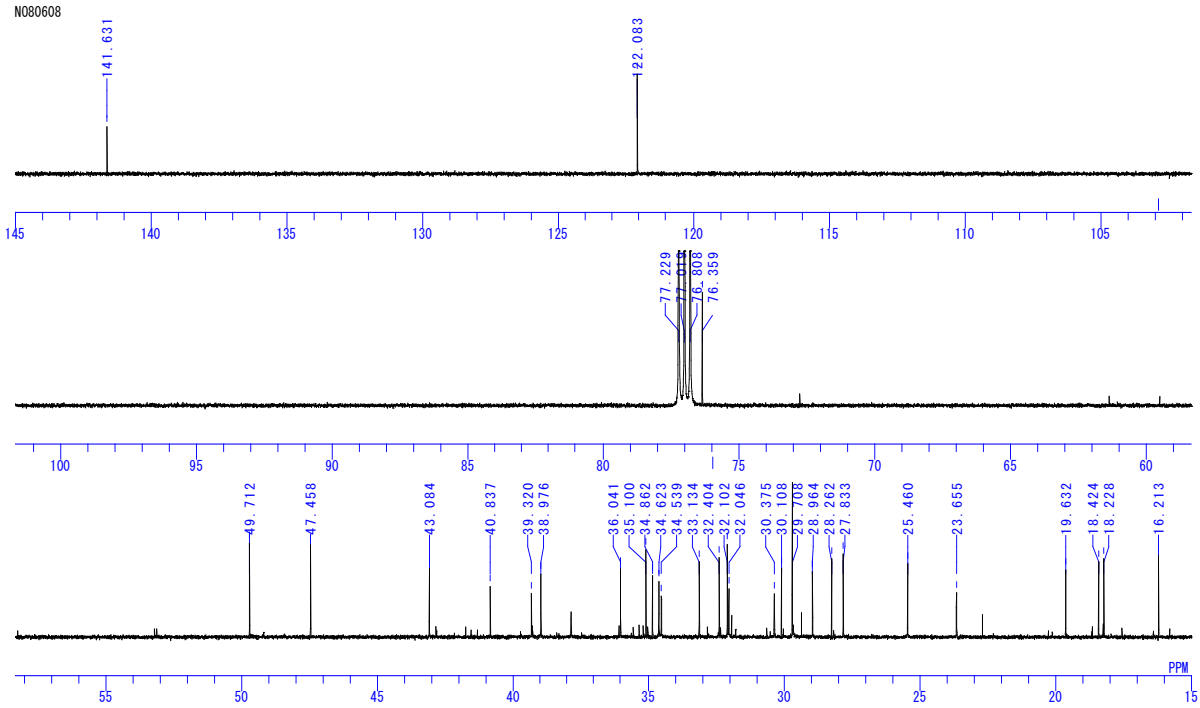
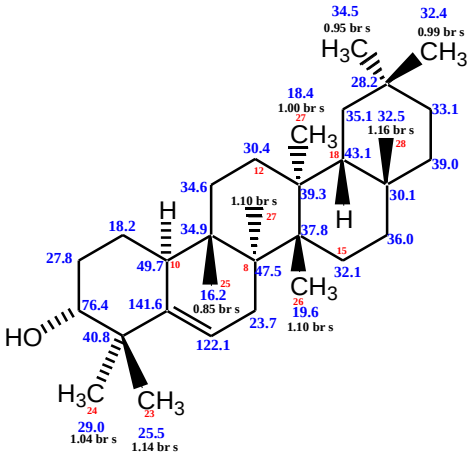


Fig. 4.10: ^{13}CMR Spectrum of Compound A and *Glutinol



C	*Glutinol	Sample A
1	18.2	18.2
2	27.8	27.8
3	76.3	76.4
4	40.8	40.8
5	141.6	141.6
6	122.0	122.1
7	23.6	23.7
8	47.4	47.5
9	34.8	34.9
10	49.6	49.7
11	34.6	34.6
12	30.3	30.4
13	39.3	39.3
14	37.8	37.8
15	32.1	32.1
16	36.0	36.0
17	30.1	30.1
18	43.0	43.1
19	34.8	35.1
20	20.2	28.2
21	33.1	33.1
22	38.9	39.0
23	28.9	25.5
24	25.5	29.0
25	16.2	16.2
26	19.6	19.6
27	18.4	18.4
28	32.1	32.01
29	34.5	34.5
30	32.1	32.4

