PHYTOCHEMICAL, ANTIHYPERTENSIVE AND ANTIMICROBIAL STUDIES ON THE STEM BARK EXTRACT OF *STEREOSPERMUM KUNTHIANUM* CHAM (BIGNONIACEAE)

BY

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BY

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MARCH, 2016

Declaration

I declare that the work in this thesis entitled **"**Phytochemical, Antihypertensive and Antimicrobial Studies on the Stem Bark Extract of *Stereospermum kunthianum* CHAM (Bignoniaceae).'' has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry Ahmadu Bello University, Zaria under the supervision of Professor M.I Sule, Professor (Mrs.) H.S. Hassan and Professor U.U. Pateh. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project report was previously presented for another degree or diploma at any University.

Umar Aliyu Hanwa	……	……
(Name of student)	Signature	Date

Certification

This thesis entitled "PHYTOCHEMICAL, ANTIHYPERTENSIVE AND ANTIMICROBIAL STUDIES ON THE STEM BARK EXTRACT OF *STEREOSPERMUM KUNTHIANUM (BIGNONIACEAE)*." By Umar Aliyu HANWA meets the regulations governing the award of the degree of Doctor of Philosophy of Science of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

_____ _____

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Dean, School of Postgraduate Studies, A.B.U. Zaria

Dedication

This thesis is dedicated to Almighty Allah the Exalted and Most High, then to my beloved family.

Acknowledgement

My sincere gratitude goes to my supervisor, Professor M.I. Sule for his patience, constructive criticism, and immense guidance through out this research work. I am also indebted to my co-supervisors Professor (Mrs.) H.S. Hassan and the Dean of the Faculty in person of Professor U.U. Pateh for their words of encouragement, advice and support. My appreciation goes to Dr.A.M. Musa as my Head of Department for yhe interpretation of spectral data and for linking me to the Professor Simon Gibbons University of London, School of Pharmacy London.Who ran the NMR spectrum of HNW2.

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I would finally like to thank any other relations old and new who had directly or indirectly contributed to the successful completion of this studies. God bless. I am indeed very grateful.

Abstract

Stereospermum kunthianum (Family: Bignoniaceae) plant is used in Hausa ethnomedicine in treating bronchitis, veneral diseases, diarrheoa and dysentery. It is also used in the treatment of ulcers, leprosy, skin eruptions, respiratory ailments, abortifacient, antihypertensive and gastritis. The plant is common in Northern Nigeria where it is used as abortifacient and as antihypertensive agent. Preliminary phytochemical investigation of methanolic extract of the stem bark of *Stereospermum, kunthianum* revealed the presence of sterol/triterpenes, coumarin, saponins, flavonoids, steroids, tannins and carbohydrates. Extensive phytochemical investigations using silica gel column chromatography, gel filtration and preparative thin layer chromatography led to the isolation of β – sitosterol from Hexane fraction, ipolamiide (an Iridoid glyciside) and a flavonoid diglycoside Quercetin 3-O-β-D-glycopyranosyl (1→6)-α-rhamnoside (rutin) from the acetone fraction. The structures of these compounds were elucidated using UV, IR and 1D and 2D NMR. The antihypertensive property was evaluated by studying the effect of the acetone fraction on the cat blood pressure. The acetone fraction, dose dependently decreased cat blood pressure and the decrease in the blood pressure was not blocked by atropine.This implied that the action of acetone was not via muscarinic receptors.The paper discs diffusion method was used to determine the antimicrobial activities of the stem bark methanol extract, hexane, ethyl acetetae and acetone fractions using the following clinical isolates, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi.* as the test microorganisms. The results of the antimicrobial study obtained indicated that the extract of the *Stereospermum kunthianum* inhibited the growth of the microorganisms within the range of 10 – 22mm diameter zone of inhibitions. This, therefore, shows that

the extract and fractions contains substance(s) that inhibit the growth of some microorganisms. Ipolamiide isolated from acetone fraction was found to be active against *Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoneae, Escherichia coli, Salmonella typhi* and *Candida ikrusei,* with zones of inhibition ranging from 21 – 29 mm against all the susceptible microorganisms.

The results of this study has added compounds ipolamiide and rutin to data base of the family of Bignoniaceae, expanded our knowledge on phytochemistry of *Stereospermum kunthianum* and provided scientific rationale for the ethnomedicinal uses of the plant.

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ABBREVIATIONS AND ACRONYMS

A. B. U. :	Ahmadu Bello University
AV:	Atrial ventricular
ACh. :	Acetylcholine
B.C.:	Before Christ
$CHCl_3$:	Chloroform
COOH:	Carboxylic acid group
Ca^{+2} :	Calcium ion
MRSA :	Methicillin Resistant *Staphylococcus aureus*
^{1}H-NMR:	Proton nuclear magnetic resonance
^{13}C-NMR:	Carbon-13 nuclear magnetic resonance
GC-MS:	Gas chromatography - Mass spectroscopy
M/Z:	Mass to charge ratio
TLC:	Thin layer chromatography
NaOH :	Sodium hydroxide
NH_4OH:	Ammonium hydroxide
C-C:	Carbon-Carbon linkage
UV:	Ultraviolet
FTIR:	Fourier transform infra-red
IR:	Infra-red
HCl:	Hydrochloric acid
WHO :	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

The use of plant derived products by man in medicine dates back to pre-historic times. It was believed that at the beginning of creation, God made plants and herbs before creating man. This was done with the injunction that man should eat from the plants and herbs for his good health. Henceforth, herbs have been in use from antiquity and were the first medicine used by man (Kloss, 1992). From time immemorial man and animals have had to distinguish between those plants which are poisonous and those that are not, and they gradually developed the knowledge of the naturally occurring drugs which was transmitted at one time orally, later on as baked clay tablets, parchments, man scripted herbals, printed herbals, pharmacopoeias and by computerized information – retrieved system (Evans, 2002). African traditional medicine has been defined as the sum total practices, measures, ingredients and procedures of all kinds, whether material or not, which from time immemorial have enabled the Africans to guard against diseases, to alleviate their suffering and to cure them (WHO, 2000). The medicine man usually an accomplished botanist represents probably the oldest professional man in social evolution. Through the process of studying nature, early man observed consistencies in the chains and parttern of natural phenomena, and thus derived and described natural laws by which he could predict nature to his own advantage. This was the empirical acquisition of knowledge. In this context, man also studied plants, some of which he found to be useful for food – these he noted well and cultivated some as food crops; the others he found were harmful or poisonous – these he rejected and passed the information on to his progeny; yet another group of plants he found to possess medicinal properties – these were noted as such and the special knowledge passed on to his progeny (Latum and Dan 1980).

Traditional medicine in many parts of the world relies on the use of a wide variety of plants species. In Africa for instance, phytotherapy still plays an important role in the management of diseases, mainly among populations with very low income (Geoffrey and Kirby, 1996). Although, it has come a long way, not much significant progress on its development and utilization has been made, which may not be unconnected to colonization, absence of Government political will, lack of the citizenry patriotism and care free attitude of African medical scientists (Elujoba *et al*., 2005). The World Health Organization (WHO) resolution in 1978 requested the complete inventory, evaluation of the efficacy and safety and standardization of medicinal plants (Farnsworth, 1998). WHO also recommended the initiation of programs designed to use medicinal plants more effectively in traditional health care systems of developing countries like Nigeria (WHO, 1978).

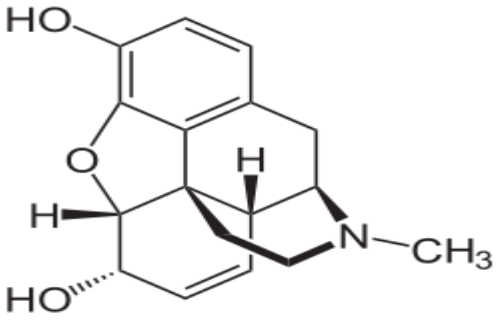
Plants represent the principal means of therapy in traditional medicine. For many centuries, plants have been used for healing in the Classical and Eastern world. Primitive societies believed in healing by similarity. A red resin, for example meant that the plant was good for the blood; a heart-shaped leaf signaled its cardiac properties, while a liver-shaped leaf was a sign of efficacy against jaundice (William and Thompson, 1978).

There were no accurate data available to assess the value and extent of the use of plants or active principles derived from countries. Traditional medicine has passed through developmental stages. That is, by passing through civilizations, from a less developed to a higher one. This is the reason why tracing the exact dates of starting the use of traditional medicines is difficult (Plowman, 1984).

But, records indicating the use of traditional medicines by the ancients civilization of Chinese, Hindus, Babylonians and the ancient Greek exist. The ancient Egyptians were in the possession of vast knowledge of traditional medicines. They have recognized the medicinal values of dates, honey, garlic and castor beans etc. a fossil remains of L*ephophora williamsii* used by Amerindians was found useful in the earliest time (Plowman, 1984).

The Chinese emperor Shen Nung published a book on herbs around 370 B.C., and published compendium uses of nearly 500 medicinal plants. Plants have undisputedly played a major role in the treatment of human traumas and diseases. The use of herbal medicines in India dates back to over 5,000 years and has become codified in the Ayurveda, which contains over 8,000 herbal remedies. However, this same ancient system of treatment is still used in over 14,000 dispenaries (Huxley, 1984).

The importance of plants in folkloric medicine may be attributed to interesting historical discoveries and subsequent development of many biologically active compounds from such claims. This include morphine (I), emetine (II), quinine (III), artemisinin (IV), the active constituent of the Chinese antimalarial medicinal herb *Artemisia annua* L., reserpine (V), strophanthidine digitoxin (VI) and physostigmine (VII). These discoveries led to the intensive search for more medicinal agents that are responsible for medicinal activity, that is, primary and secondary metabolites example carbolydrates and amino acids, alkaloids, phenolic groups (flavonoids, tannins), glycosides and anthraquinones (Evans, 1997).



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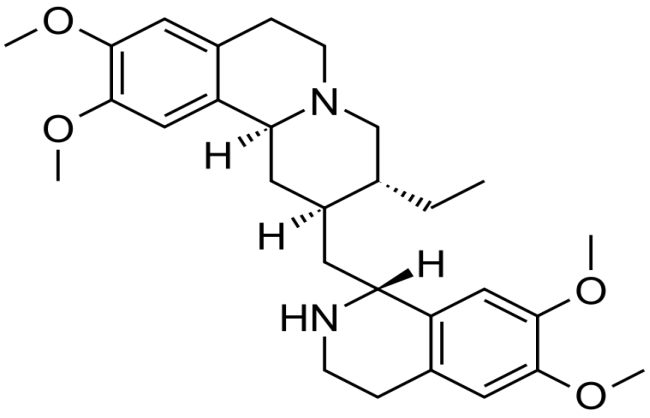

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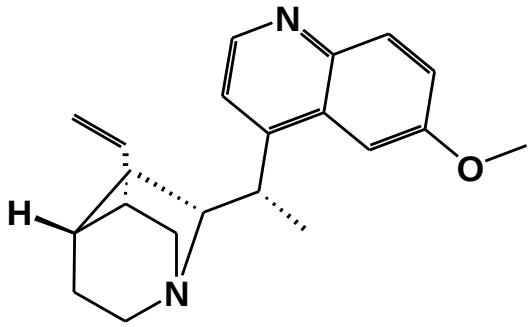

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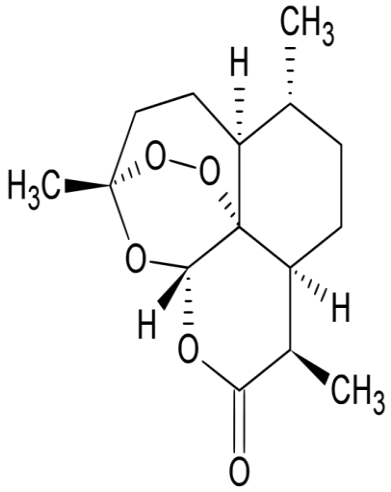

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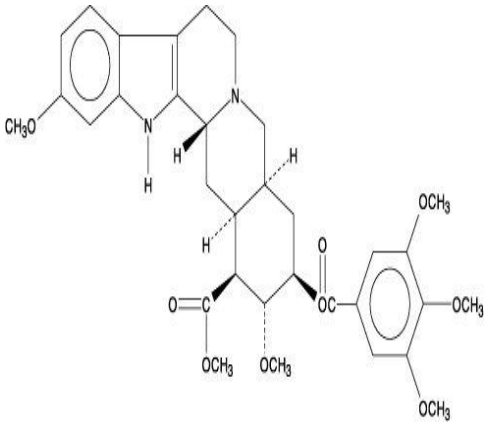

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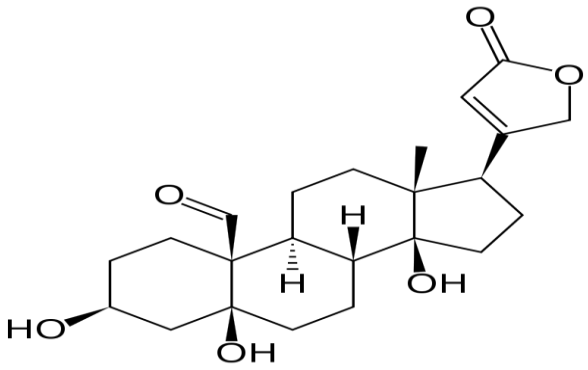

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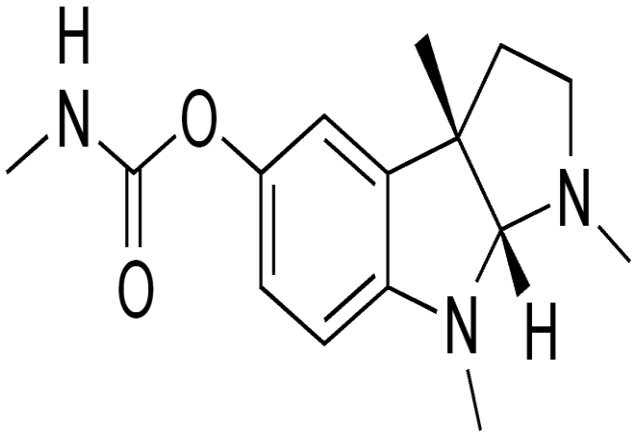
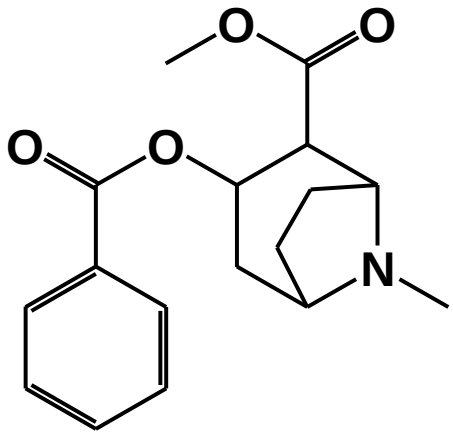


(VII)

In many countries, modern medicine has displaced plants with many synthetic products, but about 30% of pharmaceutical preparations are obtained directly or indirectly from plants. It is noteworthy that, apart from traditional uses, natural products may also be used as building blocks for the synthesis of "semi-synthetic" drug. This is the case for plant saponins which can be extracted and easily altered chemically to produce sapogenin for the manufacture of steroid drugs. Also a very important compound from medicinal plant that has led to the development of synthetic drugs is the cocaine (VIII) from *Erythroxyllum coca*. It provided the basis for the synthesis of procaine and other related local anaesthetic agent. (Hamann, 1988).



(VIII)

It was noted that, due to the economic condition of the people, there is a greater patronage of the traditional medicine. A traditional medicine has the very task of healing about 75 – 80% of the world population and therefore the aim was to improve its quality and effectiveness (WHO, 1978). It is by far cheaper and accessible than the orthodox and modern medicines, which in most cases are synthetic or semi synthetic with the accompanying serious adverse effects, for instance, loperamide hydrochloride (imodium) is effective against a wide range of secretory stimuli and can be utilized in the control and symptomatic relief of acute diarrhoea that is not secondary to bacterial infection.

Adverse effects associated with its use include abdominal pain and distention, constipation, dry mouth, hypersensitivity, nausea and vomiting. In the developing nations especially Africa, some people in the rural areas have the belief that some diseases such as epilepsy, and other mental illness, jaundice and cancer have no cure in orthodox medicine, they resorted in using traditional medicines for such diseases (Charles and Stilzel, 1997).

In the developing nations, traditional medicines has become part of their primary health care, in the event of this development, scientists are making efforts to confirm the efficacy of the traditional medicines and also standardized them. It is obvious that the drug bill for many countries still represents a sizeable proportion of their total health expenditure. These countries have to purchase the drugs at exorbitant and often inflated cost from multinational companies, which spend a disproportionately large amount on advertising in developing countries (Sofowora, 1982).

In the developed counties, plant derived drugs are well used. For example, in the United state of America (USA), 25% of all prescriptions dispensed from the community pharmacies from 1959 to 1980 contained plant extract or active principle prepared from higher plants (Farnsworth and Morriz, 1975).

In Africa, the use of medicinal plants can be traced down to our ancestors, who wholly depend on herbs for rearing and maintaining the health of their children. Whole plants or parts are used right from the period of conception up to adulthood. Amongst the traditional healers were some with specialized areas of Discipline e.g. The Orthopedics, midwifery, eye diseases, psychiatry, dentistry e.t.c all of which involve the use of herbs to cure each ailment. Others include therapeutic, Occultism, Obstetrics, gynecologists, circumcision, cupping or venesection. Hunters, especially in African countries have been reported as the ancient custodians of some effective traditional herbal

recipes. This knowledge could have been acquired by the hunters as a result of studying the behavior of wounded animals in the course of hunting. If the animal ran away and chewed leaves from a specific plant and did not die, the hunter noted the plant as an antidote for poison or as a pain reliever (Sofowora, 1982). Similar observations were made in villages where domestic animals chewed a particular portion of a specific plant when ill and later recovered or when another animal accidentally chewed a portion of the same plant and died. There are some plants or herbs commonly used to either suppress or cure large number of mental diseases. The root portion of the plant *Rauwolfia vomitora* has been used through out Africa to treat mentally disturbed patients. This is for its ability to calm the patient and to make him more amenable to other forms of treatment. The roots of *Rauwolfia vomitora* and *R. serpentina* Benth have been confirmed to contain alkaloid reservepine which is known to possess anti-hypertensive and sedative activity (Sofowora, 1982).

The use of traditional medicine in Nigeria for the treatment of various ailment has been fully accepted, this is because it serves as an alternative means of curing different diseases and as such its importance cannot be over emphasized. The herbal products are cheap, relatively affordable, reliable and available for sale within the nooks and crannies of our communities. The traditional medicine in most countries involves the use of medicinal herbs in their crude forms. The herbs are prepared in form of decoctions, infusion or powder to be taken and which has been in practice to date ((Sofowora, 1982 ; Owonubi, 1989). The traditional herbs are an inspirational part of our health because it is the total sum of knowledge, skills and practice based on the theory, belief and experience used in maintaining good health as well as curing diseases using medicinal plants. Some varieties of plants have been used medicinally based on this fact, the plant kingdom is hence described as the sleeping giant of drug development (Verpoorte *et al.*, 1987), while the forest in general was being referred to as "God's own pharmacy" (Treben, 1986).

It is evident, that the plant kingdom still holds many species of plants containing substances of great medicinal value which have just been discovered or yet to be discovered. Large numbers of plants are constantly being investigated for their possible pharmacological values (Evans, 2002).

Plant may be regarded as a biosynthetic laboratory that produces or synthesizes a large variety of chemical compounds during its metabolic activities. These include compounds that are utilized by man and other animals to exert physiological effects on them (Richard, 1998). Some of the plants being studied where found to possess an antimicrobial activity which include *Plumbagozeylanica* L., *Mitracarpus scaber* Zucc, *Euphorbia hirta* L,*Terminalia L*., *Alstonia boonei*De Wild and *Eupatorium odoratunm* L.(Ekpendu *et al.*, 1989).

Some plants are known to combat and expel or destroy the intestinal worms e.g. *Carica papaya* L. So also *Combretum micrathum* leaf has been reported to be an effective Antihelmintic. The National Institute for pharmaceutical Research and Development has also confirmed the Antihelmintic property of *Berlina grandiflora* (Enwerem, 1999). Therefore, medicinal plants are referred to as those in which one or more of their parts contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. The medicinal properties of drug plants are dependent on the presence of a variety of chemical substances known as the secondary metabolite. These secondary metabolites are constituent synthesized by the plant in addition to its basic metabolites, which may be concentrated in different part of the plant. Some of these compounds include saponin, glycosides, flavonoids, anthraquinones, alkaloids, steroid and terpenoids. Others include coumarins, tannins, gum and mucilage, volatile oil and organic acids. These chemical constituents are dominant in different drug plants given it a unique chemistry which differs from one plant to another at varying concentrations. The phytochemical examination of all

these constituents has been made possible and easier by the improved method of extraction, separation, isolation and characterization (Farnsworth, 1996).

1.1 Secondary Metabolites in Plants

Right from time immemorial, plant secondary metabolites have contributed immensely to the social and economic well being of man. This involves the provision of food, medicines, additives, fragrance and other pesticidal needs. Man has obtained a lot of advantages in areas of defense, attractiveness and medicinal compounds present in the roots, flowers, leaves, sap and bark of different plant species around the world. Conventional methods are being used to extract and isolate those compounds from the whole plant or different plant parts. Those compounds are derived from the primary metabolites such as acetic acid, carbohydrate and fatty acid. These compounds have a limited distribution within a section of a plant and of certain species or even varieties of some species. They are present in small quantities and often very tedious to isolate. Although the functions of all these compounds in plants metabolism are not certain, many of them appear to have an ecological functions such as attracting pollinating insects or repelling the herbivorous animals (Richard, 1998).

It is evident, that early man has used plant extract to heal as well as to kill dangerous animals. Quite a number of these extracts and some organic compounds isolated from culture of micro-organism have been used for curing different diseases. Such organic compounds isolated from the natural sources are referred to as the metabolites. A significant characteristic of plants has the ability to produce endless varieties of metabolites (Evans 2002; Gbodossou 2005).

1.1.1 Flavonoids

Flavonoids are large category of natural plant products that derive from γ – pyrone. It is a large group of biologically active natural products, distributed widely in higher plants, but also found in some lower plants, including algae (Cody *et al.*, 1986).

More than 1300 different flavonoid compounds have been isolated from plants. Individual flavonoids in a group differ from each other by the number and position of the hydroxy, methoxy, and sugar subtituents. As a rule, flavonoid compounds occur in plants as glycoside, galactose and rhamnose, and pentose such as arabinose and xylose as the most commonly found sugars. The sugars can be attached or in combination with each other. Glycosylation renders these compounds water – soluble and permits their accumulation in the vacuoles of cells (Cody *et al.*, 1986).

The flavonoid compounds can be regarded as $C_6 - C_3 - C_6$ compounds, in which each C_6 moiety is a benzene ring. The variation in the state of oxidation of the connecting C_3 moiety determined the properties and the class of each such compound. For the growth, development and defence of plant, flavonoids are important. Beverages,such as red wine, tea, coffee all contain large amount of flavonoids (Cody *et al.*, 1986).