*Chen *et al*, 2003

Fig. 4.11: HMQC Spectrum of Compound A

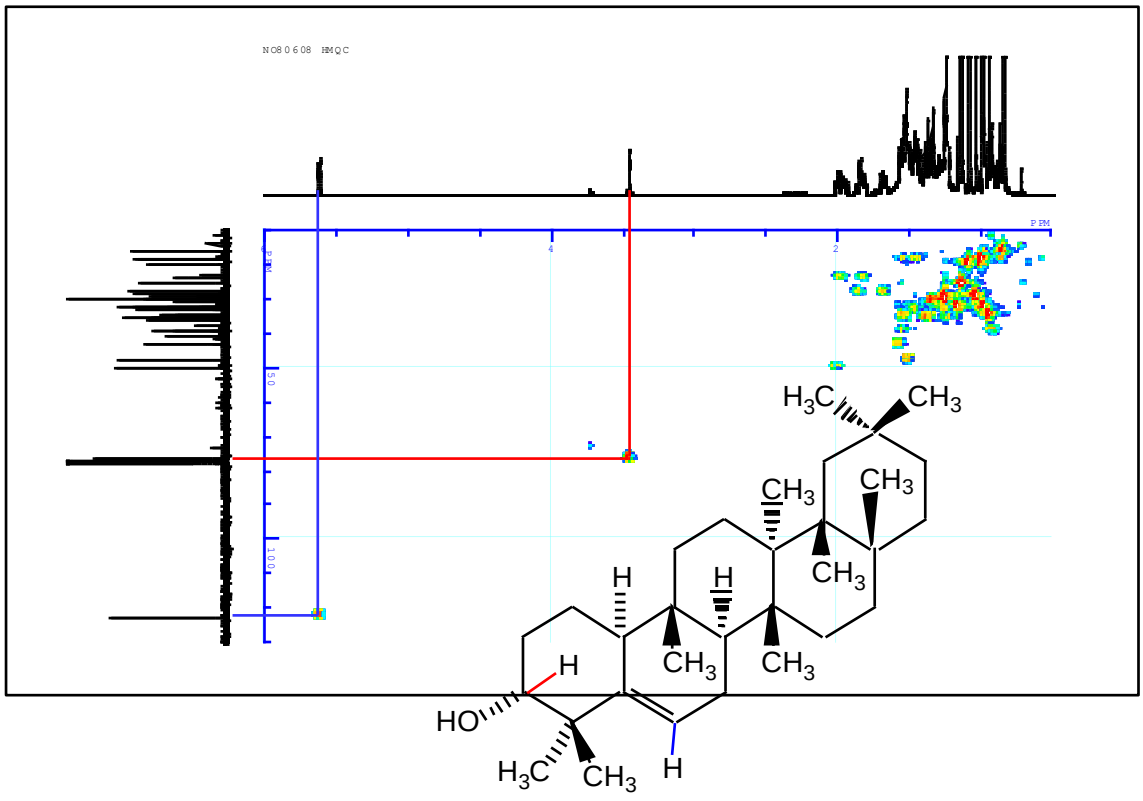


Fig. 4.12: Expanded HMQC of Compound A

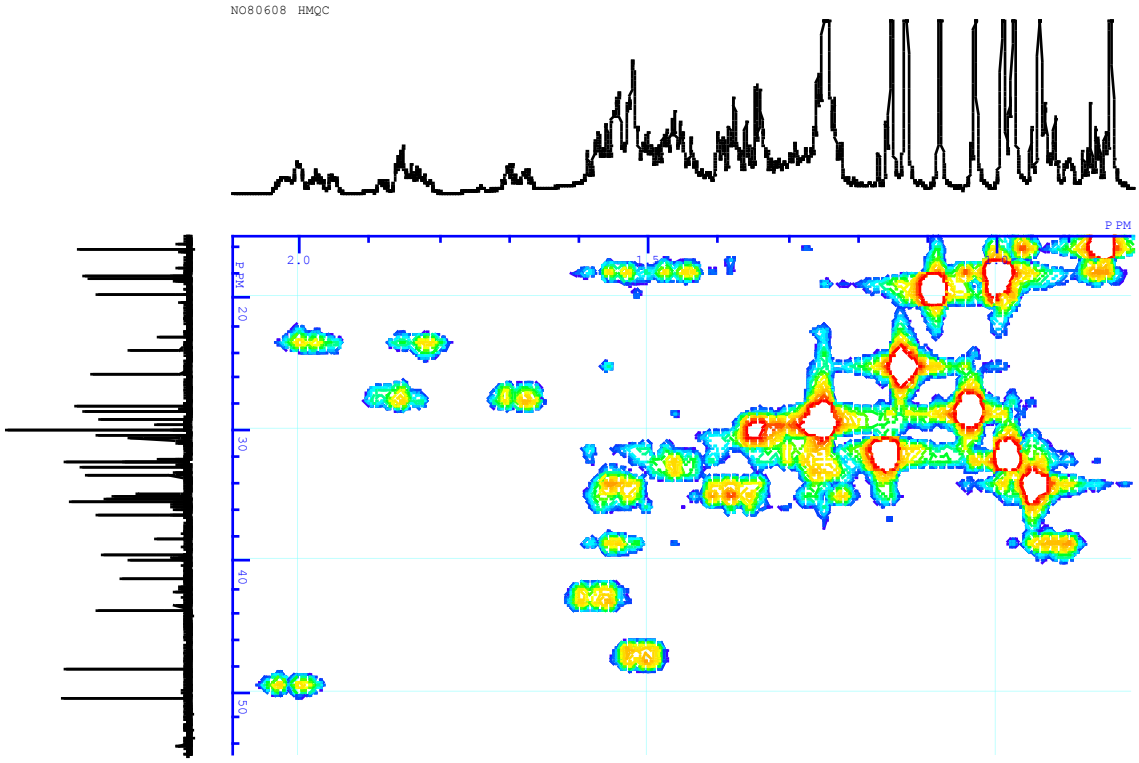


Fig. 4.13: HMBC Spectrum of Compound A

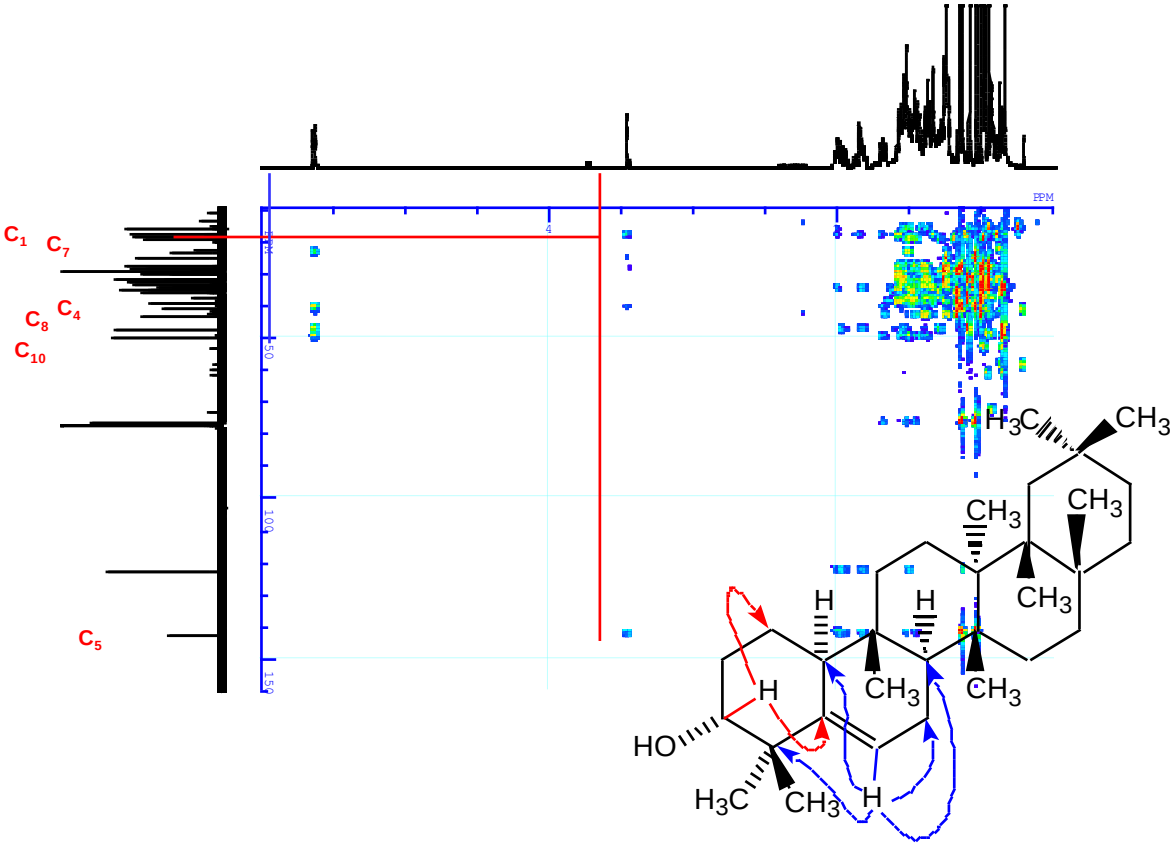


Fig. 4.14: Expanded HMBC Spectrum of Compound A

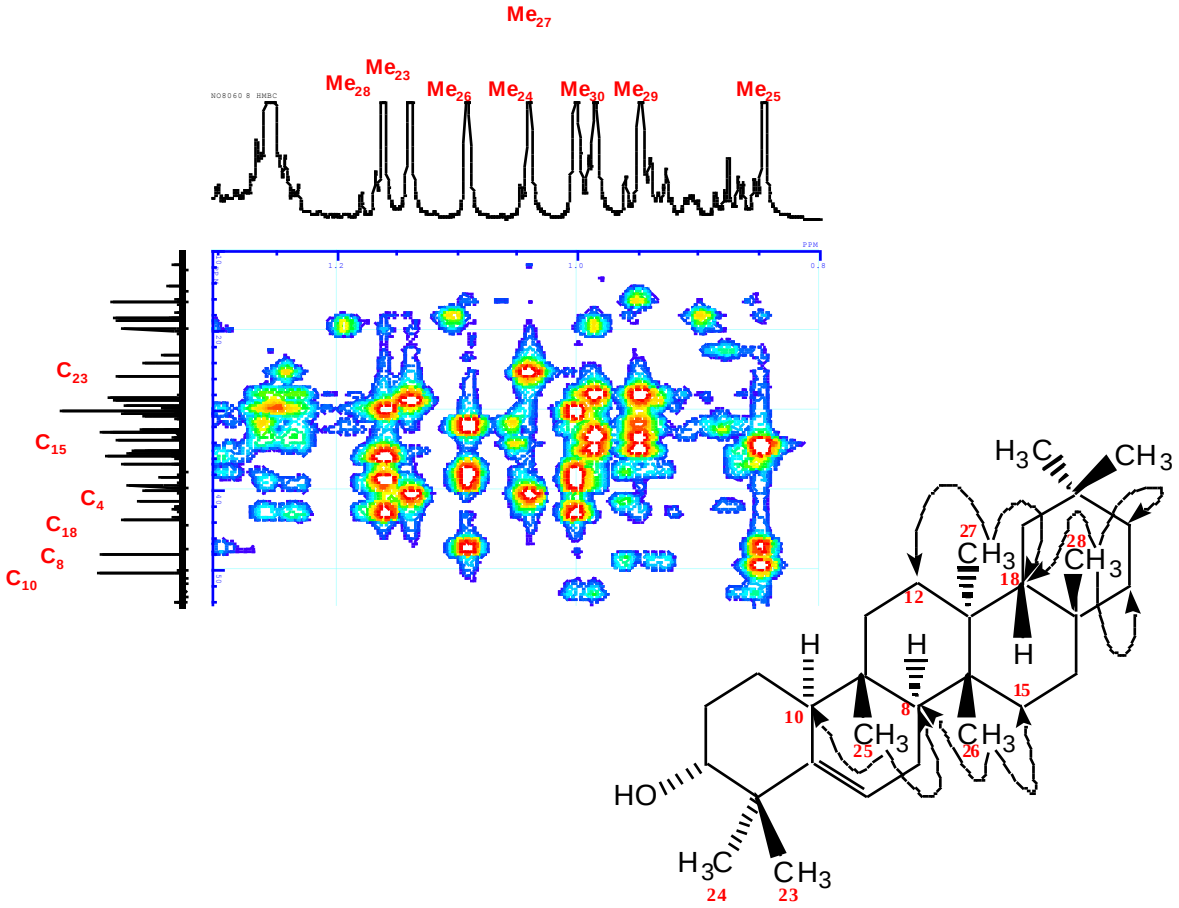


Fig. 4.15 ^{1}H-^{1}H COSY Spectrum of Compound A

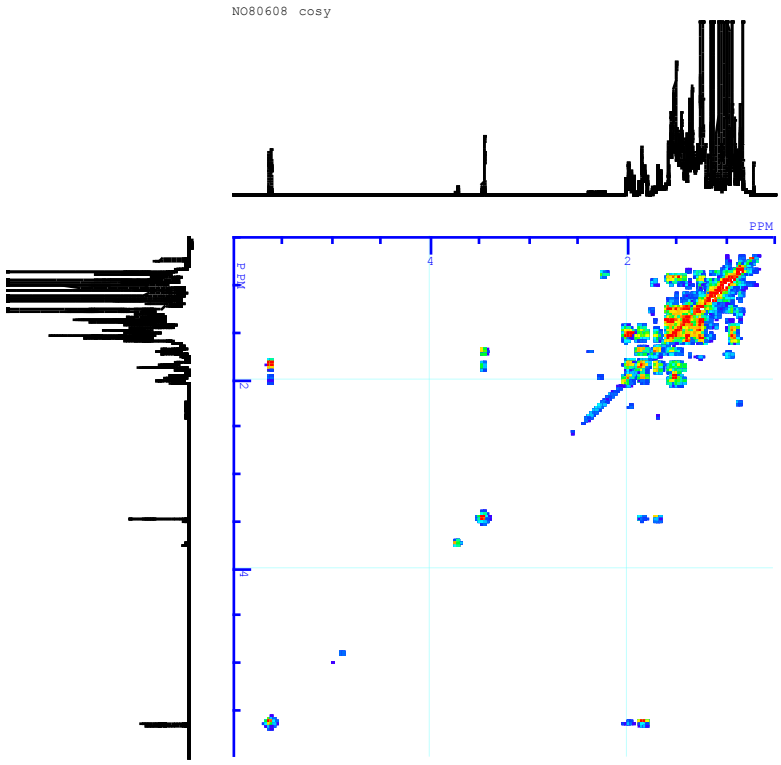


Fig. 4.16 Expanded ^{1}H-^{1}H COSY Spectrum of Compound A

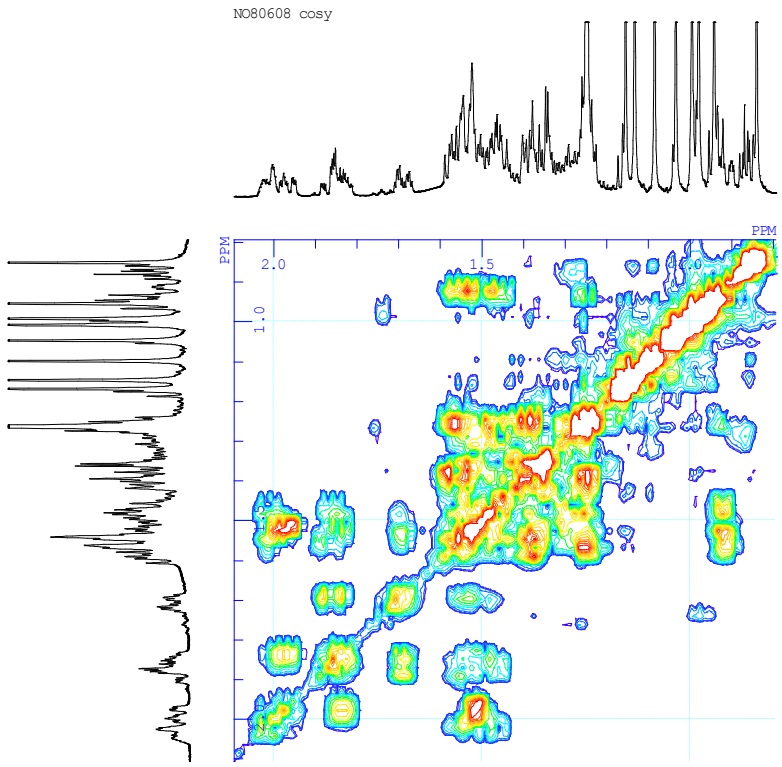


Fig. 4.17: NOESY Spectrum of Compound A

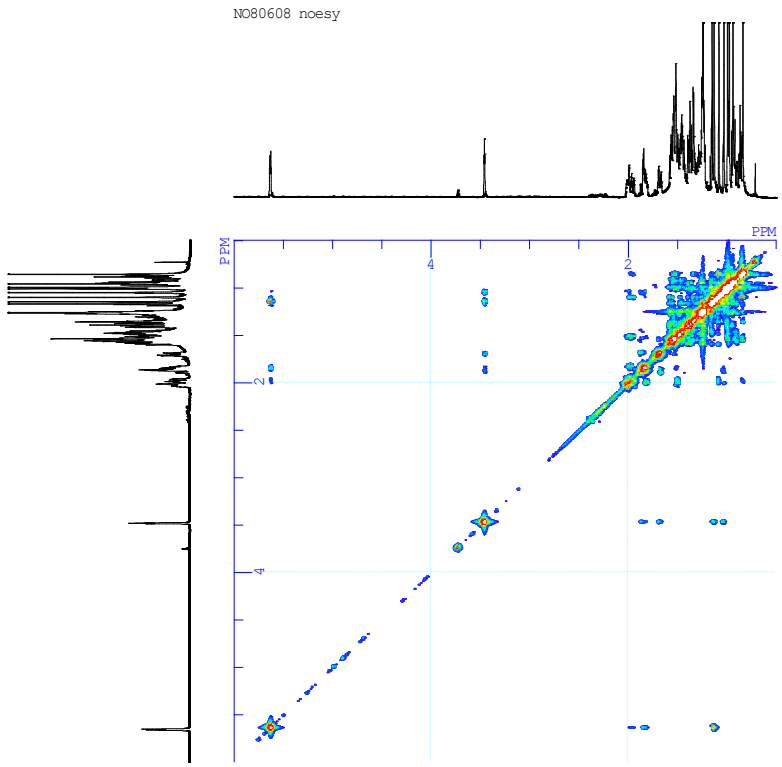
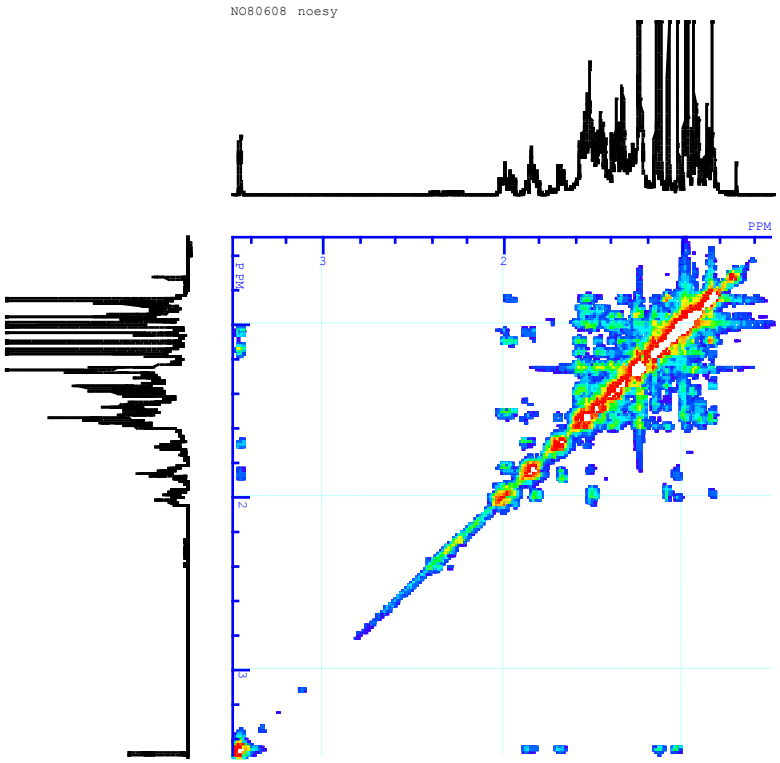
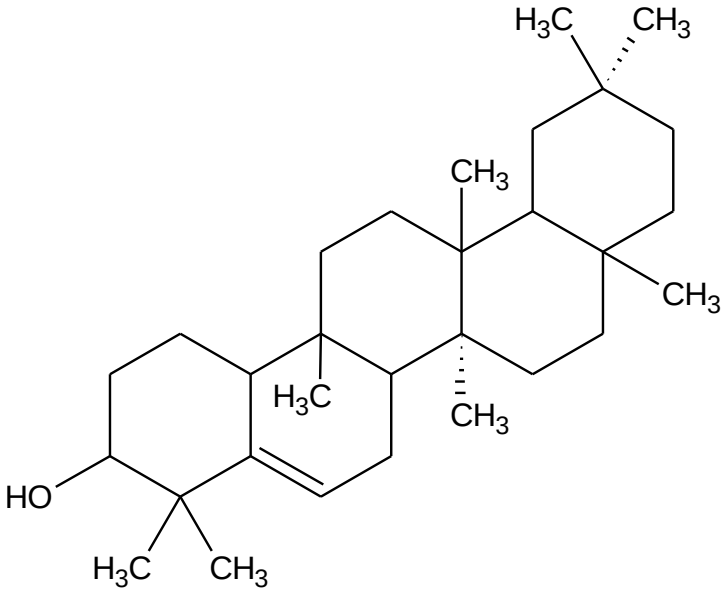


Fig. 4.18: Expanded NOESY Spectrum of Compound A



When these spectroscopic data on compound A are analyzed together, it is conclusive that compound 3 epi-glutinol with the following structure.