Flavonoids with six membered rings being the δ-pyrone are termed as flavones and flavonols . While those with the dihydro derivatives are termed as flavononol and flavonones . Flavonoids may also be aglycones or glycoside e.g. baicalein – glycoside a flavone – glycoside, rutin a flavonol glycoside. Flavonols and flavones are the most widely occuring flavonoids, of this quercetin , myricetin , kaempferol, chrysin, and apigenin are widely distributed. Flavonones and flanonols dihydroflavones and dihydrochalcones are limited naturally, therefore, are considered minor flavonoids (Carlos *et al*., 1999).

1.1.2 Chemical structure (Biosynthesis)

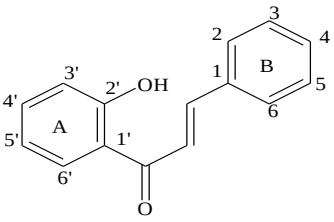
All flavonoids possess a three – ring diphenyl propane (C_6 - C_3 - C_6) core structure in which each C_6 moiety is a benzene ring. The variation in the state of oxidation of the connecting C_3 moiety determined the properties and the class of each such compound and their main subclasses are depicted in figure 1.1.

Flavonoids are of mixed biosynthesis, consisting of units derived from both shikimic acid and polyketide pathway , Figure 1.2 (Heinrich *et al*., 2004). The polyketide fragment is generated by three molecules of malonyl – CoA, which combine with the C_6- C_3 unit (as a CoA thioester) to form a triketide starter unit, The triketide starter unit undergoes cyclisation by the enzyme chalcone synthase. In addition, cyclisation can occur to give a pyranone ring containing flavanone nucleus, which can either have the 2,3 bond oxidised to give the flavones or be hydroxylated at position 3 to give the flavonol group. Flavonol may be further oxidised to yield anthocynins.

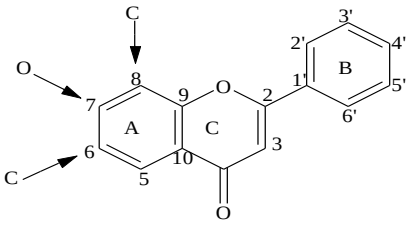
The basic structure is usually modified by means of hydroxylation and/or methylation at positions C-3, C-5, C-7, C-3□, C-4□ and/or C-5□. Occassionally, aromatic or aliphatic acids, sulphate, prenyl or isoprenyl groups are attached to the flavonoids backbone (Barron and Ibrahim, 1996 and Flamini *et al*., 2001).

In the plants, flavonoids are mostly present as glycosides. The purpose of glycosylation is to render a molecule less reactive and more water soluble, thus glycosylation in plants can be regarded as a form of protection to prevent cytoplasmic damage (Cuyckens and Claeys, 2004). O-glycosides have sugar substituents bound by a hydroxyl group of the aglycone, whereas in the case of C-glycosides, the sugar is connected to the aglycone through carbon-carbon. Thereotically, the glycan residue can

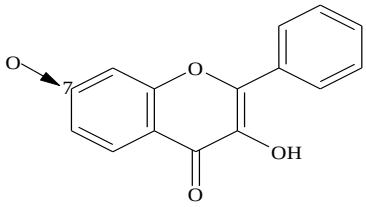
be attached to any of the aglycone's hydroxyl groups, but certain positions are apparently favored, such as 7-hydroxly group for flavones, flavanones, flavonols and isoflavones and positions C-3 for flavonols and anthocyanidins (Cuyckens *et al*., 2004 and Williams, 2006). 5-O-glycosides are rare for compounds with a carbonyl group at C-4, since the 5-hydroxyl group participates in hydrogen bonding with the adjacent carbonyl (Prasain *et al*., 2006). C-glycosylation has almost exclusively been found at positions 6,8 and only in two cases at position 3 (Jay *et al*., 2006). The most frequently found monosaccharides in glycosidic combinations are glucose, and rhamnose, and less frequently arabinose, xylose and glucoronic acid (Williams, 2006 and Jay *et al*., 2006). In addition, one or more of the sugar hydroxyls were reported to be esterified with aromatic or aliphatic acids (Abdel – Shafeek *et al*., 2000).



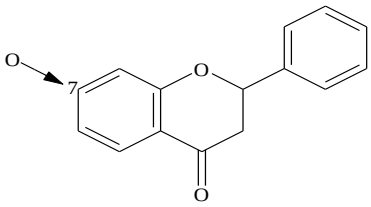
Chalcone



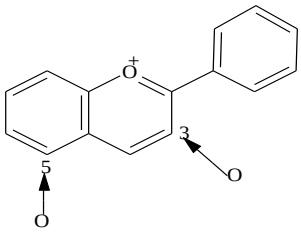
Flavone



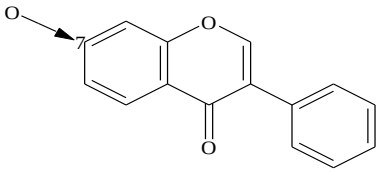
Flavonol



Flavanone



Anthocyanin



Isoflavone

Fig : 1.1: Structure of the main flavonoid classes. Common O- and C-glycosylation positions are indicated by arrows (Barron and ibrahim, 1996)

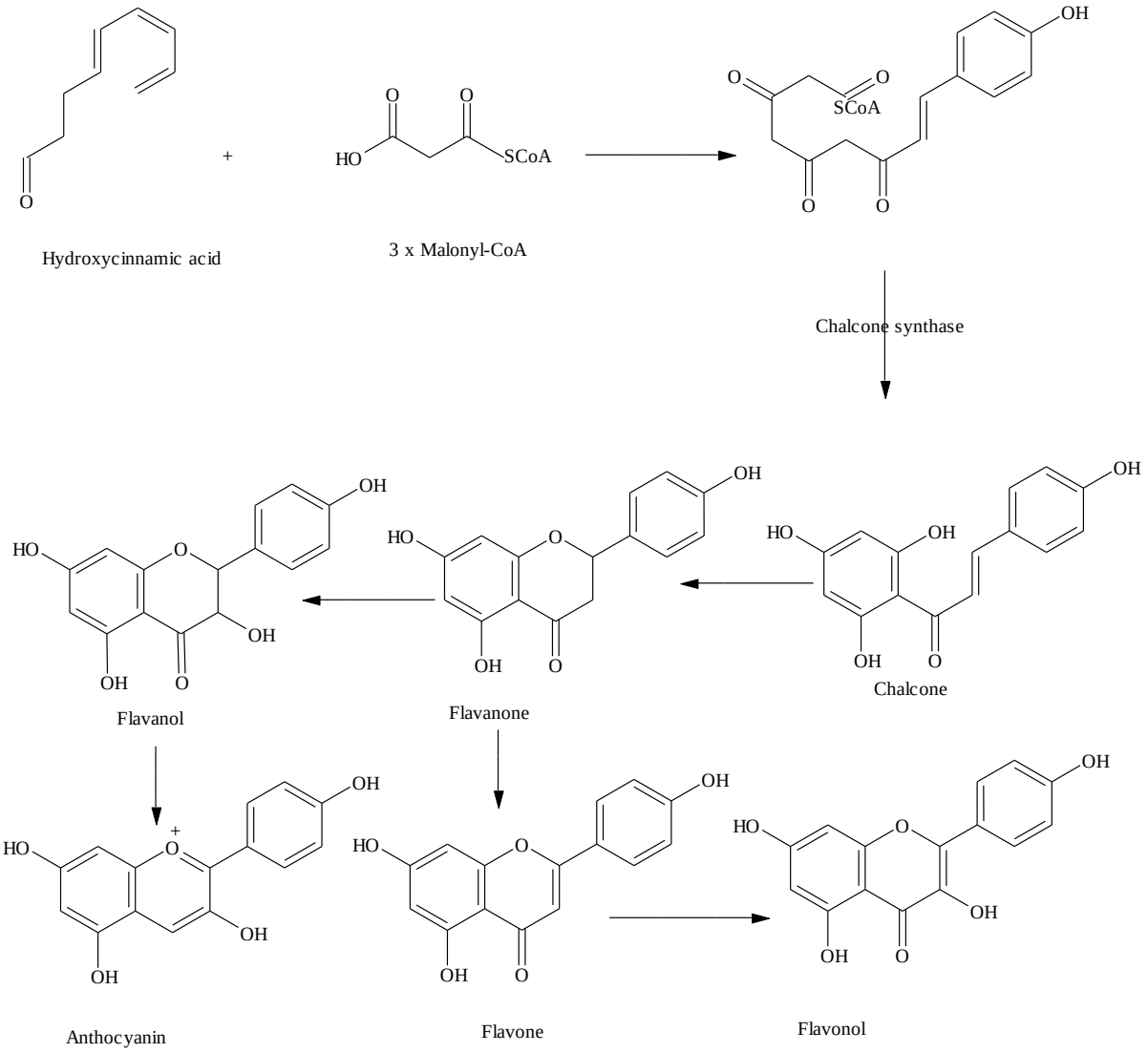


Fig : 1.2: Biosynthesis of the major flavonoid classes (Heinrich *et al*., 2004).

1.1.3 General considerations on qualitative flavonoids analysis

Over the years, many sample pre-treatment have been developed to determine flavonoids in plants. For isolation of flavonoids, solvent extraction (SE) – which may be followed by solid phase extraction (SPE) – is still the most widely used technique, mainly because of its ease of use and wide applicability. Soxhlet extraction is used frequently to isolate flavonoids from solid samples. From liquid samples, analytes are isolated using liquid-liquid extraction (LLE) or (SPE). As regards SE and soxhlet, in most cases aqueous methanol or acetonitrile is used as solvent. In the case of LLE, the extraction solvent is usually ethyl acetate or diethyl ether containing small amount of acid. LLE is usually directed at the isolation of glycones, while the other methods can have the isolation of both aglycones and conjugates as their goal. In flavonoids analysis, LC based methods are by far the most important ones. Less common procedures involve GC, capillary electrophoresis (CE) or thin-layer chromatography (de Rijke *et al*., 2006).

1.1.4 Separation and detection of flavonoids

LC of flavonoids is usually carried out in the reversed –phase mode, on C_{8}- or C_{18}- bonded silica columns. However, also other phases, such as silica, sephedex and polyamide are used. Gradient elution is generally performed with binary solvent system, eg with water or acetonitrile as organic modifier. LC is usually performed at room temperature, but temperature up to 40^0C are sometimes recommended to reduce the time of analysis and because thermostated columns give more repeatable elution times (Tura and Robards, 2002).

All flavonoid aglycones contain at least one aromatic ring and consequently, efficiently absorb UV light. The first maximum, which is found in the 242 - 285nm range, is due to the A-ring and the second maximum, which is in 300 - 500nm range, to the substitution pattern and conjugation of B-ring. Simple substituents such as methoxy, methyl and non-dissociated hydroxyl groups generally effect only minor changes in the position of the absorption maxima. Already several decates ago,

UV spectrometry was, therefore, a popular techique to detect and quantify flavonoids aglycones. Also, UV detection became the preferred tool in LC-based analyses and, LC with multiple-wavelenght or diode-array UV detection is a fully satisfactory tool in studies dealing with, eg screening, quantification of the main aglycones and/or a provisional sub-group classification (Mabry *et al*., 1970).

In flavonoids analysis, fluorescence detection is used only occasionally, because the number of flavonoids that exhibit native fluorescence is limited. For these compounds, the limits of detection in LC and CE are typically about an order of magnitude lower than with UV- detection. Moreover, their fluorescence facilitates selective detection in complex mixture. (de Rijke *et al*., 2002). Classes of flavonoids that shows native fluoscence include the isoflavones, flavonoids with an OH group in the 3-position. Since most flavonoids are electroactive due to the presence of phenolic group, electrochemical detection can also be used (de Rijke *et al*., 2006).

1.1.5 Structure elucidation

The characterisation of flavonoid glycosides by mass spectrometry comprises the determination of (i) glycosylation types (O-,C- or mixed glycosides), (ii) types of the sugar units (hexoses, deoxyhexoses or pentose), (iii) distribution of the sugar residue, (iv) order of the glycan sequence, (v) interglycosidic linkages, (vi) glycosylation position and (vii) nature of the aglycone. Flavonoids O-glycosides most often contain one or two sugar units, but tri- and tetraglycosides are also not uncommon (Williams, 2006). By definition, two sugars can be attached to the flavonoid aglycone either at two different position (di-O- glycosides, di-C-O- glycosides) or at the same position (O-diglycosides, C,O-diglycosides) forming a dissaccharide.

The sugar units in flavonoid oligosaccharides connect through interglycosidic linkages. Although, the glycosidic hydroxyl group (at position 1) can produce glycosidic bonds thereotically with 4

hydroxyl groups (attached to C-2,C-3, C-4 and C-6) of the other sugar units, the 1→2 and 1→6 linkages are preferred. Thereotically, the glycan residue can be attached to any of the aglycones's hydroxyl groups, but certain positions are apparently favored, such as the 7-hydroxyl group for flavones, flavonols, flavanones and isoflavones and position C-3 flavonols (Cuyckens *et al*., 2004 and Williams 2006).

1.1.6 UV spectra of flavones and flavonol

UV spectroscopy is particularly applicable to flavones and flavonol, because of the conjugation of both the A and B of the carbonyl group. The methanol spectra of flavones and flavonols exhibit two major absorptions in the region (240-400nm). These two peaks are commonly referred to as band I which usually occured between 300-380nm and band II at 240-280nm. Band I is considered to be associated with absorption due to B ring, (cinnamoyl system) and band II with absorption due to the benzoyl system (Mabry, 1969).

1.1.7 Infrared spectroscopy of flavonoids

Infrared spectroscopy is valuable for the identification of flavonoids. This is due the fact that, each peaks of absorption is associated with the presence in the molecule of a specific chemical group, apart from the possibility of identification, comparison with spectrum of a reference compound may lead to the production of or help to determine the structure of an unknown constituent.

(a) Free hydroxyl groups are responsible for absorption in the region of 3300cm^{-1}, when they are hydrogen bonded, in 3 and 5 – position, the frequency is lowered to 3100cm^{-1}.

(b) Absorption around 2930cm^{-1} is as a result of methyl group.

(c) Carbonyl absorption of the heterocyclic usually occurs at 1685cm^{-1} in the case of flavonones, where the carbonyl groups are conjugated only with the A ring. This is lowered to1650cm^{-1} in case of hydroxyl 5 – flavonone.

For flavones, the carbonyl group is conjugated with both A and B rings. The absorption due to the carbonyl group in 5, 7 – dihydroxyl flavones is 1655cm^{-1} while for 7 – dihydroxyl flavone is 1630cm^{-1}.

In the case of flavonols, the carbonyl absorption is lowered by about 30cm^{-1} compared with that of the homologous flavones. But aromatic double bonds absorps between 1500cm^{-1}and 1655cm^{-1} a second peak will be present at 1585cm^{-1} when the double bond is in conjugation with benzene ring. Phenolic hydroxyl group have a strong absorption at about 1360cm^{-1} with secondary peak at 1200cm^{-1}. Peak at 1165cm^{-1} is due to metadihydroxyl substitution (5, 7 - dihydroxyl).

1.1.8 Analysis of flavonoids structure by nuclear magnetic resonance

Proton signals obtained in NMR spectra of trimethylated flavonoids generally occur in the range of 0 – 8ppm, but this on a scale of δ.

(i) A – Ring protons

C – 6 and C – 8 protons. The C – 6 and C – 8 protons of the flavones, flavonols and isoflavones that contain the common 5,7 – dihydroxy- substitution pattern give rise to two doublets. (J = 2.5) in the range of 6.0 – 6.5 δ, with the H-6 protons appearing at higher field than the signal for H- 8. When a sugar is attached to the oxygen at C- 7, the signals for both H- 8 and H - 6 are shifted down field (Mabry, 1969).

When a sugar is attached to the oxygen at C- 7, there is desheilding of the H- 8 and H- 6 protons. For flavones and dihydroflavonols, which contain the 5, 7 – dihydroxy substitutions, the signals for the A ring protons appears at higher field than in the corresponding flavones and flavonols.

C – 3 protons. The C – 3 protons of flavones appears at singlet at around δ 6.3ppm. For trisubstituted flavones/flavonols for A- ring, e.g. 5,6,7 or 5,6,8, the long A- ring protons produces a singlet, which is often in the same region as the C-3 protons (Mabry, 1970).

(ii) B – ring protons

C – 2□, C – 3□, C- 5□ and C- 6□ protons in 4□-oxyenated flavonoids appears in the range of 6.7 – 7.9 δ scale, which is down field from the A- ring protons. The signal pattern is usally characteristic of the substitution pattern and the level of oxidation of C – ring . For C – 4□ oxygenation, a four – peak pattern of two doublets is observed. The doublets at C-3□ and C-5□ are shielded by C-4□ oxygen subsistent hence always appear at higher field than C- 2 and C- 6, and generally fall, in the range of δ6.65 - 7.1. For all types of flavonoids and usually appears doublets.

(iii) **C-Ring protons**

C-3 and protons in flavones:

Variation in the chemical shifts of the C-ring protons among the different flavonoids classes depending on the oxidation level of the C-ring. The C-3 protons in flavones give a sharp singlet near 6.3ppm similar to the region of ring A protons. In the C-2 of the isoflavones, the signal occurs in the range of δ7.6 - 8.7ppm, a ragion down field from A and B ring protons (Mabry, 1970).

C-2 and C-3 of flavonones and dihydroflavonols. The signals for the C-2 protons of flavonones appear as quatet (two doublets) near 5.2ppm due to the coupling of C-2 protons with the two C-3 protons. For the dihydroflavonols, the C-2 protons signal occurs as doublets near 5.2ppm, while the C-3 protons appear at higher field of δ = 4.5ppm.

(iv) **Sugar protons**

The C-1 proton of a sugar directly attached to the flavonoid hydroxyl group depends on the nature of the flavonoid and the position of stereochemistry of attachment. Glucoside on C-3 hydroxyl group can be distinguished from one at C-4, C-5 Or C-7. For C-4, C-5 and C-7 glucoside, the C-1 proton appears near 5.8ppm while in flavonol 3-glycosides, the C-1 proton appear down field at 5.0ppm.

For C-1 proton of glucose in 3-0- glucosides appears as doublets while for 7-O-glucoside, the glucosyl C-1 proton appears as complex multiplet. In flavonoid rhamnoside, both 3- and 7-O-rhamnoside, C-1 protons signal occurs in the range 0.81 - 1.2ppm and is a distinguishing feature . The rhamnose methyl proton appears as doublet at 0.85ppm in quecetin 3-α O-rhamnoside while in quecetin 3-β – glucoside- 7-α-rhamnoside, the rhamnose methyl protons appears as a complex signal at 1.2ppm. In flavonoid rhamnosyl glucose, either as rutinosyl or the neo-hespeeridosyl moities, e.g. in flavonoid 7-O- and 3-O- rutinoside, rhamnose is characterised by the C-1□□□, protons signal near 4.2 – 4.4ppm, and a broad peak for the C-6□□□proton (methyl) at 0.7 - 1.0ppm. whereas in the flavonoid 7- and 3-O-neohesperidosides, the rhamnose C-1□□□protons absorbs at 4.9 - 5.0ppm, while the methyl group appears as doublet at 1.1 - 1.3ppm. Methoxyl and Acetoxyl protons: Methoxyl proton in flavonoid appears in the region 3.7 -4.1ppm, while acetoxyl protons occur in the range 1.65 - 2.50ppm (Mabry, 1969).

1.1.9 Beneficial health effects of flavonoids

Up until today, flavonoids have been found only in plants. Being not only colored pigments, but also enzymes inhibitors and stimulants, metal chelators and reducing agents, they are important for the plant's normal growth, development and defence (Harbone, 1988 and Carlos *et al*., 1999).

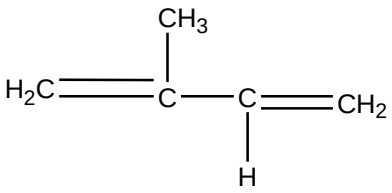
In addition, several papers including epidemiological studies and meta-analyses report on their beneficial effects on human health. The most important examples are ; flavonoids reportedly reduce

risk of cancer (Steinmetz and Potter, 1991 and Birt *et al*., 2001) and cardiovascular diseases (Tijburg *et al*., 1997 and Bazzano *et al*., 2002) as they hinder the invasion of metestases (Piantelli *et al*., 2006), slow the division of tumour cells (Fotsis *et al*., 1997 and Caltagirone *et al*., 2000), reduce hypertension (Moline, 2000 and Garcia – Saura *et al*., 2005) as well as protect and stregthen vascular walls (Geleijnse *et al*.,1999 and Reed *et al*., 2002). In addition, flavonoids showed prospective benefits in the treatment and/or ease of symptoms of several serious illness such as HIV AIDS (Ono and Nakane, 1990), Morbus Alzeheimer's (Commenges *et al*., 2000 and Kim *et al*., 2005), rheumatic diseases (Guardia *et al*., 2001 and Ostrakhovitch and Afanas, 2001), diabetes (Varma *et al*.,1977 and Miyake *et al*., 1998), asthma bronchiale (Havsteen, 2002) and gastro-intestinal ulcers (Lewis and Hanson, 1991, Borelli and Izzo, 2000 and Awaad *et al*, 2008) due to their immun-modulatory, antimicrobial, antioxidant, and anti-inflammatory, pain-killing, and smooth muscles relaxant effects (Pietta ; 2000, Havsteen, 2002 and Cushnie and Lamb, 2005). Flavonoids also render valuable help in minor problems eg wounds, bites, burns or common cold (Mors *et al*., 2000 and Havsteen, 2002).

1.2 Terpenoids

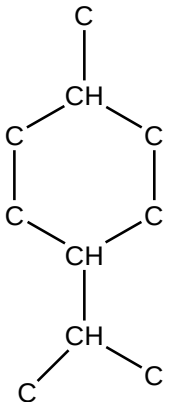
These are most diverse and chemically interesting groups of natural products. Terperoids or terpenes are a group of compounds that are made up of one or more units called isoprene (ix). They are widely distributed through out the plant kingdom. Essential oils constitute the simpler mono and sesquiterpenoids. The di- and triterpenoids are found in tree gums and resin while the tetraterpenoids exemplified to as carotenoids and polyterpenoids exemplified by rubber are also found commonly in plants (Hansel, 1972).

Terpenoidal compounds are classified based on the isoprene units present. The molecular formula employed for the classification is $(C_5H_8)n$, where n is used as the basis for classification.



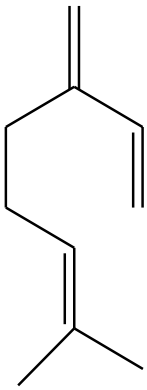
(ix)

Terpenoidal compounds include monoterpene, triterpene and polyterpenoid chiefly found in the latex. Monoterpenes are compounds made up of two isoprene units that are joined head to tail. The monoterpenes can either be monocyclic or bicyclic, but most monoterpenes are derivatives of P-cymene structure (x).

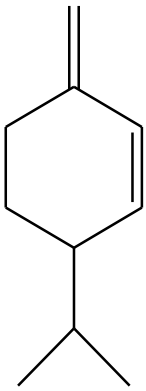


(x)

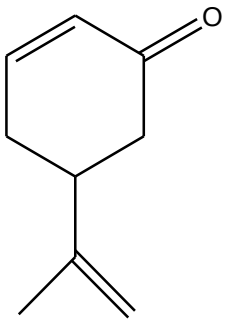
Monoterpenes are exemplified by myrcene (xi), β- phellandrene (xii) and carvone (xiii). In general, there are four methods of extraction and isolation of monoterpenes. Viz:- expression; steam distillating extraction by using volatile solvents and adsorption in purified fats (enflurage) (Finar, 2003).