Fig. 4.19: Structure of compound A (Glutinol)



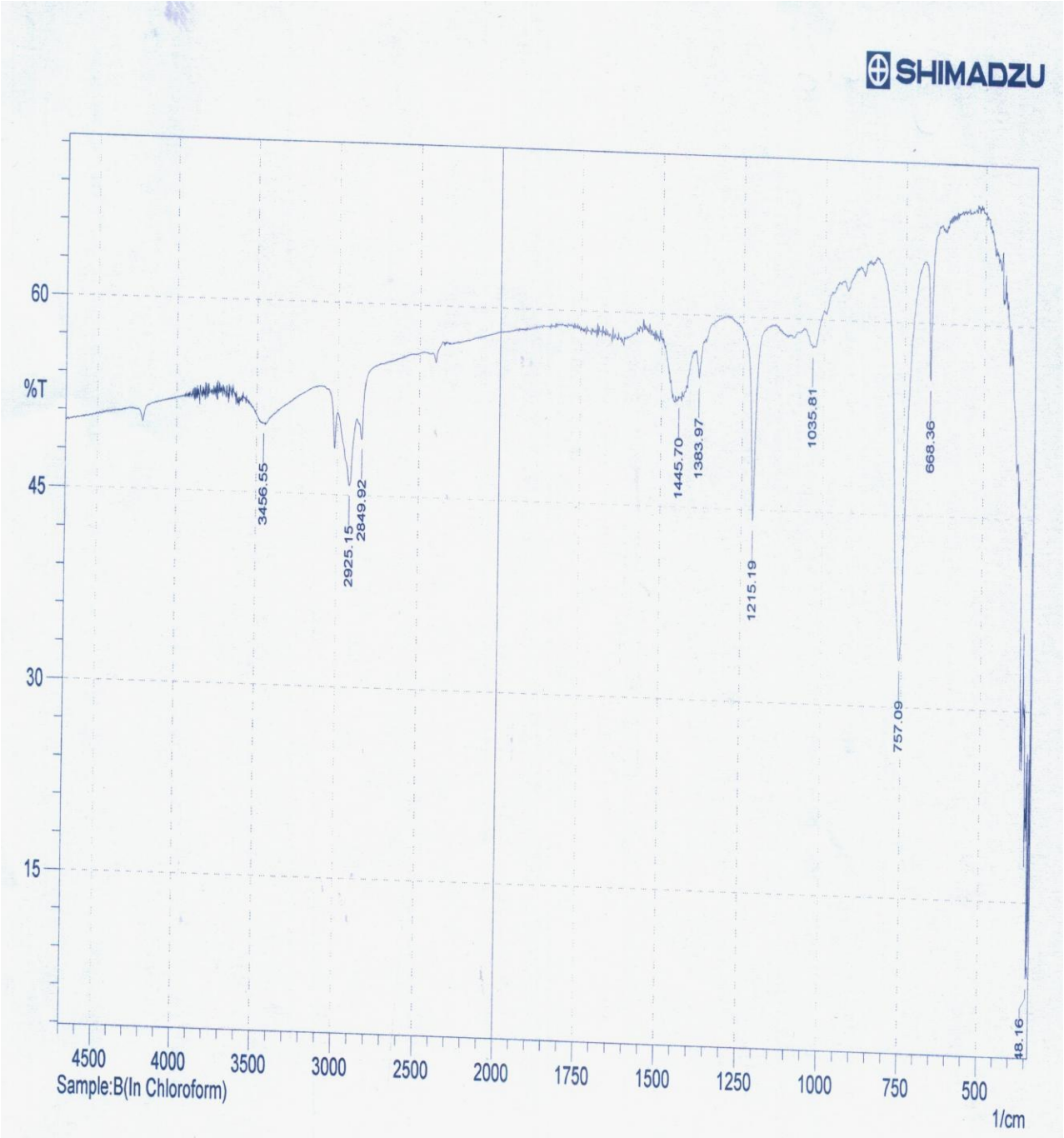
CI

The mass spectrum of compound A suggests the typical fragmentation pattern for ursane type of triterpenoids. The RDA reaction that was triggered by the double bond at position 5 revealed a fragment ion signal *m/z* = 218 and a further methyl cleavage from this fragment formed the signal *m/z* = 203 (Fig. 4.12a).

4.2.5 Spectroscopic Identification of Compound B

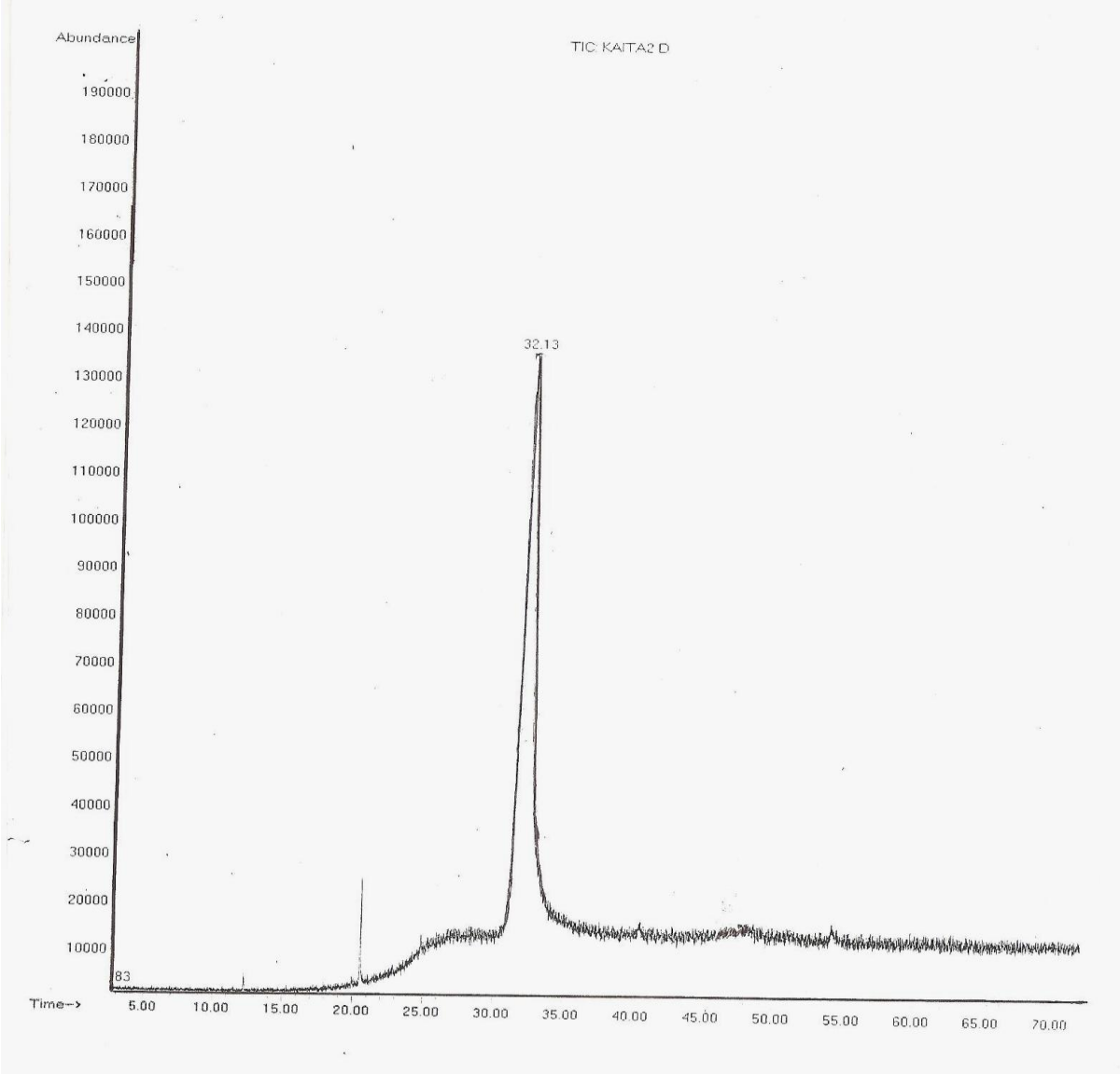
Infra-red spectroscopy: The IR spectrum of compound B (Fig. 4.7) indicates very similar peaks with that of compound A (Fig. 4.1). There are absorption peaks 3456.55cm^{-1} (O-Hstr for alcohols), 2925.15cm^{-1} and 2849.92cm^{-1} are for (SP^3 C-Hstr) vibrations. The absorption peak at 3100cm^{-1} could be ascribed to the SP^2 C-Hstr vibrations. The weak signal at 1680cm^{-1} could be as a result of C = C Str vibrations for probably the only olefinic bond in the molecule.

Fig. 4.20: Infra red spectrum of Compound B



Gas chromatography/Mass Spectrometry (GC-MS). The gas chromatogram of compound B (fig. 4.8) indicates a retention time for the compound to be 32.13min. This retention time is shorter than that observed for compound A. The single peak obtained confirms the state of high purity of compound B and the chromatogram indicates that this compound is in a state of high abundance.

Fig. 4.21: Gas Chromatogram of Compound B



The mass spectrum of compound B (Fig. 4..22) is very similar to that obtained for compound A. The molecular ion peak is 426 also suggestive of the molecular formula $C_{30}H_{50}O$. The *m/z* value of most of the fragments are very similar to those a compound A, indicating that the compounds (A and B) are either isomers or of very similar structural formulae. The fragmentation pattern (Fig. 4.23) also resembles that reported for pentacyclic triterpenoids that have an olefinic bond at position 5. The fragment peaks obtained, for compound B are very consistent with those obtained for Rhoiptelenol as given in Table 4.6 (Fraga *et al*, 2003).

Fig. 4.22: Mass spectrum of compound B

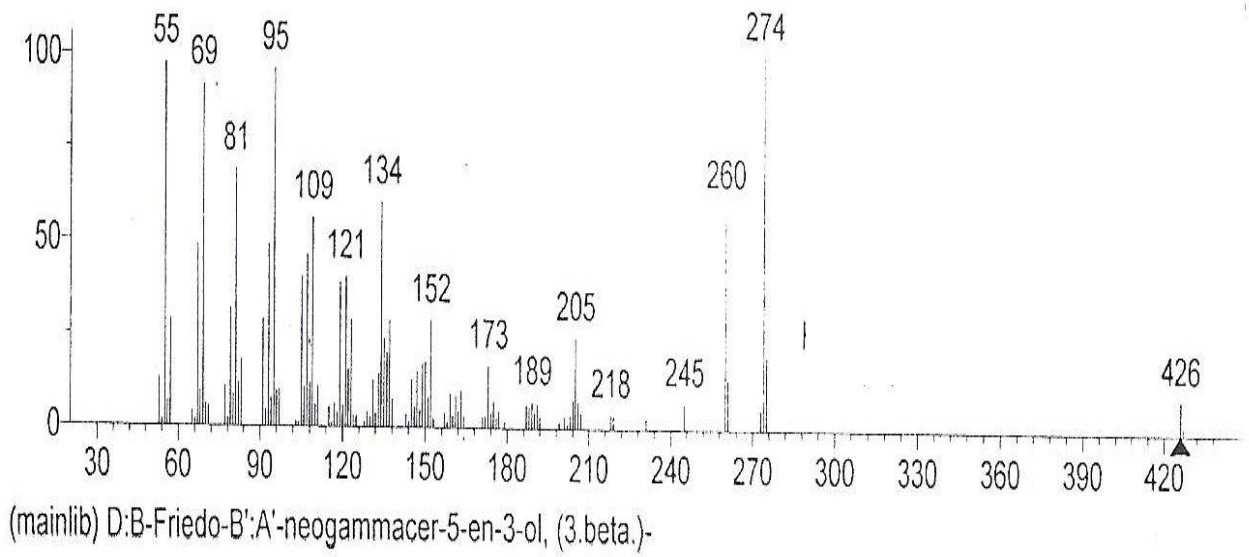
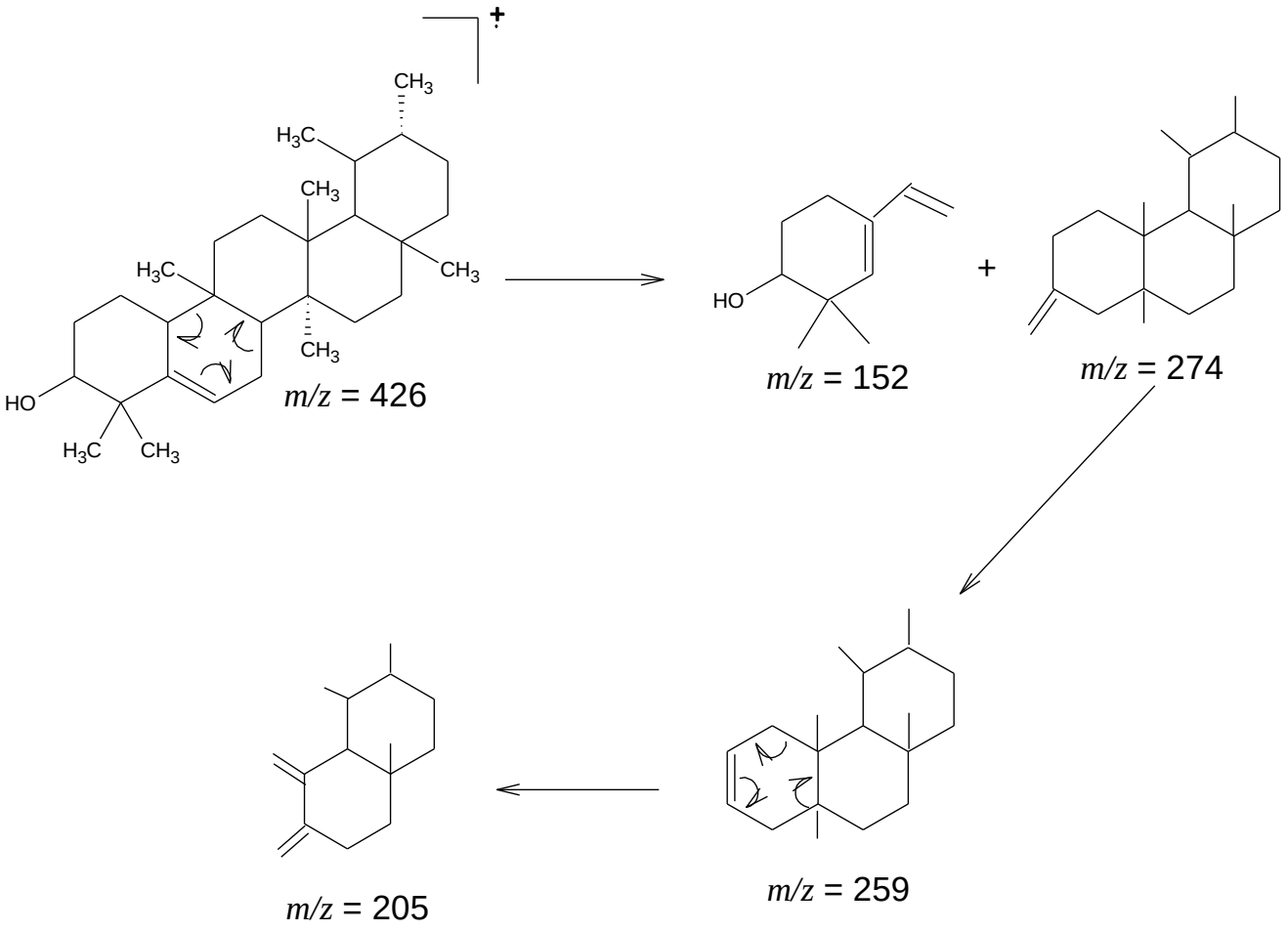


Fig: 4.23 Possible Fragmentation Pattern of Compound B



Compound B	Rhoiptelenol
426 [M]+ (6.7)	426 [M]+ (3.1)
274 (75)	274 (74.2)
260 (50)	259 (74.8)
245 (3.5)	245 (6)
205 (3.00)	205 (27.4)
173 (20.1)	173 (18.0)
152 (35.3)	152 (21.0)
134 (66.5)	134 (54.0)
121 (48.5)	123 (46.6)
109 (57.5)	107 (56.0
95 (100)	
81 (75.8)	
69 (90.0)	

Fraga *et al* (2003)

Table: 4.6 Mass Spectrum of Compound B and Rhoiptelenol

H-NMR *Spectroscopy*: Figures 4.20 and 4.21 are the ^{1}H-NMR spectra of compound B. There are eight singlets at δ 0.841, 0.862, 0.880, 0.947, 0.963, 0.977, 1.144 and 1.262 respectively. These are attributable to the eight methyl protons in the molecule. The sharp peak at δ1.262 (20H) is suggestive of methylene protons and the peaks at δ3.46, and 5.25 are indicative of the presence of an alcoholic proton and a vinyl proton respectively. The ^{1}H NMR Spectrum of the literature values reported for Rhoiptelenol (Katajima *et al*, 1994 and Fraga *et al*, 2003).

Fig. 4.24: ^{1}H-NMR Spectrum of compound B

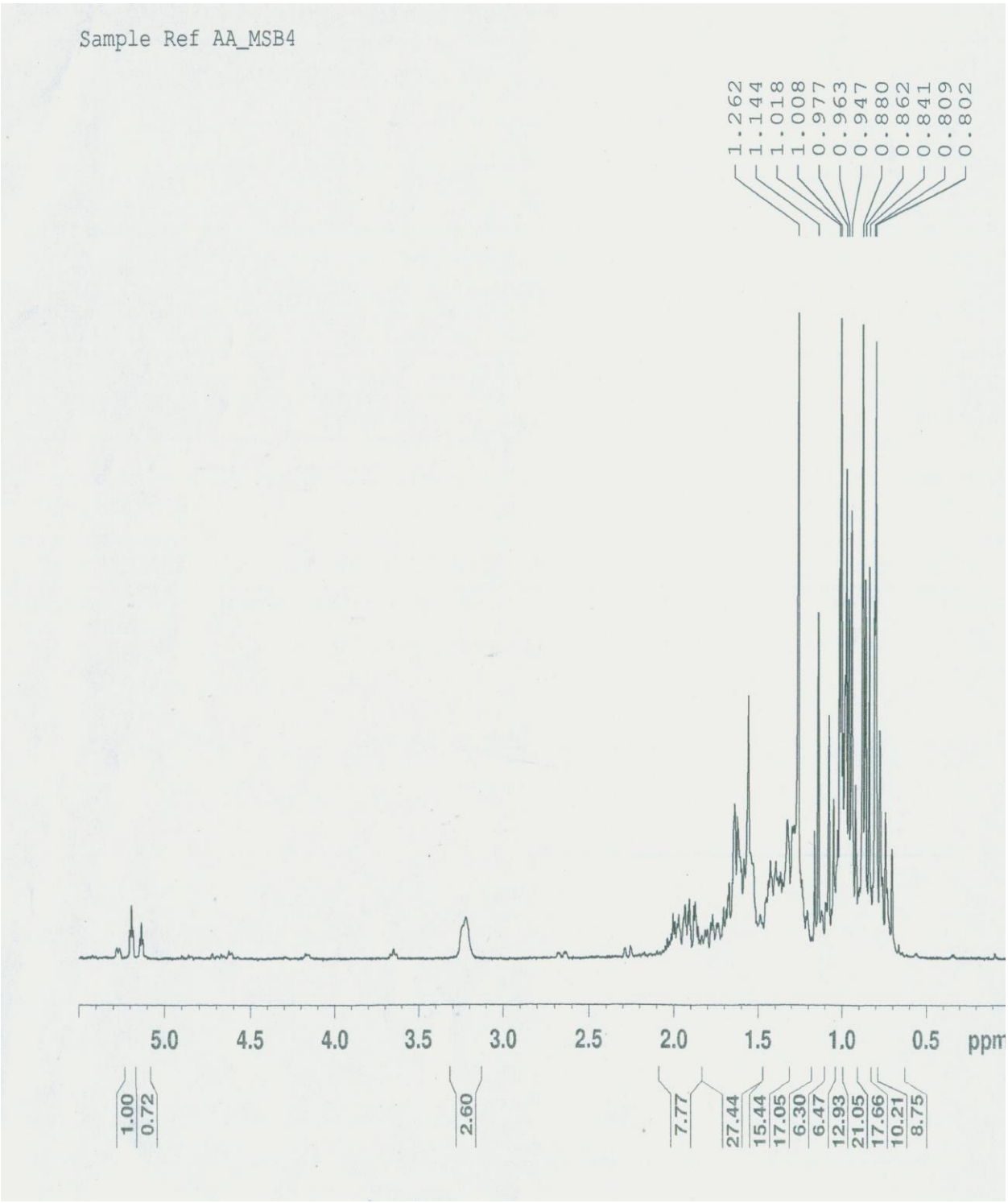
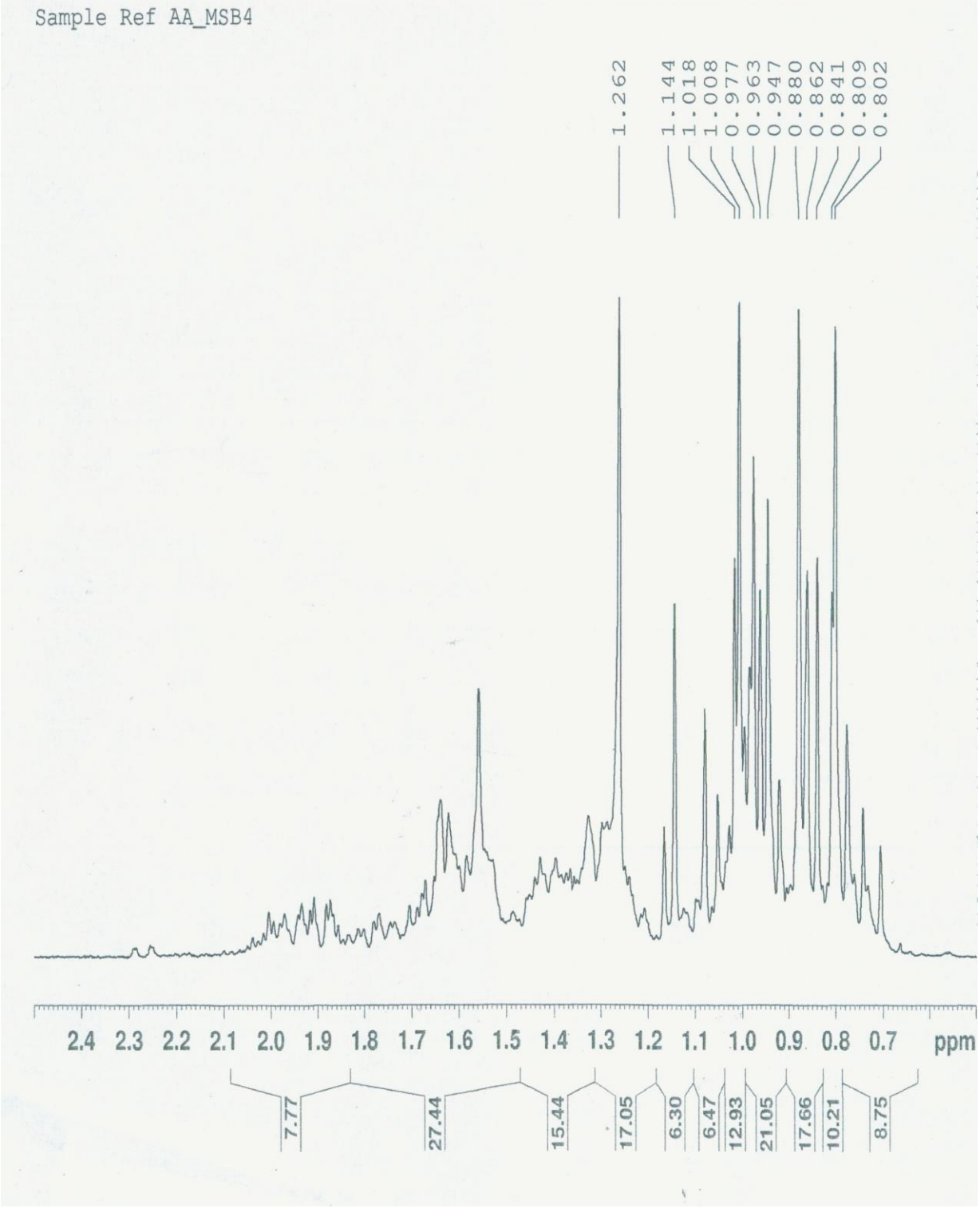
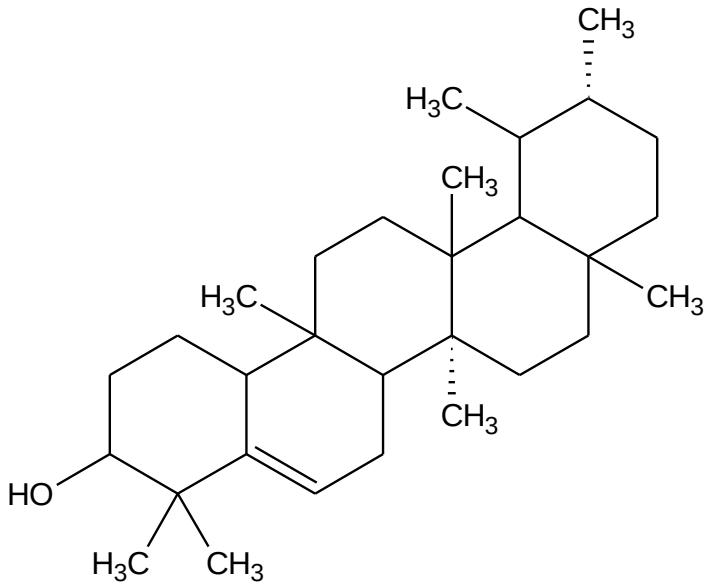


Fig. 4.25: Expanded ^{1}H-NMR Spectrum of compound B.



The first distinguishing feature between compound A and B is the GC chromatogram. The retention time for compound B (fig. 4.21) is shorter (32.13min) as against 50.43min for compound A (fig. 4.2). Basar (2005) has earlier reported that β-Amyrin has a shorter retention time in the GC chromatogram than the α-Amyrin. This was explained by the shift of the CH_3 from group an axial conformation at C-19 to C-20 which causes an increase in the planarity of the molecules that relates to the retention time. In view of the above spectroscopic evidence, compound B is Rhoiptelenol (CII) with the following structure.

Fig. 4.26: Structure of Compound B (Rhoiptelenol)



CII

A similar fragmentation pattern for the mass spectrum of Rhoiptelenol as applicable to the oleanane type of pentacyclic triterpenoids with an olefinic bond at C5 can be proposed (Fig. 4.23).