(xi)

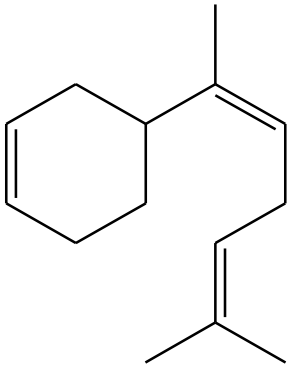


(xii)

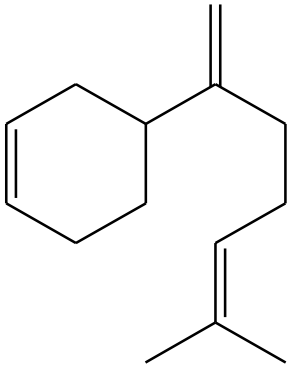


(xiii)

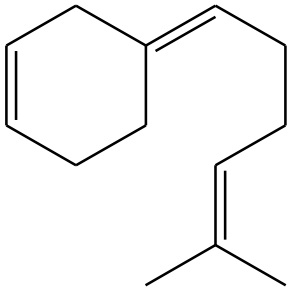
Sesquitepenes are made up of three isoprene Units. They can be acyclic, monocyclic, bicyclic or tricyclic. Examples of sesquiterpene are α – bisabolene (xiv), β – bisabolene (xv) and λ- bisabolene (xvi) (Finar, 2003).



(xiv)

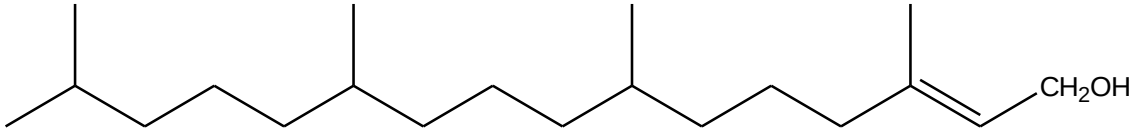


(xv)

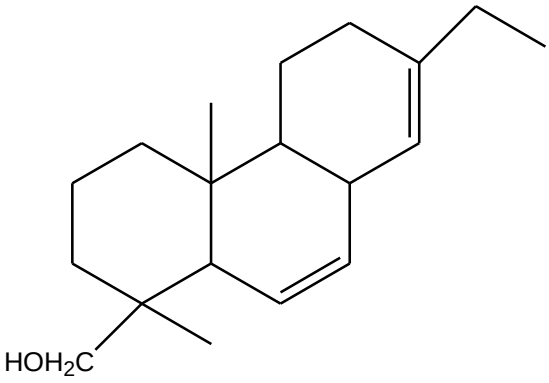


(xvi)

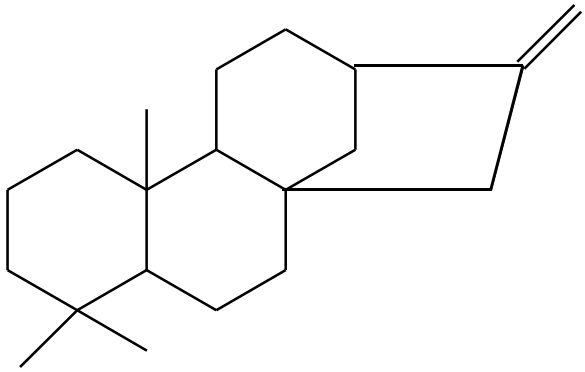
Diterpenoids contain four isoprene units and are mostly found in plants. They are produced from the hydrolysis of chlorophyll and it also forms part of the molecules of vitamins E and K. Diterpenes can be classified based on the number of rings present. Although, the acyclic diterpenenoid occurs, the cyclic are found as monocyclic, tricyclic and tetracyclic in nature. Examples of diterpenes includes phytol (xvii); abietic acid (xviii) and phythocladene (xix)



(xvii)

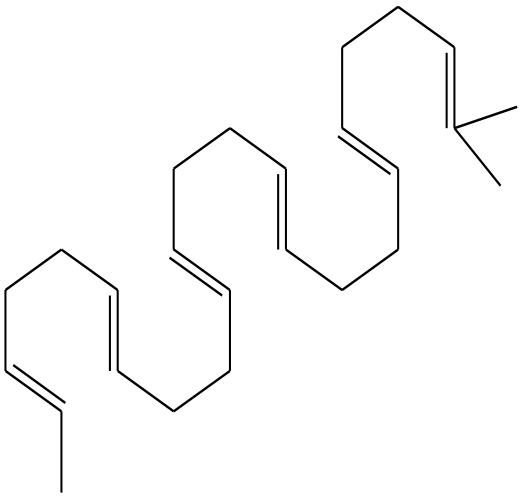


(xviii)

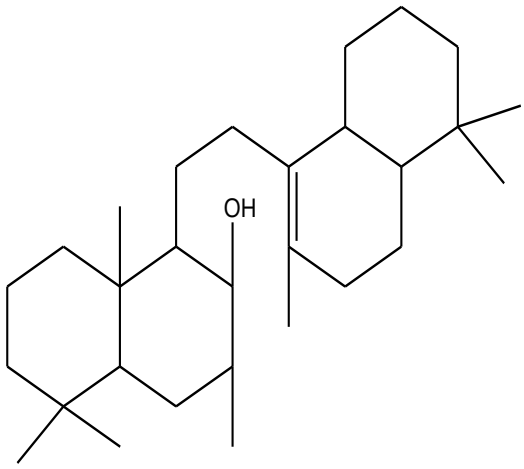


(xix)

Triterpenoid compounds are made up of six isoprene units and can be acyclic or cyclic. The cyclic triterpenoids are usually tricyclic, tetracyclic or pentacyclic. Examples among others are squalene (xx) and Ambrein (xxi) (Finar, 2003).



(xx)



(xxi)

Polyterpenoids are compound with more than 8-Isoprene Units contained in a linear pattern (Finar, 2003).

1.2.1 Extraction of terpenes

A large number of terpenes are usually non-polar compounds and may therefore be separated from polar plant constituents by extraction with solvents like benzene or ether. Associated matters like lipids, esters and waxes may be removed by saponification in alcoholic alkali, followed by extraction with ether (Robinson, 1972). Terpenoid glycosides however are usually insoluble in non-polar solvents, they are most conveniently extracted from plants with 70-95% ethanol. Extraneous lipids are removed from the alcoholic solution by partition with benzene. Acidic terpenes when present as free acids are soluble in non-polar solvents, but on saponification will pass into alkaline phase.

1.2.2 Spectral properties of terpenes

[1] Infra-red (IR) spectroscopy

Infra-red spectroscopy is used to prove bond vibrations and bending in molecules. Therefore, this is a simple method to reveal the types of groups present in compounds. Functional group region in the range from 400 – 600cm^{-1} can be divided into three bands:

- OH, NH, C – H
- C≡C, C≡N, X=C=Y (C, O, N, S)
- C=O, C=C

Finger print region has very little information about functional group and used only to compare two compounds. The range is 1550-660cm^{-1}.

IR spectra can be measured either as solution (in $CHCl_3$) or in the solid state mixed with KBr or as thin liquid film. The range of measurement is from 4000-600cm^{-1}. The frequency is from 6×10^{11} to 4000×10^{11} Hz.

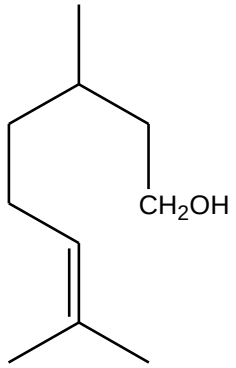
The two major regions of the spectrum that are importances in terpenes are:

i. O-H stretching (3650-2650cm^{-1}). This region may be used to determine the presence of hydroxy group and related species.

ii. Carbonyl stretching (1820-1640cm^{-1}). This is a strong absorption region, and the region is further divided into two (a) saturated carbonyl (1750-1700cm^{-1}) and (b) unsaturated α, β- Carbonyl (1700-1600cm^{-1}).

Infra-red spectra are particularly useful for detecting the presence of isopropenyl group in terpenes usually between 895-885cm^{-1}. For example, the spectrum of citronenollol (xxii) show a maximum at 890cm^{-1} which corresponds to the presence of the Isopropenyl group while that of isopropylidene is 850-790cm^{-1}.

Myrcene (xi) with λmax 224 had no band at 890cm^{-1}. This shows the complete absence of the isopropenyl group.



(xxii)

Heteroannular dienes and α, β- unsaturated Ketones can be distinguished by the infra-

red spectra.

[ii] Ultraviolet spectroseopy (UV)

Energy of Ultraviolet/Visible is 7.5 × 10^{14} to 300 × 10^{14} Hz, which proves outer core electron transitions. The absorption spectrum is measured in dilute solution.

There are three factors for a good solvent. Firstly, the dilute solvent must not absorb the UV radiation in the same region as substance whose spectrum is being determined. Secondly, the

solvent should not have effect on the fine structure of an absorption band. The last factor is the ability of the solvent to influence the wavelength of UV light that will be absorbed via stabilization of either the ground or the excited state (Finar, 2003).

The maxima and minima of the absorption are recorded in nm and the intensity is recorded in term of log ε with ε = A/c.l; (A: absorbance, c: molar concentration, l: length of sample cell).

It is useful to identify conjugated system of the compound. The 95% ethanol is the common solvent used for UV. UV is used in identification by measuring the solution as neutral and adding basic and acidic solution to the unknown or under different pH. The change of the maxima to higher or lower λmax indicates the class of compounds. The value of UV and visible spectra in identifying unknown constituents is indicative of the compounds.

The following observations can be used to identify functional group, double bonds, and aromatics systems.

- Two bands of medium intensity (ε = 1000 to 10000), both with λ_{max} above 200nm indicated the presence of an aromatic system.
- A single band of low intensity (ε = 10 to 100) in the region of 250 to 360nm, with no absorption at shorter wavelength (200 to 250nm).

Conjugation can be detected by ultraviolet spectroscopy technique in terpenoid chemistry. For simple acyclic dienes, λmax is 217-218nm, while in heteroannular dienes λmax is 230-240nm, and for homoannular dienes λmax is 250-265nm (Finar, 2003).

The absorption of the diene system is affected by substitution and empirical rules, which was later modified for calculating the λmax from the molecular structure of a compound. A monoterpene myrcene (xi) based on the rules have a λmax of 219nm while the observed λmax is 224nm. Similarly, carvone (xiii) has a calculated λmax of 237nm, while the observed λmax is 237nm.

[iii] Nucleaar magnetic Resonance (NMR) Spectoscopy

NMR has become a very important spectroscopic method and the premier organic spectroscopy available to Chemists to determine the detailed chemical structure of the compounds they were isolating from natural products. NMR spectroscopy is routinely used by chemists to study chemical structure of simple molecules using simple one- dimensional techniques (1D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules. Suggested tactics for solving structure using MNR are the following (Breitmaier, 2002)

(a). ^{1}H-NMR

^{1}H-NMR has been used to detect and identify double bonds, to determine the nature of end groups, the member of rings present and to ascertain the orientation of methyl groups present. For example, Abscisin, a plant hormone and a monocyclic sesquiterpenoid has a ^{1}H-NMR spectrum as follow: methyl singlets at δ=1.1 and δ=1.1.7; two vinyllic methyl groups singlets at δ = 1.1, and 2.1; and a methylenic group adjacent to the carbonyl group at δ 2.41.