4.2.6 Spectrometric Identification of compound X

The mass spectrum of compound X is presented in figure 4.27. The results suggested a molecular weight of the compound as 486, with a molecular formular of $C_{31}H_{50}O_4$. The fragment ions obtained are signifying the retro-diels-alder fragmentation pattern for ursane type of pentacyclic triterpenoids that have an olefinic bond at C12 (fig. 4.28). The base peak at *m/z* 468 was that of the molecular ion signal. Two other fragment ion signals were observed with *m/z* values of 468 and 452 before the RDA fragmentation reaction to yield *m/z* 220 and 232 fragments (fig. 4.28). The latter probably fragmented to *m/z* 192 fraction with an elemental composition of $C_{14}H_{24}$.

Fig. 4.27: High Resolution Mass Spectrum of Compound X.

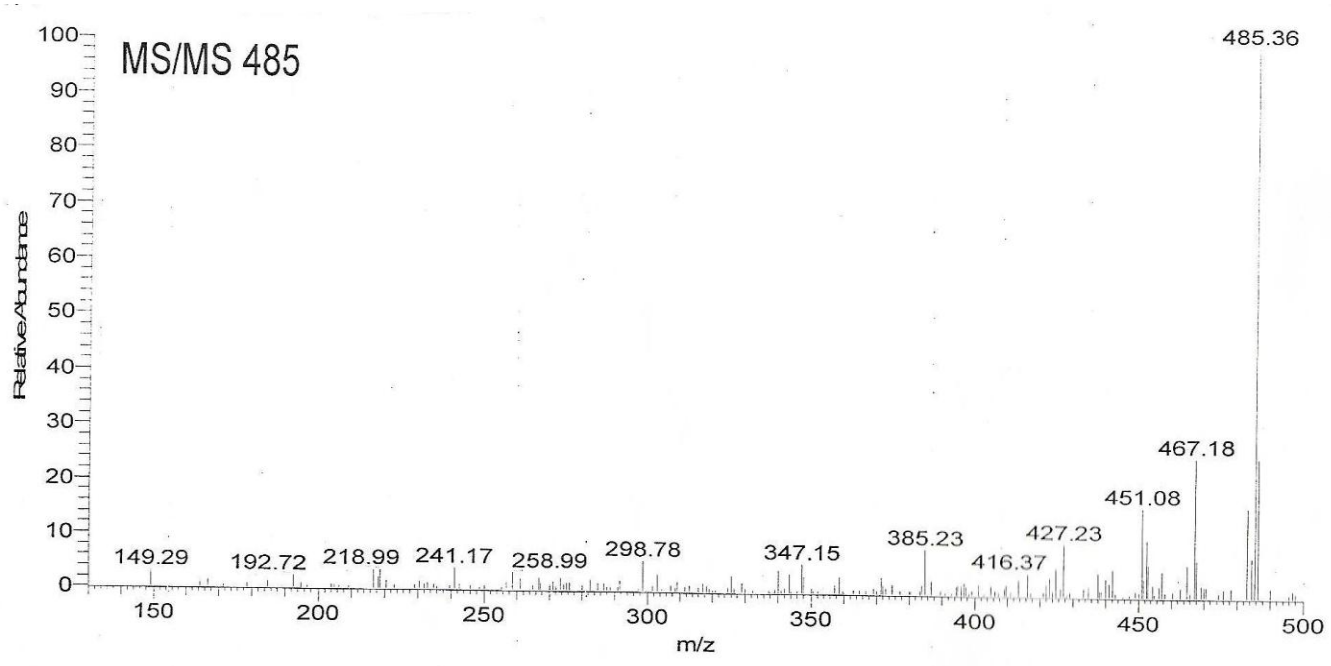
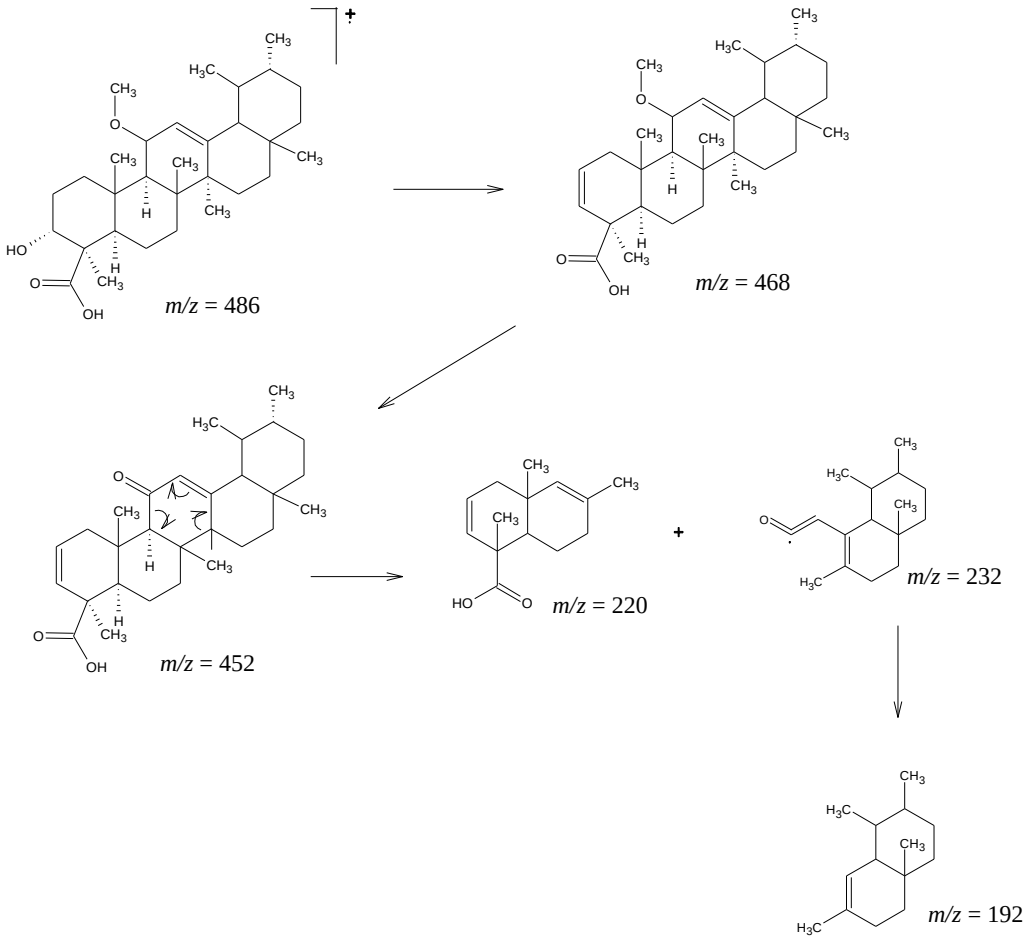
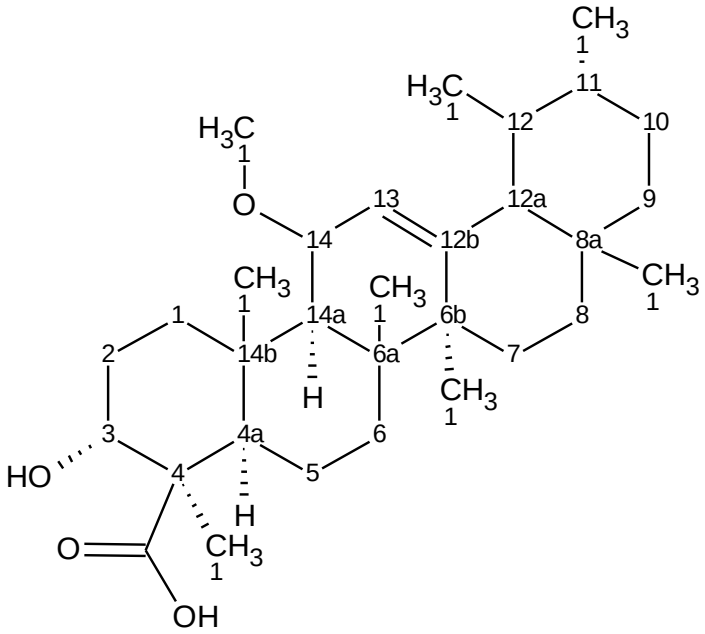


Fig. 4.28: Possible fragmentation pattern of 11-methoxyl-β-Boswellic acid.



In view of the results obtained from the chemical tests carried out and those from the high resolution mass spectrometry, and in consideration to literature reports from, Budzikiewiez *et al*, 1963 and Basar, 2005, it can be understood that compound X (CIII) is 3-hydrox-14-methoxy-4, 6a, 6b, 8a, 11, 12, 14b-heptamethy-1,2,3,4,4a, 5,6, 6a, 6b, 7, 8 , 8a, 9, 10, 11, 12, 12a, 14, 14a, 14b, -icosahydrodibenzo [a,i] phenanthrene-4-carboxylic acid (11-methyoxyl-β-Boswellic acid) or commonly known as 11-methoxy-β-Boswellic acid (Fig. 4.29).

Fig. 4.29: Structure of 11-Methoxy-β –Boswellic Acid



4.3 PHARMACOLOGICAL SCREENING OF EXTRACTS

4.3.1 Toxicity studies

The acute toxicity studies (LD_{50}) carried out on the acetone and methanol extracts of the leaves of *Commiphora kerstingii* using mice and rats as animal specimens is as shown in table 4.7. The results indicate that the acetone extract is more toxic when compared to methanol extract.

	LD_{50} in	
EXTRACT	Mice	Rats
Acetone	2154.10mg/kg	>5000mg/kg
Methanol	>5000mg/kg	>5000mg/kg

Table 4.7: The acute toxicity values (LD_{50}) of the crude extracts of *Commiphora kerstingii* in mice and rats.

4.3.2 Analgesic studies

The anti-nociceptive effects of the acetone and methanol extracts of *Commiphora kerstingii* leaves on the acetic acid induced contractions in mice are given in tables 4.8 and 4.9 respectively. Both extracts exhibited significant inhibition ($P<0.001$) of the acetic acid induced abdominal contractions, which was comparable to that of the positive control (ketoprofen). The acetone extract was found to be particularly more potent as even at low dosage of (100mg/kg) it inhibited abdominal contraction by 81.74% as against the 80.86% inhibition expressed by Ketoprofen (10mg/kg) positive control (table. 4.8and 4.9).

Treatment (mg/kg)	Mean number of abdominal constrictions	Percentage of inhibition
N/Saline (10mg/kg)	19.17± 0.98	-
100	3.50± 0.56[c]	81.74
200	2.67±0.49[c]	86.07
400	1.83±0.31[c]	90.45
Ketoprofen (10mg/kg)	3.67± 0.42[c]	80.86

C = P < 0.001 (student t-test), n =6

Table 4.8: Effect of acetone extract of *Commiphora kerstingii* leaves on acetic acid induced abdominal writhings in mice.

Treatment (mg/kg)	Mean number of abdominal constrictions	Percentage of inhibition
N/saline (10mg/kg)	19.17± 0.98	-
500	2.67± 0.71[c]	86.07
1000	2.50±0.62[c]	86.96
2000	2.17±0.60[c]	88.68
Ketoprofen (10mg/kg)	3.67± 0.42[c]	80.86

C = P < 0.001 (Student t-test), n = 6.

Table 4.9: Effect of methanol extract of *Commiphora kerstingii* leaves on acetic acid induced abdominal writhings in mice.

Fig. 4.30 Effects of acetone extract of *Commiphora kerstingii* leaves on acetic acid-induced abdominal writhing in mice.