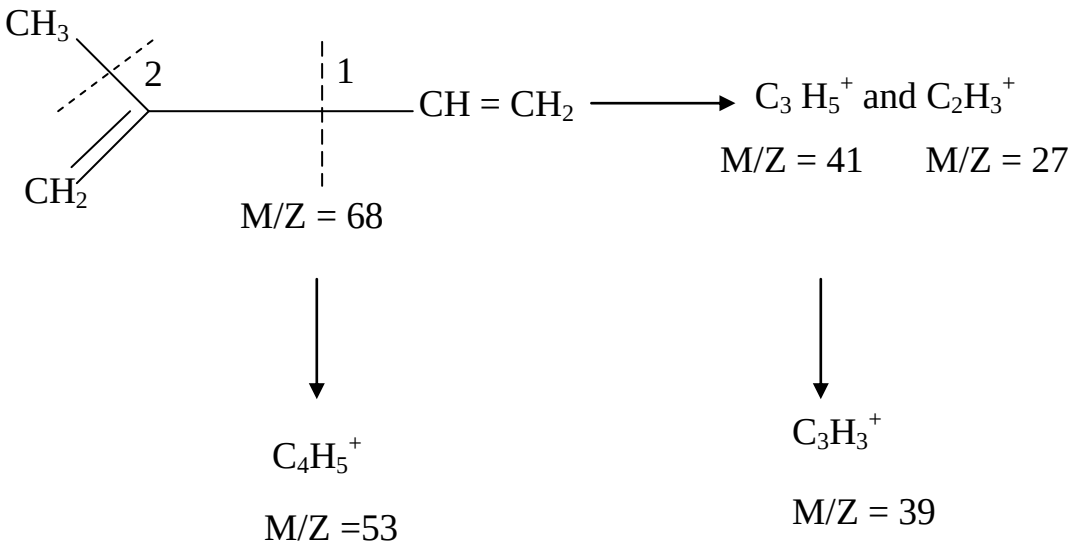
(b). ^{13}C-NMR

The ^{13}C-NMR is a more useful tool than ^{1}H-NMR for the elucidation of carbon skeleton of a compound (Stothers, 1972). This is because most of the carbon compounds of moderate complexity give rise to separate signals, while the proton spectrum on the other hand permits the identifications of only a few positions in the compound (Finar, 2003).

[iv] Mass spectrometry (MS)

Mass spectrometry is being increasingly used as a means of elucidating the structure of terpenoids, thus, it is possible to determine the molecular weight, molecular formular, the nature of various founctional groups and the relative position of double bonds. Since isoprene is the building unit of terpenoids, the following include the peaks which are usually observed in the spectra of terpenoids

in general: 68 ($C_5H_8^+$), 67 ($C_5H_7^+$), 53 ($C_4H_5^+$), 51 ($C_4H_3^+$), 41 ($C_3H_5^+$), 39 ($C_3H_3^+$), 29 ($C_2H_5^+$) and 27 ($C_3H_3^+$). Paths that account for some of these fragments are:



1.2.3 Pharmaceutical importance of terpenes

The volatile terpenes especially the monoterpenes have wide application in perfumery industries in production of perfumes; the sesquiterpenes have use in pharmaceutical practice especially with the introduction of the Chinese antimalaria artemisinin which is an unusual sesquiterpene lactone, it is used in the treatment of *Plasmodium falciparum* resistant malaria and also in cerebral malaria. The diterpenoids are also of current interest because they are future drugs either as isolated from plant or as modified derivatives eg Forskohlin (coleanol) a diterpene isolated by Indian workers from *Coleus forskohlii.* Forskohlin has been shown to have hypotensive spasmolytic and cardiotonic effect. Preparations of the species have long been used in Hindu and Ayurvedic traditional medicine for treatment of heart diseases, abdominal colic (Ammum and Muller, 1985).

1.3 Saponins

The groups of natural products which have in common the property of foaming when shaken with water are called saponins. They are widely distributed in the vegetable kingdom and have been reported to be present in at least 500 genera of plants. Chemically, saponins are glycosides which yield on hydrolysis (a) and one or more sugar units and (b) sugar-free aglycones which are derived from polycylic ring systems and are commonly referred to as sapogenins (Basu and Rastogi, 1967).

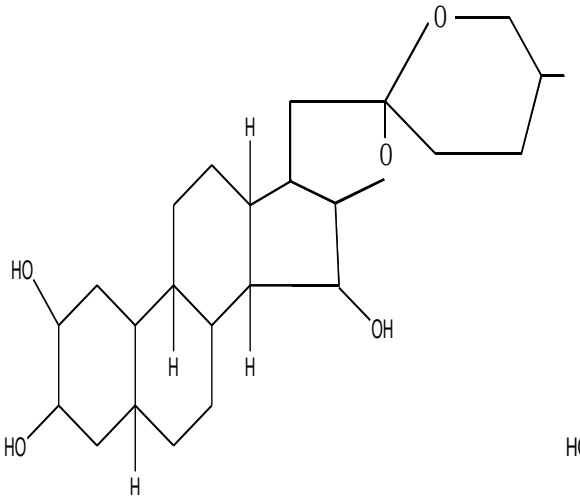
These groups share in varying degrees two common characteristics.

a. They foam in aqueous solution; that is have the property of foaming in water

b. Intravenous injection of their aqueous solution into animals results in haemolysis (Olaniyi *et al.*, 1993).

Classification of saponins into two broad groups is based on the chemical nature of the sapogenins, these are:

1. Saponins of cholane group. As in sterols, bile acids and cardiac aglycones, saponins of cholane group are derived from the same tetracyclic ring system. Some example includes digitogenin *(Digitalis purpure and D. lanata)* (xxiii) Tigogenin (xxiv); sarsasapogenin (xxv)



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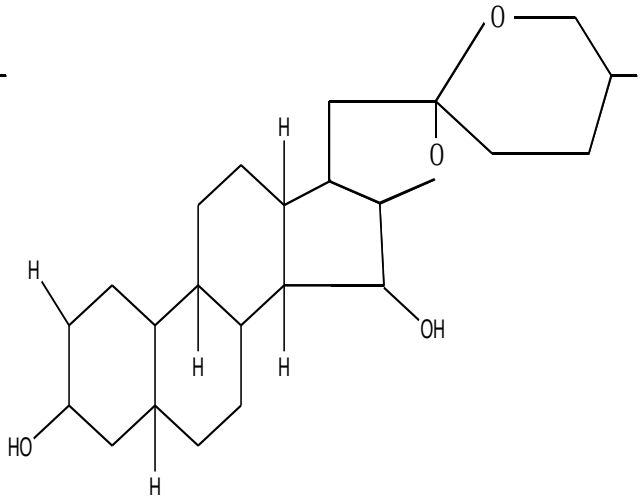

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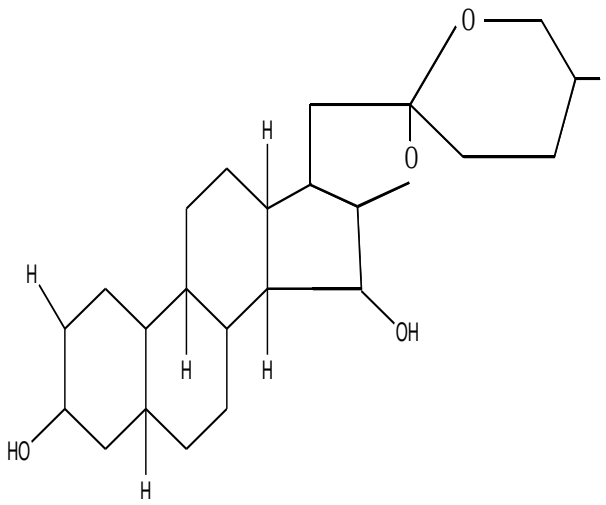
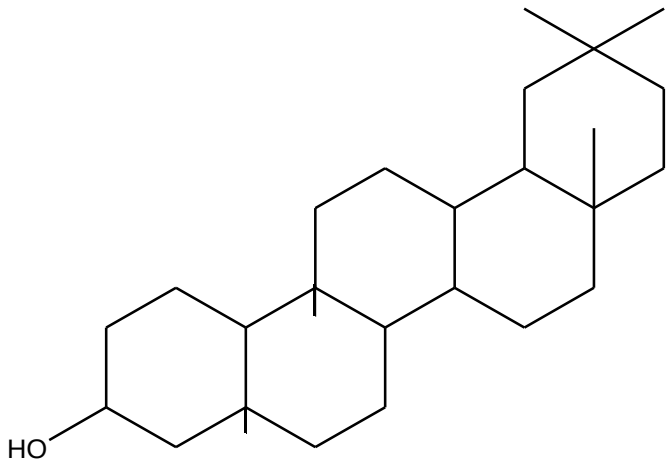


(xxv)

These groups are C-27 steroid carrying a spiroketal side chain which exhibits several characteristics strong I.R. bands in the regions of 1350 – 875cm^{-1} (Basu and Rastogi, 1967).

2. Saponins of triterpenoids group. The groups are extensively distributed in the plant kingdom and constitute the majority of the saponins found in nature. A number of these saponins differ only in the number and the type of units in the carbohydrate moiety linked to a particular sapogenin. These groups, with few exceptions, belong to β-amyrin (xxvi) group and are usually simple alcohols and acids. But, sapogenins can be aldehydic and ketone (lactone) functions occasionally (Basu and Rastogi, 1967)



(xxvi)

Saponins and their genins are usually detected by means of the characteristic colour they produce with various reagents, eg Liebermann- Burchard, thionylchloride, phosphomolybdic acid, silico-tungstic acid etc, and the haemolysis of blood. For the purpose of detection on paper chromatograms, some of the above reagents have been modified. A quantitative and highly sensitive micro method in which filter paper disc wetted with saponin are embedded in blood gelatin has been devised. Other reagents used for the detection are sodium metaperiodate, alkaline potassium permanganate mixture, antimony trichloride, chloroform-chloroform vanillin. Various solvent system have been reported to provide satisfactory separations, viz ethyl acetate: Pyridine: Water (3:1:3); butanol: acetic acid: water (6:1:3) and butanol: 1m NH_4OH: 95% alcohol (60:30:5:13) etc, chloroform: tetrahydrofuran: pyridine (10:10:2) saturated with formamide and chloroform: methanol: 1m NH_4OH: 95% alcohol (60:30.5:13) saturated with formamide and water (65:35:10) have proved to be satisfactory. For paper Chromotography, TLC as well as separation of saponins on Cellulose and silica gel columns (Basu and Rastogi, 1967).

A comparative study of the saponin content in saponaria, polymomium, primula, etc, has shown that the highest yields are obtained by collecting the plant material just before the flowering time. The ease with which saponins are hydrolysed into sapogenins and sugars (of which there may be up to 12 units) varies from case to case; usually refluxing with 5 – 10% mineral acid is necessary for a complete breakdown. In some cases, hydrolysis can be carried out with enzymes, eg hydrolysis of alfalfa saponin has been achieved with a fungus preparation (*Aspergillus*) (Basu and Rastogi, 1967).

The sugar components have been usually identified by the conventional method of paper chromatography in various solvents. The sugar units are linked to the genin by glycosidic linkage; and ester linkage is rarely encountered and only D-glucose has so far been found in such combination.

The following eight sugars have almost exclusively been found to be involved in glycosidation: D-glucose, D-galacturonic acid, D-glucoronic acid, D-Xylose, L- arabinose, L-Furanose, L-rhamnose and D-galactose. The structure of the sugar chains in a few cases have been elucidated by the usual method of methylation followed by hydrolysis and the identification of the individual methylated units. It has been found that the sugar moiety is very often linked at C-3 OH of the aglycone, but in some, this linkage is at other positions, eg in Musennin at C-16 OH and in asiaticoside at C-28 COOH (Basu and Rastogi, 1967).

1.3.1 Pharmaceutical importance of saponins

Saponins occurred widely in nature, this has evoked considerable interest in their uses and there are considerable data accumulated on their physiological action and other properties. They tend to alter the permeability of the wall hence exert a general toxicity in all organized tissues, their haemolytic and anti-lipemic activity and capacity to lower serum chlolesterol level can be their important characteristics. The ability of saponins to reduce surface tensions have been utilized in making emulsion stabilizer (Basu and Rastogi, 1967)

1.4 Tannins

The term "tannin" was first applied to denote substances present in plant extracts which were able to combine with protein of animal hides, to prevent their putrefaction and convert them into leather. On this note, tannin is a substance which is detected qualitatively by a tannin test (the goldbeater's skin test) and is determined quantitatively by its adsorption on standard hide powder. This definition excludes simpler phenolic substance, often present with tannins, such as gallic acid, catechins and chlorogenic acid, although, they may under certain conditions give precipitate with

gelatin and be partly retained by hide powder. Such substances of relatively low molecular weight are called ‘pseudo-tannins’ (Trease and Evans, 1997).

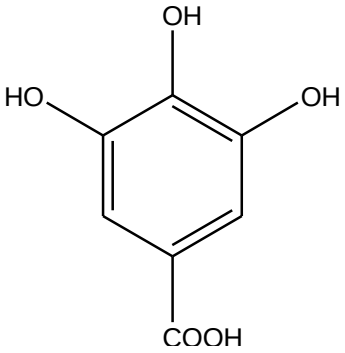
Most tannin has molecular weights of from 1000 to 5000. To be effective for tannage, the polyphenol molecule most be neither so large as to be unable to enter the interstices between the collagen fibrils of the animal skin nor so small that it is unable to cross – link between the protein molecules of adjacent fibrils at several points (Trease and Evans, 1997).

The characteristics properties of tannins derive from the accumulation within a moderately sized molecule of a substantial number (1-2 per 100 molecular weight) of phenolic groups many of which are associated with O-dihydroxy and O-trihydroxy orientation within a phenyl ring.

Two main types of groups of tannins are usually recognized, these are the hydrolysable tannins and the condensed tannins (proanthocyanidins) (Trease and Evans, 1997).

1.4.1 Hydrolysable tannins

These may be hydrolysed by acids or enzymes such as tannase. They are formed from several molecules of phenolic acids such as Gallic (xxvii) and hexahydroxy diphenic acids (xxviii) which are united by ester linkages to a central glucose molecule. The above examples were formerly known as pyrogallol tannins, because on dry distillation, Gallic acid and similar components are converted into pyrogallol.



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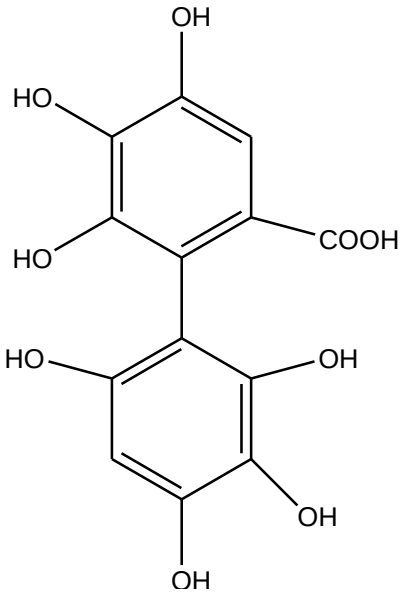


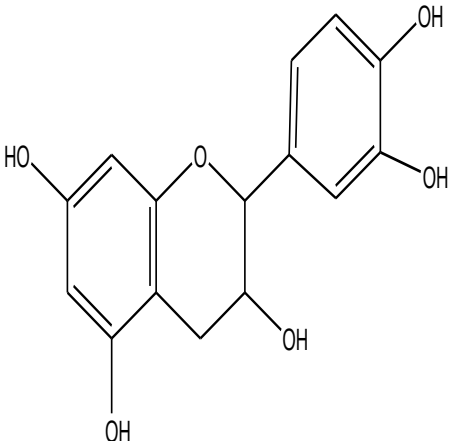
(xxviii)

The hydrolysable tannins can be hydrolysed by boiling with dilute hydrochloric acid, the alcoholic components of the esters is usually sugar. Hydrolysable tannins are often complex mixture containing several different acids esterified to different positions with sugar molecule and these render it water soluble. They are sometime named after the acid derivatives eg Gallitannins for tannin from Gallic acid and Ellagitannin for tannin from Ellagic acid. They can also be hydrolysed by an enzyme called tannase. They are soluble in water, dilute alkalis, acetone, alcohol but insoluble in chloroform and benzene (Trease and Evans, 1997).

1.4.2 Condensed tannins (Proanthocyanidins)

Unlike hydrolysable tannins, these are not readily hydrolysed to simpler molecules, and they do not contain a sugar moiety. They are related to the flavonoids pigments and have polymeric flavan-3-ol structures. Catechins, (xxix) which also occur with tannins and flavan-3,4-diols (xxx) (leucoanthocyanidins) are intermediates in the biosynthesis of the polymeric molecules.

Condensed tannins have no sugar linkage molecules; they consist of larger phenolic group chemically linked directly by carbon-carbon (C-C) linkage.



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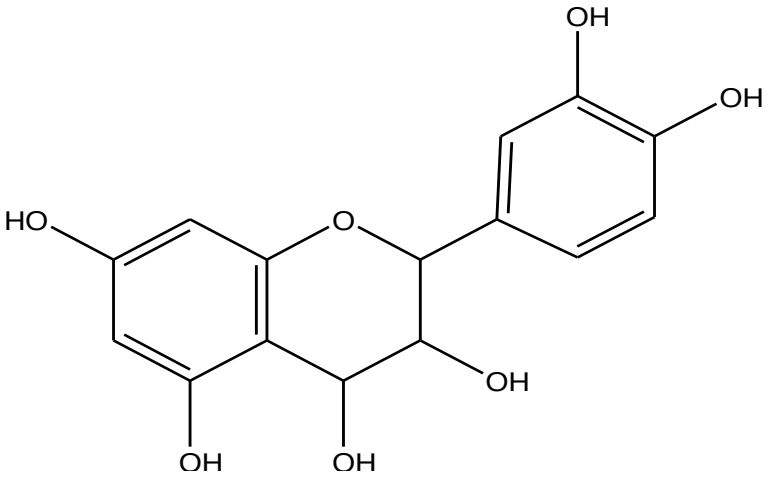


(xxx)

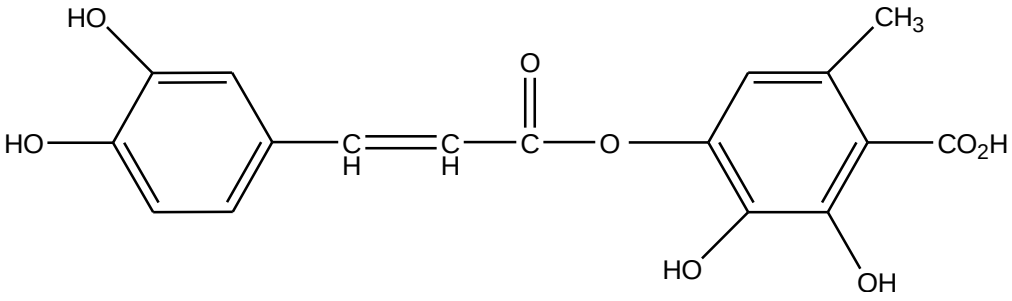
On treatment with acids or enzymes condensed tannins are converted into red insoluble compound known as Phlobaphenes. Phlobaphenes give the characteristics red colour to many drugs such as red cinchona bark, which contain these phloba-tannins and their decomposition products on dry distillation, they yield catechol and these tannins like catechol itself, their solutions turn green with ferric chloride (Trease and Evans, 1997).

1.4.3 Complex tannins

The term ‘complex tannins’ is applied to a newly discovered group of tannins which are from both a hydrolysable tannin (mostly a C-glucoside Ellagitannin) and a condensed tannin. The union occurs through a C-C bond between the C-1 of the glucose unit of the Ellagitannin and the C-8 and C-6 of the flavan – 3-ol devivative (Trease and Evans, 1997).

1.4.4 Pseudotannins

Pseudo tannins are compounds of lower molecular weight than true tannins and they do not respond to the goldbeater's skin test. Example of pseudotannin is the chlorogenic acid tannins (xxxi).



(xxxi)

1.5 Steroids

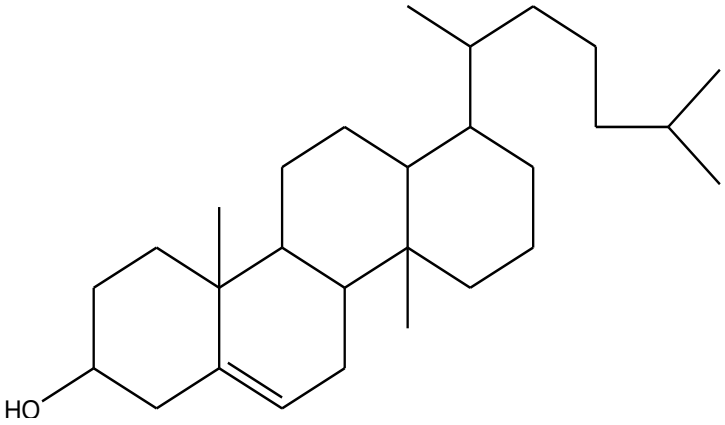
The steroids form a group of structurally related compounds which are widely distributed in animals and plants. Included in the steroids are the sterols [from which the name steroids is derived] , Vitamin D, the bile acids, a number of sex hormones, the adrenals cortex hormones, some carcinogeric hydrocarbons, certain sapogenics etc. The structures of the steroids are based on the 1, 2- cyclopentenophenanthrene skeletons. All the steroids give, among other products, Diels' hydrocarbon on dehydrogenation with selenium at 360^{o}C. Steroid could be defined as any compound which gives Diels' hydrocarbon when distilled with selenium (Finar, 2003).

1.5.1 Sterols

Sterols occur in animal and plant oils and fats. They are group of crystalline steroidal alcohol containing between 27 and 30 carbon atoms all possess 3β-hydroxyl group and an exocyclic double bond usually in 5, 6 position, together with a side – chain which exhibits various degrees of branching and unsaturation (Dence, 1980).

There are three groups of sterols:-

(a) **The animals sterols** :- Cholesterol (xxxiv); cholestanol (xxxv); are the animal sterols. The animal sterols are referred to as zoosterol



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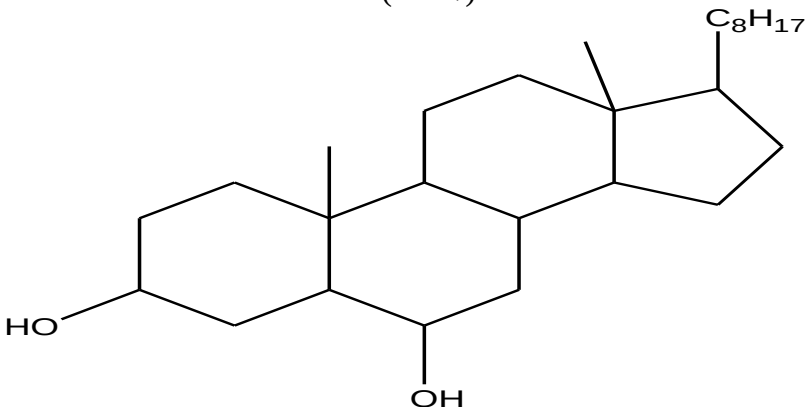
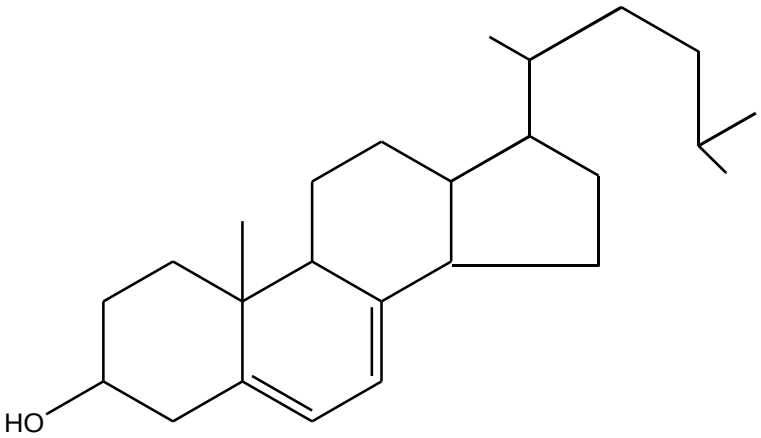


(xxv)

(b) **Plant sterols: -** Ergosterol (xxxvi) and stigmasterol (xxxvii) are the principal plant sterols. The difference in structure between animal and plants sterol is the presence of an additional alkyl in the C-17 side chain of the plant sterols. This group is referred to as phytosterols

(c) **Yeast sterols**:- Yeast sterols is classified under plant sterol sometime. It is an important example of plant steroids. Eg Cardiac ghycosides, digitoxin occur generally largely in a conjugated form with one or more sugar residue. This is referred to as mycosterols.