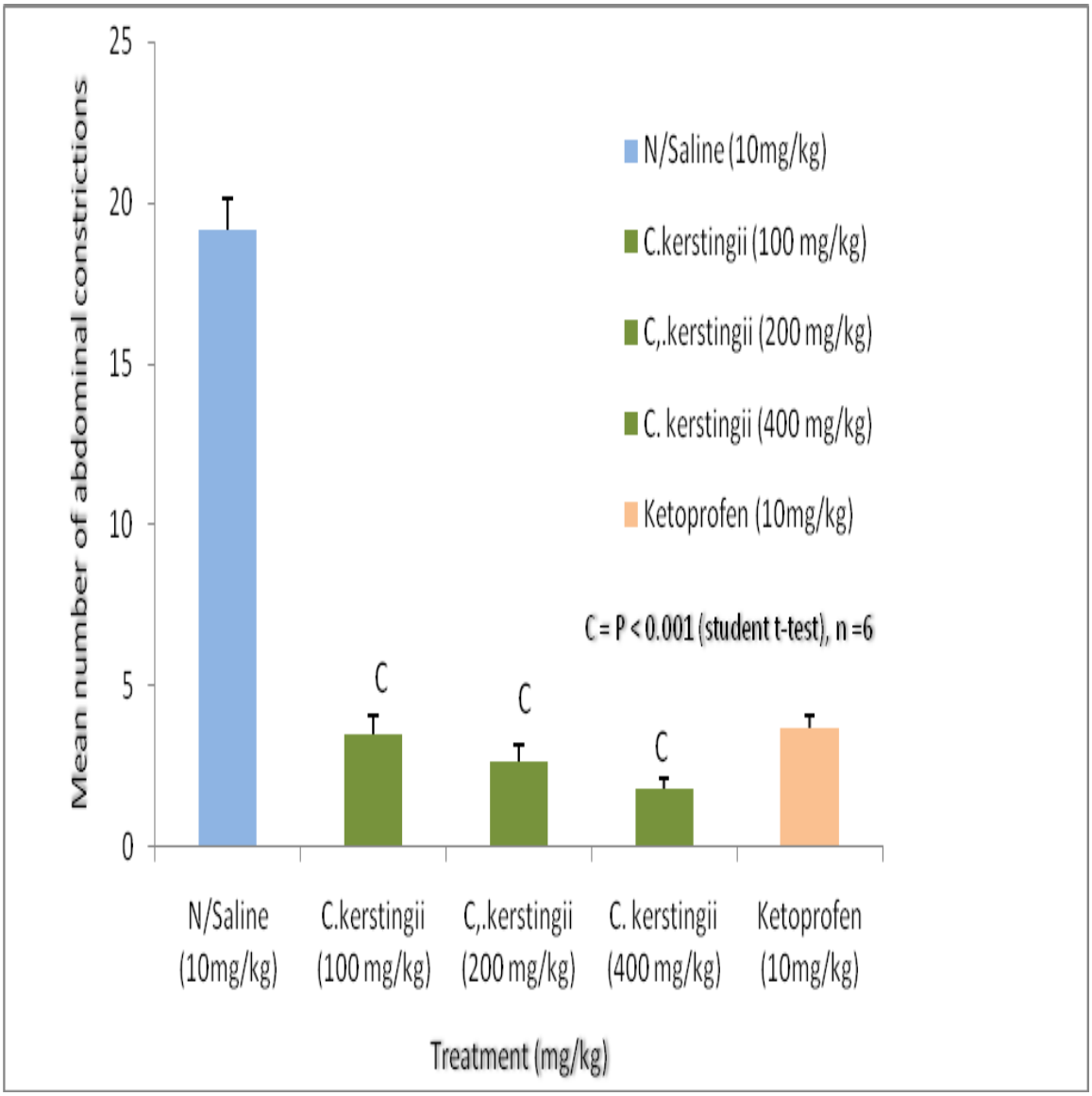
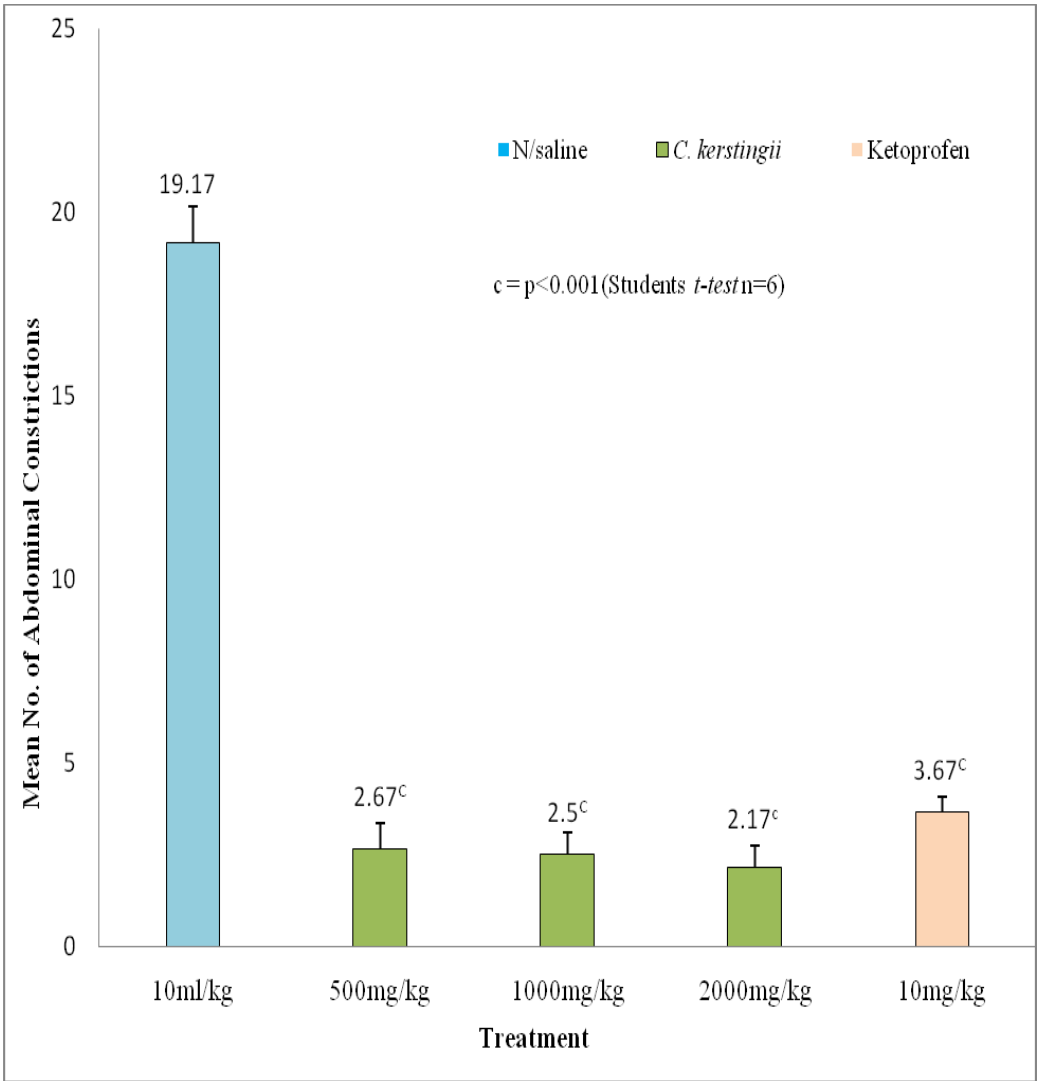


Fig. 4.31: Effects of methanol extract of *Commiphora kerstingii* leaves on acetic acid-induced abdominal writhing in mice.



4.3.3 Anti-Inflammatory Studies

The effects of crude acetone and methanol extracts of the leaves of *Commiphora kerstingii* on the carrageenan-induced paw oedema in rats are presented in tables 4.10 -4.13 as well as in figures 4.32 and 4.33 respectively. The results reveal that both extracts caused inhibition of the carrageenan-induced inflammation over a period of 4 hours.

These results also indicate that the acetone extract exhibited higher percentage inhibition of carrageenan-induced inflammation when compared to the methanol extract. In particular at a dosage of 200mg/kg, the acetone extract significantly inhibited ($P < 0.001$) the inflammation in rats for the periods of 2 to 4 hours. These values compare favourably with that of ketoprofen, 10mg/kg.

The methanol extract also significantly inhibited ($P < 0.001$) carrageen-induced paw oedema in rats particularly at the third hour.

Treatment	Paw diameter (cm)			
	1 HOUR	2 HOURS	3 HOURS	4 HOURS
N/saline (10mg/kg)	0.18± 0.03	0.29±0.04	0.28±0.02	0.210.02
100	0.073±0.01[b]	0.102±0.01[b]	0.053±0.01[c]	0.067±0.01[c]
200	0.095±0.01[a]	0.098±0.02[c]	0.077±0.01[c]	0.072±0.02[c]
400	0.078±0.01[b]	0.098±0.02[c]	0.083±0.02[c]	0.110± 0.01[b]
Ketoprofen (10mg/kg)	0.075±0.01[c]	0.130±0.01[c]	0.0163±0.02[c]	0.085±0.01[c]

a,b and c = P < 0.05, 0.01 and 0.001 respectively student t-test (n =6)

Table 4.10: Effects of acetone extract of the leaves of *Commiphora kerstingii* on the carrageenan-induced *paw* oedema in rats.

Treatment	Paw diameter (cm)			
	1 HOUR	2 HOURS	3 HOURS	4 HOURS
N/saline (10mg/kg)	0.180± 0.03	0.290±0.04	0.280±0.02	0.210±0.02
500	0.085±0.02[a]	0.140±0.01[b]	0.145±0.01[c]	0.025±0.01
1000	0.083±0.02[b]	0.160±0.02[c]	0.130±0.01[c]	0.15±0.02
2000	0.062±0.011[b]	0.110±0.01[c]	0.117±0.01[c]	0.105± 0.02[b]
Ketoprofen (10mg/kg)	0.075±0.01[c]	0.130±0.01[c]	0.0163±0.02[c]	0.0850±0.01[c]

a,b and c = P < 0.05, 0.01 and 0.001 respectively student t-test (n =6)

Table 4.11: Effects of the Methanol extract of the leaves of Commiphora kerstingii on the *carrageenan*-induced paw oedema in rats

Treatment (mg/kg)	% inhibition at various times (hours)				Mean % of inhibition
	1hr	2hrs	3hrs	4th hrs	
N/Saline (1ml/kg)	-	-	-	-	-
100	59.4	64.8	81.1	68.1	68.3
200	47.2	66.2	72.5	65.7	62.9
400	56.7	66.2	70.4	47.4	60.2
Ketoprofen (10mg/kg)	58.3	55.2	41.8	59.5	53.0

Table 4.12: Percentage of inhibition by acetone extract of the leaves of *Commiphora kerstingii* and ketoprofen on carrageenan induced oedema in rats.

Treatment (mg/kg)	% inhibition at various times (hours)				Mean % of inhibition
	1hr	2hrs	3hrs	4th hrs	
N/Saline (1ml/kg)	-	-	-	-	-
500	52.8	51.7	48.2	88.1	60.2
1000	53.8	44.8	53.6	28.6	45.2
2000	65.6	62.1	58.2	50.0	59.0
Ketoprofen (10mg/kg)	58.3	55.2	41.8	59.5	53.0

Table 4.13: Percentage of inhibition by methanol extract of the leaves of *Commiphora kerstingii* and ketoprofen on carrageen induced oedema in rats.

Fig 4.32: Effect of Acetone extract of *C. kerstingii* Leaves on carragenann-induced Paw oedema in Rats

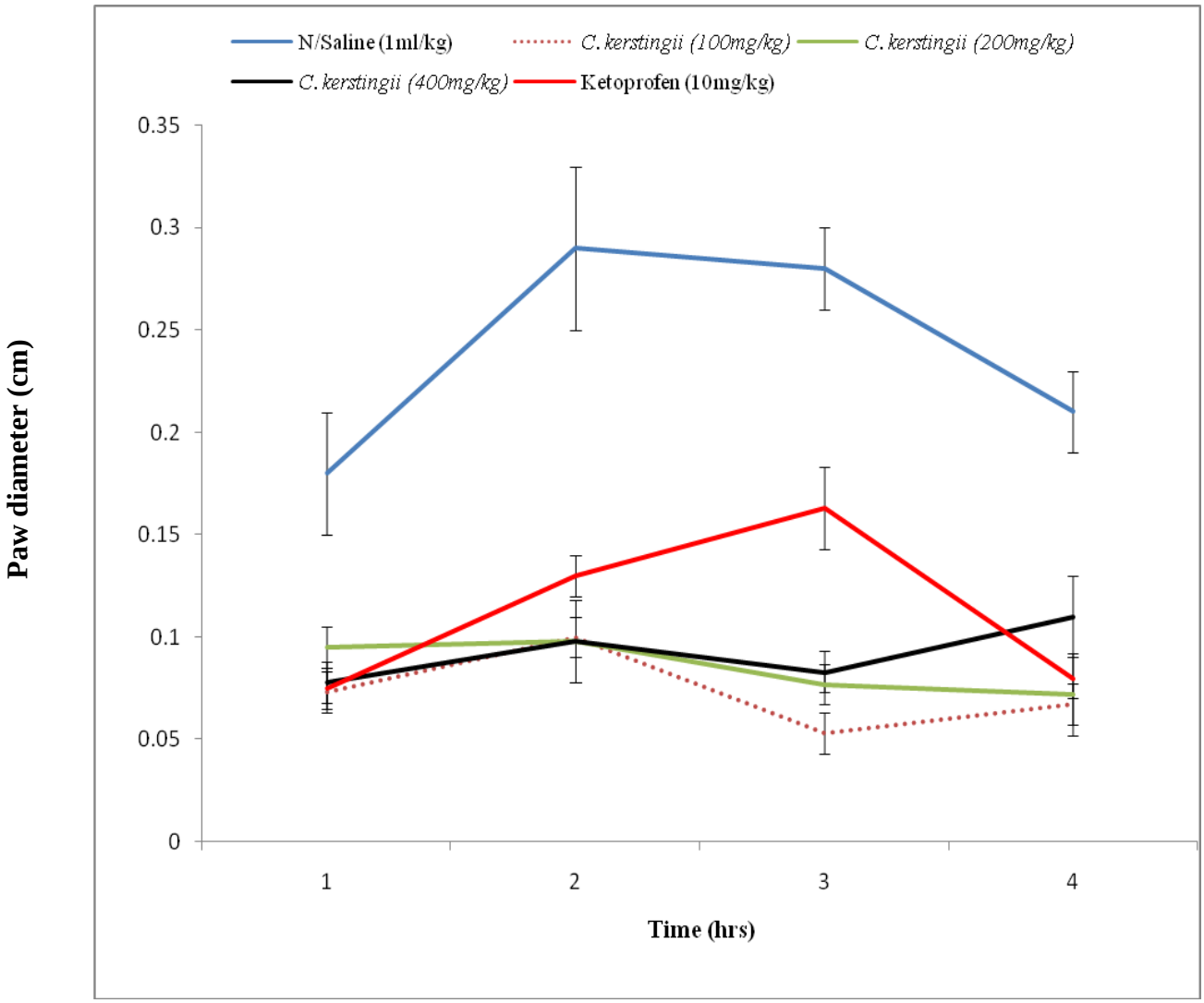
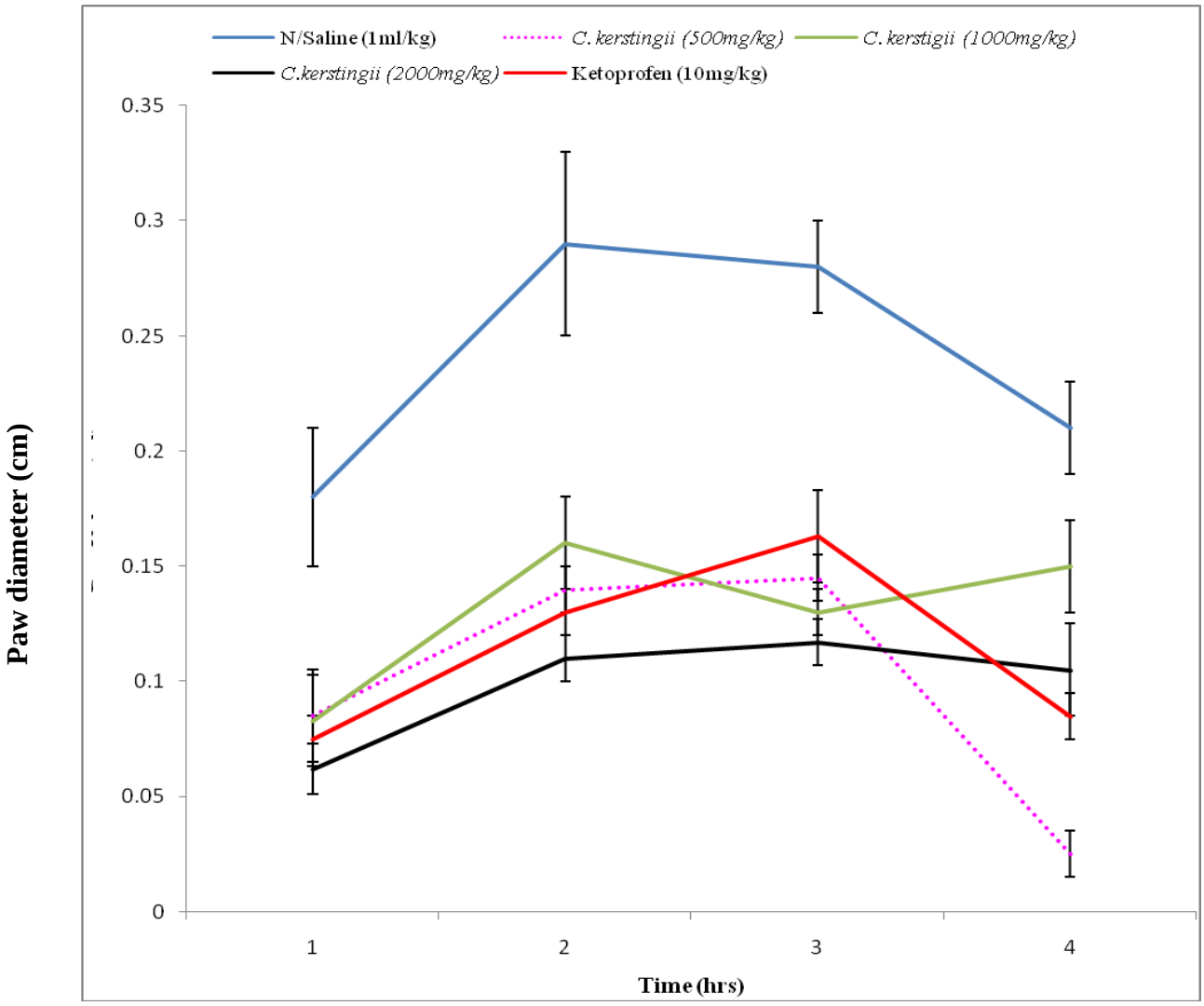


Fig 4.33: Effect of Methanol extract of *C. kerstingii* Leaves on Carrageenan-induced Paw oedema in Rats



4.4 ANTI-MICROBIAL ASSAY

4.4.1 Anti-microbial activity of the crude Extracts

The anti-microbial sensitivity results for the crude acetone and methanol extracts of the leaves of *Commiphora kerstingii* are presented in tables 4.14 and 4.15 respectively. Generally the acetone extract has higher sensitivity against the tested organism than the methanol extract. Even at the concentration of 12.5mg/mL the zone of inhibition by the acetone extract for *klebsiella pneumonia, Streptococcus pyogenes and Pseudomonas aeruginosa* are 30, 32 and 35mm respectively, values in both cases of which are higher than those for the control. The methanol extract also has a high sensitivity against *Pseudomonas aeruginosa* (30mm) which is greater than that for the control. However, even at 200mg/mL it does not inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* which are gram positive and gram negative bacteria respectively.

Test organism	Gram ±	Concentration of extract ($x10^3$ ug/mL)			Ampiclox (5mg/mL)
		50	25	12.5	
Staphylococcus aureus	+		20		20
Streptococcus pyogenes	+			32	26
Escherichia coli	_	17			16
Salmonella typhi	_		20		19
Klebsiella pneumonia	_			30	21
Pseudomonas aeruginosa	_			35	26

Table 4.14: Anti-microbial activity of acetone extract of the leaves of *Commiphora kerstingii* at various concentrations (zone of inhibition in mm).

Test organism	Gram ±	Concentration of extract (x10^3 ug/mL)				Ampiclox (5mg/mL)
		200	50	25	12.5	
Staphylococcus aureus	+	0				20
Streptococcus pyogenes	+			25		26
Escherichia coli	_	0				16
Salmonella typhi	_		16			19
Klebsiella pneumonia	_			24		21
Pseudomonas aeruginosa	_					26

Table 4.15: Antimicrobial activity of methanol extract of the leaves of *Commiphora kerstingii* at various concentrations (Zone of inhibition in mm)

4.4.2 Minimum Inhibitory concentration (MIC) of the crude extracts

The minimum concentration of the crude extracts that inhibits the growth of the various test organisms employed in this research is presented in table 4.16. The acetone extracts has lower MIC values for 5 of the 6 tests organisms studied than the methanol extracts. The MIC values for acetone and methanol extracts are comparable for *Pseudomonas aeruginosa*.

Test organisms	MIC in mg in L^{-}	
	Acetone Extract	Methanol Extract
Staphylococcus aureus	25.0	> 200.0
Streptococcus pyogenes	12.5	25.0
Escherichia coli	50.0	> 200.0
Salmonella typhi	25.0	50.0
Klebsiella pneumonia	12.5	25.0
Pseudomonas aeruginosa	12.5	12.5

Table 4.16: Minimum Inhibitory concentration (MIC) of the crude acetone and methanol extracts of the leaves of *Commiphora kerstingii*.

4.4.3 Minimum bactericidal concentration (MBC) of the crude extracts

The results for the minimum concentration of the crude extracts that kills the tested micro-organisms are given in table 4.17. The results revealed that the acetone extract is a more effective bactericidal drug than the methanol extract on *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Salmonella typhi and Klebsiella pneumonia.* The Bactericidal properties of the acetone and methanol extracts are comparable on *Pseudomonas aeruginosa.*

Test organisms	MBC in mg in L^{-}	
	Acetone Extract	Methanol Extract
Staphylococcus aureus	50.0	> 200.0
Streptococcus pyogenes	25.0	50.0
Escherichia coli	100.0	> 200.0
Salmonella typhi	50.0	100.0
Klebsiella pneumonia	25.0	50.0
Pseudomonas aeruginosa	25.0	25.0

Table 4.17: The minimum bactericidal concentration of the crude acetone and methanol extracts of the leaves of *Commiphora kerstingii*.

4.5 ANTI OXIDANT PROPERTIES

The acetone and methanol extracts exhibited moderate anti-oxidant properties. Compound A and B however have no radical scavenging properties. Substances which have a potential radical scavenging effects show white inhibition zones over a violet background when spread with DPPH.

CHAPTER FIVE

DISCUSSIONS

5.0 GENERAL DISCUSIONS

5.2 PHYTOCHEMICAL METHODS

5.2.1 Extraction

The two solvents separately employed for the extraction of the powered leaves have extracted reasonable quantities in both cases. Acetone is a slightly polar solvent therefore it is expected to have extracted both non-polar components such as chlorophyll, pigments and oils as well as some polar solutes. This is responsible for the high yield of the acetone extract.

Methanol is however strongly polar and would have extracted almost all the polar constituents of the leaves. Furthermore, methanol dissolves polar metabolites as well as medium and low polarity compounds by co-solubilization. In addition, methanol has higher penetration into cell membranes, permitting the extraction of endocellular components, while non-polar solvents, such as petroleum ether, wash out exocellular components only (Pateh, 2006).

5.1.2 Preliminary Phytochemical Screening

The preliminary phytochemical studies carried out on the leaves of *Commiphora kerstingii has* unveiled the presence of a lot of primary and secondary metabolites. The leaves are particularly very rich in saponins, steroids terpenoids and tannins especially in the acetone extract. There are also considerable amounts of carbohydrates. Glycosides are also slightly present. The presence of these metabolites has vindicated earlier reports that a number of plant species of the same family with *C. kerstingii* have most of these chemical constituents (Hanus *et al.*, 2005; Basar, S., 2005 and Kubmarawa, *et al.*, 2008). In particular Kubmarawa, *et al*., (2008) reported the presence of

saponins, tannins and volatile oils from the ethanolic extract of the stem bark of *Commiphora kerstingii*. Similarly Musa , A.A. (2008) recently reported the presence of alkaloids, saponins, anthraquinones, cardiac glycosides, tannins and flavonoids from the methanolic extract of the stem back of *Commiphora kerstingii*.

Both digitalis and cardiac glycosides, steroidal aglycones, and cardinolide aglycones have been discovered to be moderately present in both the acetone and methanol extracts of the leaves. The presence of steroids and terpenoids in the acetone extract only is not unexpected as most of the constituents are essentially less polar in nature. The vast number of these chemical constituents in the leaves of *Commiphora kerstingii*, some of which could be potentially bioactive vindicates the ethnomedical usage of the plant in the treatments of some ailments.

5.2 PHYSICAL AND CHEMICAL PROPERTIES OF ISOLATED COMPOUNDS

The R_f values of compounds A, B and X of 0.46, 0.42 and 0.56 obtained from TLC chromatography using ethyl acetate/n-hexane (1:9) solvent system suggests that these compounds are largely non-polar in nature. Both samples are soluble in $CHCl_3$.

The compounds, on derivatization with anisaldehyde and with vanillin/sulphuric acid reagents gave characteristic colours of triperpenoids. This means that the compounds are terpenoidal in nature.

5.3 PHARMACOLOGICAL STUDIES

The acetone and methanol extracts of the leaves of *Commiphora kerstingii* have shown significant anti-nociceptive activities. The acetic acid-induced writing method also called the abdominal constriction response is very sensitive as it can detect anti-nociceptive effects of

compounds (substances) at a dosage that may be inactive in other methods such as tail-flick test (Koster *et al*, 1959, Collier *et al*, 1968 and Bentley *et al*, 1981). The significant anti-nociceptive activity demonstrated by the acetone and methanol extracts of the leaves of *Commiphora kerstingii* may be due to the terpenoidal constituents of the plant. Flavonoids, tannins triterpenoids possess analgesic and/or anti-inflammatory activities (Ahmadiani et *al*, 1998 and Ahmadiani *et al*, 2000). In particular Freire *et al*, (1991) has reported that the administration of Glutinol (30mg/kg, p.o.) reduced writhing induced by acetic acid in mice by 40% and the carrageenan-induced paw oedema in rats by 73%. The more effective analgesic properties of the acetone extract of the leaves of *Commiphora kerstingii* suggests a higher concentration of the active ingredients.

Both extracts have also shown a dose dependent inhibition of the carrageenan-induced inflammation in rats than the negative control. The percent inhibition in oedema volume of the extract is dose dependent. This anti-inflammatory property of the extracts may be due to the presence of Glutinol and Rhoiptelenol as well as Bowellic acids and their derivatives. Glutinol in particular has earlier been reported by Freire *et al*, (1991) to reduce writhing induced by acetic acid in mice by 40% and the carrageenan induced paw oedema in rats by 73%. Recio *et al* (1995) also reported on the structure activity relationship between anti-inflammatory activity and natural triterpenoids and indicated that an acid functional group increased the effects.

These results provide evidence for the anti-inflammatory and analgesic properties of the leaves of *Commiphora kerstingii*, as having a potential for local therapeutic applications in pain and inflammatory diseases.

The results of the anti-oxidant properties carried out indicated that even though the acetone and methanol extracts exhibited moderate radical scavenging activities, compounds A, B and X have no such similar properties. This suggests that both compounds A, B and X are not phenolic in nature since earlier reports have shown that there is a high correlation between anti-oxidant activity and phenolic compounds (Odabasoglu, *et al*, 2004). Compounds like tannins are most likely to exhibit antioxidant activity and are likely responsible for the moderate anti oxidant properties shown by the acetone and methanol extracts. This radical scavenging properties of the crude acetone and methanol extracts is in agreement with an earlier report by Musa (2008) that the methanolic extract of the stem bark of *Commiphora kerstingii* exhibited significant anti oxidant activity.

5.4 ANTI-MICROBIAL ASSAY

The results of the anti-microbial assay obtained indicated the existence of compounds with antimicrobial activity in the crude acetone and methanol extracts of the leaves of *Commiphora kerstingii*. The results showed a good correlation of the reported use of the plant in traditional medicine against infectious diseases. For instance the inhibition of *E. coli* by the acetone extract has justified the use of the plant for the treatment of diarrhea and dysentery in traditional medicine. This is in agreement with an earlier report by Kubarawa *et al* (2007) on the stem bark of *Commiphora kerstingii*.

The acetone extract of the leaves of the plant was most potent against all the test organisms tested with MIC values ranging from 12.5-50.0mgmL^{-1}. The lowest MIC (highest potency) exhibited was 12.5mgmL^{-1} for both acetone and methanol extracts against *P. aeruginosa* and 12.5mgmL^{-1} for the acetone extract against *K. pneumonia*. The antibacterial activities of the acetone extract

showed good response on both gram-negative and gram-positive bacteria tested. This is inspite of the fact that the gram-negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents (Ndukwe *et al*, 2005). The acetone extracts is therefore most promising since it exhibited marked activity or a broad spectrum of activity towards gram-positive and gram-negative bacteria. The results suggest either good potency or high concentration of the active ingredient.

The acetone extract also has a strong activity against *S. aureus with* a MIC value of 25.0mgmL^{-1}. This organism is known to play a significant role in such skin diseases (Srinivasan *et al*, 2001). This suggests that the plant can be a good source of the compound (s) that can be effective against such skin infections. Furthermore, both *P. aeruginosa and E. coli*, the two organisms that cause infections that are very difficult to combat due to their multi-drug resistance (Salie *et al*, 1996, Afolayan and Aliero, 2006), were found to be susceptible to particularly the acetone extract. This gives an indication that compound (s) can be obtained from the plant leaves that can be used against infections caused by these organisms.

The potency of the extracts based on the zones of inhibition as compared to a standard antibiotic (Ampiclox at 5mgmL^{-1}) indicates that the acetone extract is of remarkably higher activity against all the organisms tested than the standard drug. Nevertheless, anti-bacterial properties of several plant extracts have been attributed to the presence of secondary metabolites such as flavonoids, saponins, tannins, steroids and terpenoids (Margaretha *et al*, 1979, Cowan, 1999 and Okoli and Iroegbu, 2005) and since all these metabolites have been certified to be present in the leaves of this plant, it is probable that the strong anti-microbial activity of the plant is as a consequence of these metabolites.

5.5 SPECTROSCOPIC STUDIES

Compound A:

The results from infra-red spectroscopy have suggested the presence of C-H, O-H, C=C and C-O bonds in the molecule. Indications are also clear that the compound does not contain aromatic rings of C= O and or N-H bonds.

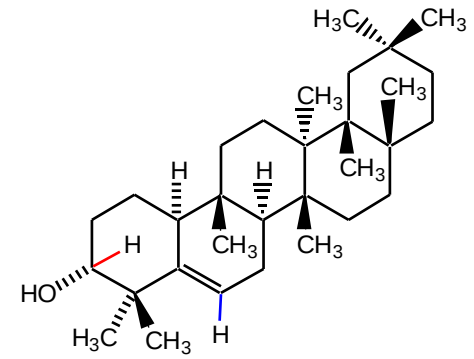
The MS spectrum suggested a molecular ion peak of 426 with a molecular formula of $C_{30}H_{50}O$. The formation of most of the fragments with *m/z* values in agreements with fractions obtainable when the compound undergoes cleavage at C5 as suggested by the retro-diels-alder (RDA) reaction suggests that the compound is a pentacyclic triterpenoid. Although mass spectra alone are insufficient in structure determination of target compounds, in the case of triterpenoids however such as Boswellic acid, and especially for assignment of peaks which could not be properly isolated, the interpretation of the mass spectra was found to be very useful (Basar, 2005). This is because members of 12-ursene and 12-Oleanene series showed very similar fragmentation pattern of the RDA reaction in mass spectra. The fragment peaks obtained for compound A, are consistent with all the peaks obtainable in the mass spectrum of Glutinol (Chen *et al*, 2003).

The ^{1}H NMR of compound A is in very good agreement with most of literature reports on glutinol. There are about 8 methylene protons, six of which appear in singlet peaks at δ 0.85, 0.95, 0.99, 1.00, 1.04, 1.09, 1.14 and 1.16. The peak at δ1.262 is probably indicative of nine methylene protons in the compound. Peaks at δ1.39, 1.58 and 1.7 are suggestive of the six methine protons in the molecule. The chemical shifts at δ3.5 and 5.6 are suggestive of alcoholic and olefinic protons respectively.

The 2-DNMR clearly indicated the connectivities between the carbon atoms in the skeleton of the molecule and between C-H bonds as well as the position of the unsaturations. In particular, HMBC spectrum indicated C-H relationships through two and three bond ($^{2}J_{CH}$ and $^{3}J_{CH}$)

connectivities. Together with observed couplings in the ^{1}H-^{1}H COSY spectrum the correlation of HMBC resulted in small subunits of the molecule which were connected to each other.

Based on the physical properties (physical state, solubility and melting point) and chemical properties (R_f values and colour with derivatising reagents) as well as spectroscopic analysis and in comparison with appropriate literature data (Sageer 2003, Gutierrez-Lugo *et al*, 2004, Basar, 2005 and Silveira *et al*, 2005) it can be concluded that compound A is 3 epi-glutinol



Structure of compound A

CI

Compound B

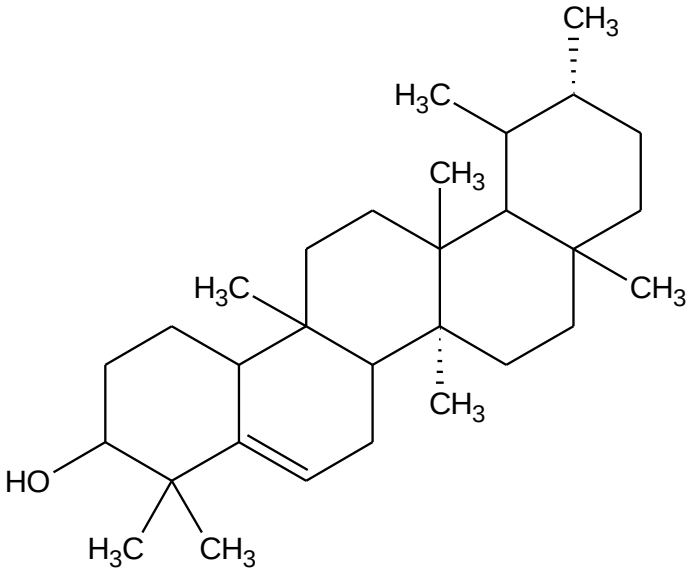
The infra-red spectroscopy of compound B has suggested the presence of C-H, O-H, C-O and C=C bonds in the compounds. There is no clear indication of the presence of C=0, N-H or aromatic systems in the compounds.

Gas chromatography indicates that compound B has a shorter retention time than compound A. The mass spectrum indicates that the compound has a molecular weight of 426 and a suggested molecular formula of $C_{30}H_{50}O$. This suggests that the compound B is an isomer of compound A. The *m/z* values of nearly all the fragments obtained are very similar to those obtained for compound A and also in good agreement with the fragmentation pattern of RDA reaction for pentacyclic triterpenoids. The similarity in the mass spectrum of compound B and its shorter retention time when compared with compound A is suggestive that compound B is β-Amyrin. The longer retention time of compound A could be attributed to the fact that compounds of ursane series have longer retention time than the corresponding Oleanane compounds. This was explained by the shift of the CH_3 group from an axial conformation at C20 in Oleanane structures to an equatorial conformation at C19 in Ursane type compounds which caused an increase in the planarity of the molecules that related to their retention time (Burnough-Radosevich, *et al*, 1985).

The ^{1}H NMR spectra obtained for compound B further conform its similarity with compound A. Compound B however has 8 methyl singlet peaks at δ 0.802, 0.841, 0.862, 0.880, 0.947, 0.963, 0.977 and 1.008. No doublet methyl protons are noticeable. The intensity of the methylene proton peaks at δ1.262 (20H) is higher than the corresponding value for compound A. This shows that there are more methylene protons in compound B. The alcoholic proton appeared at δ 3.25 and the Olefinic proton peaks at δ5.25. The peaks at δ1.58 are suggestive of the methine protons in the compound. The absence of methyl doublet peaks in the ^{1}H NMR spectrum of compound B

confirms the facts that there is no methyl group at the equatorial conformation of C19, but an axial conformation of C20.

In view of the physio-chemical properties of compound B (Physical state, solubility, melting point, R_f value and colouration with derivatizing reagents), the spectral properties and in consideration with relevant literature information (Sageer, 2003, Gutierrez-Lugo *et al*, 2004, Basar, 2005, Silveira *et al*, 2005 and Chen *et al*, 2003) it can be concluded that compound B is Rhoiptelenol.

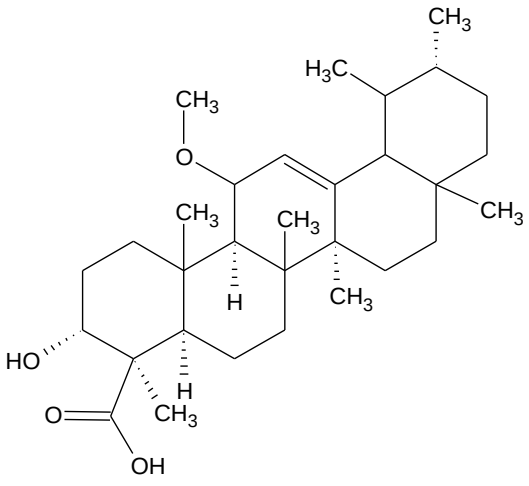


CII

Compound X

The chemical tests have suggested that compound X is a triterpenoic acid. This is not unexpected because literature reports from numerous sources (Hanus *et al*, 2005, Basar, 2005, etc) have indicated the presence of tetracyclic and pentacyclic triterpenoic acids and derivatives from mainly the resinous exudates of *Commiphora* and *Boswellia* species.

The high resolution mass spectrum obtained for compound X has further vindicated these reports. Most of the fragment ions obtained are consistent with RDA fragmentation pattern chacteristics of pentacyclic triterpenoids with olefinic bond at C12. Even though mass spectra alone are insufficient for structure elucidation of target compounds, Basar (2005) has reported that its application to the characterization of pentacyclic triterpenoids such as Bowelled acid is very useful. It is therefore suggestive that compound X is 11-methoxy-β-Bowelled acid.



CIII

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.0 PREVIEW

6.4 SUMMARY

The presence of alkaloids, carbohydrates, saponins, steroids terpenoids and tannins were indicated in the crude acetone and in most cases methanol extracts of the leaves of *Commiphora kerstingii*.

The acetone extract is slightly toxic. Both extracts however exhibited effective anti-nociceptive, anti inflammatory and anti oxidant properties. Furthermore, the acetone extract in particular exhibited a broad spectrum of activity against gram positive and gram negative bacteria. The antibacterial assay has generally revealed that compounds that have good potency against *S. aureus, S. pyogenes, E. coli, S. typhi, K. pneumonia and P. aeruginosa* can be obtained from the leaves of *Commiphora kerstingii*.

Extensive phytochemical studies led to the isolation and characterization of pentacyclic triterpenoids as Glutinol and Rhoiptelenol as well as 11-methoxy-β-Bowelled acid from the acetone extract of the leaves of *Commiphora kerstingii*.This is the first time that such compounds are being reported to be present in a *Commiphora* specie. Some of the pharmacological properties observed especially from the acetone extract of leaves of the plant have been attributed to probably the presence of these terpenoids.

6.5 CONCLUSION

The presence research findings have shown that the leaves of *Commiphora kerstingii* are extensively rich in secondary metabolites. These plant leaves therefore have a high potential for a vast number of bioactive compounds. The effective anti inflammatory and analgesic properties as well as a good potency and broad spectrum of activity against both gram-positive and gram-negative bacteria by the extracts of the plant are all as a result of the bioactive constituents. These findings have provided scientific basis to the ethnomedical usage of the plant. This research findings especially on the leaves of the plant is to the best of our knowledge, a break through to the data bank in the literature.

6.6 RECOMMENDATIONS

The research findings have prompted some vital areas for further studies. These include:

i. Isolation and characterization of compounds of bioactivity especially from the acetone extract of the leaves of the plant.

ii. Investigation of these bioactive compounds for pharmacological studies such as anticancer, antiviral, anti-asthma, anti-diarrhea and antispasmodic properties.

iii. Isolation and characterization of compounds responsible for the good potency of the extracts against most of the gram-negative and gram-positive bacteria tested.

iv. Extensive chronic toxicity studies on the plant leaves in order to avoid any serious deleterious effect as a result of prolonged usage.

v. Thorough bioassay studies of the active plant constituents so as to arrive at the most suitable form of usage and the correct dosage forms and regiments.

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