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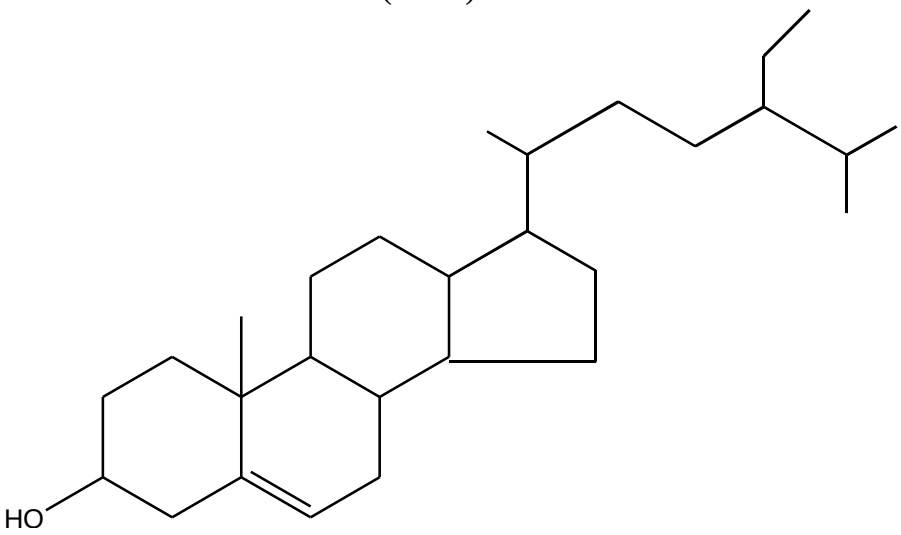


(xxxvii)

1.6 Role of Medicinal Plants in Treating Cardiovascular Diseases

The cardiovascular system is the chief transport system of the body which consists of the heart, blood and a closed system of blood vessels – arteries, arterioles, capillaries, venules and veins. Blood ejected out of the heart through one set of vessels returns to the heart by different set. The heart function as the primary pump, large arteries as conduits, the arterioles as resistance vessels, the capillaries as exchange mechanisms and the veins as capacitance vessels. Each time the heart beats it pumps blood into the arteries. Blood pressure is the force of the blood exerted on the walls of the arteries. The pressure is highest when the heart beats pumping the blood into the aorta and is

called systolic pressure. When the heart is at rest between beats the pressure falls and is called diastolic pressure (Nick *et al*., 1998).

Medicinal plants have been an integral part of society. They have been used for food and as a means of medication since the beginning of civilization. Medicinal plants have made many contributions to commercial drugs preperations manufactured today.

For cardiovascular diseases, herbal treatments have been used in patients with the following:

i. Congestive cardiac failure – Digitoxin from *Digitalis purpurea*, Digoxin from *Digitalis lanata*

ii. Hypertension – Reserpine from the roots of *Rauwolfia serpentine*

iii. Angina pectoris – *Crataegus oxyacetha, Cratuegus monozyna*

iv. Cerebral and Peripheral vascular diseases – *Ginkgo biloba*

v. Venuos insufficiency – *Aesculus hippocastanum* (Nick *et al*., 1998).

1.7 Antihypertensive Agents

Antihypertensive medications are some of the most important and commonly used drugs in medical practice. Hypertension affects at least 50% of person over the age of 60 and is an important cause of morbidity from cardiovascular and cerebrovascular disease. Antihypertensives are a class of drugs that are used to treat hypertension a condition in which Systolic pressure exceeds 160mm (21.3kPa)Hg or Diastolic pressure exceeds 95mm (12.7kPa)Hg. For normal blood pressure, Systolic pressure is equal to or less than 140, while Diastolic pressure is equal to or less than 90, preferably 120/80mmHg (Law *et al*., 2003). Antihypertensive therapy seeks to prevent the complications of high blood pressure, such as stroke and myocardial infarction. Evidence suggests that reduction of the blood pressure by 5 mmHg can decrease the risk of stroke by 34%, of ischaemic heart disease by 21%, and reduce the likelihood of dementia, heart failure, and mortality from cardiovascular disease (Law *et al*., 2003).

An ideal antihypertensive agents must have all or most of the following characteristics:

1. Be active on oral administration
2. Have a useful duration of action
3. Be effective in all hypertensive patients
4. Have minimum side effects
5. Prevent pathological organ changes
6. Reverse pathological organ changes that have occurred
7. Have reasonable cost (NIH, 2014).

There are more than 50 medications in use in the therapy of hypertension. The drugs are classified into seven major classes, these include

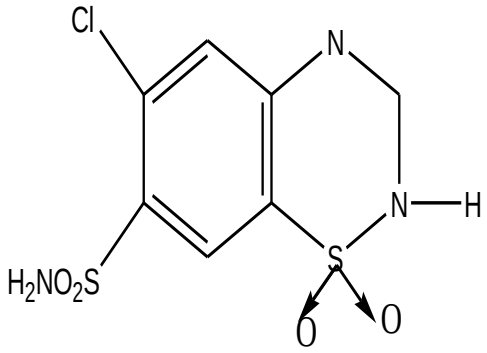
1. Diuretics
2. Sympatholytic drugs
3. Calcium channel blockers
4. Angiotensin Converting Inhitors
5. Angiotensin II Receptors Antagonists
6. Direct Renin Antagonists
7. Various Vasodilators (NIH, 2014)

Antihypertensive agents are classified by mechanism of action than chemical class; this is due to the fact that they vary widely in their chemical structure.

1. Diuretics: These can be categorised into four groups
    a. Thiazide diuretics and related agents
    b. Loop diuretics
    c. Potassium-sparing diuretics

d. Cabonic anhydrase inhibitors

The diuretics lower blood pressure by depleting the body of sodium and reducing blood volume. Eg, hydrochlorothiazide (xxxviii) (Hydrdiuril, Esidex), Furosemide (Lasix) (NIH, 2014).



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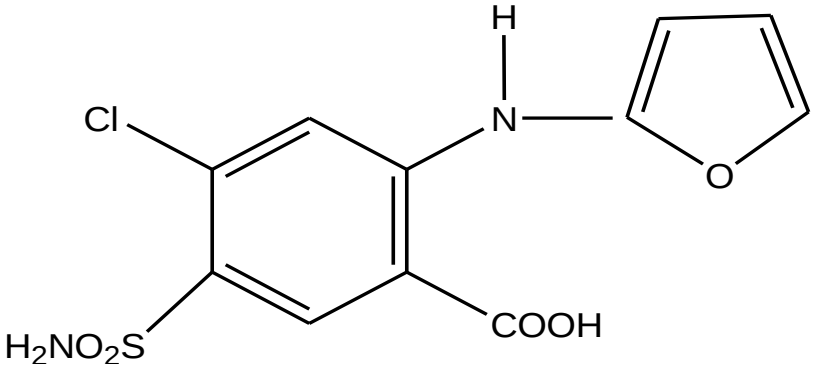
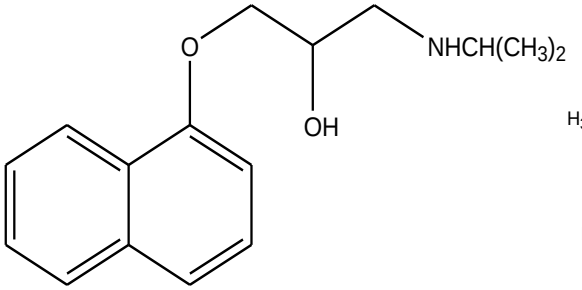


(xxxix)

Hydrochlorothiazide is a benzothiadiazide diuretic while Furosemide (xxxix) is a loop diuretic derivative of 2- aminobenzenoic acid. These natiuretic diuretics can provide adequate treatment for mild or moderate essential hypertension, while in the case of severe hypertension, they are used in combination with sympatholytic and vasodilators drugs. Hydrochlorothiazide belong to a chemical class or Benzothiazidiazole while Furosemide is of the class of anthranilic acid.

2. Peripheral Sympatholytic: this class of antihypertensive drugs include non-selective (β-adrenergic blockers) eg Propranolol (xl) (Inderal), selective (β-blocker) eg atenolol (Tenormin), Open chain guanidine moiety eg guanethidine (Ismelin), cyclic guanidine moiety eg Prozosin (xli)

(Minipress) and Rauwolfia alkaloids eg reserpine (Serpasil). These drugs interfere with adrenergic function by blocking postganglionic adrenergic receptors, eg Propranolol and Prazosin, limiting the release of neurotransmitters from adrenergic neurons eg guanethiline or depleting interneuronal catecholamine storage sites, eg resepine (NIH, 2014)



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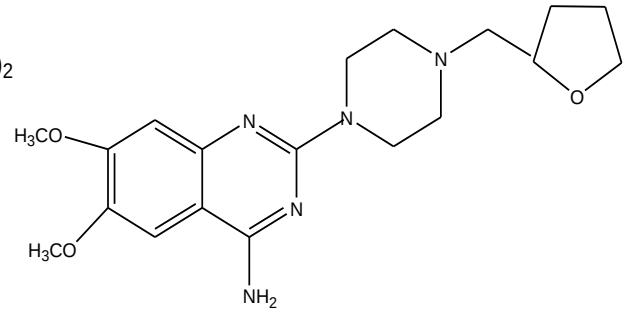
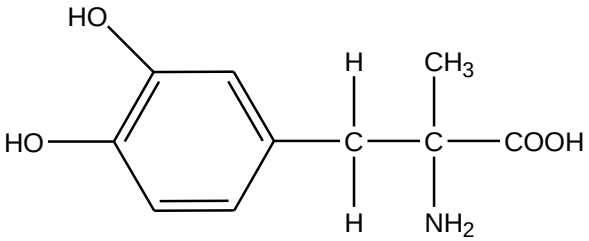


(xli)

Propranolol is highly lipophillic, well absorbed after oral administration and 90% bound to plasma protein, while Prazosin is a highly selective competitive antagonist of α_1- adrenoreceptor, it is readily absorbed on oral administration, bioavailability 55-60%, it is highly bound to plasmaproteins and extensively metabolized in the liver (NIH, 2014).

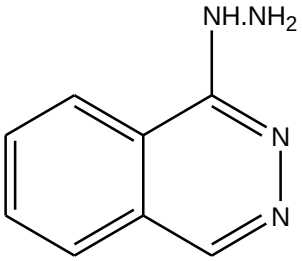
3. Central α_2 – Sympatholytic: They mediates their effects by stimulating presynaptic α_2 – inhibiting receptors, resulting in a negative sympathetic outflow and lowered peripheral resistance, e.g Methyldopa (xlii) (Aldomet) and Clonidine (Catapress). Methyldopa is 2-amino-2-methyl- 3-(3, 4- dihydroxyphenyl) propanoic acid. It is an intermediate acting drug use in the treatment of moderate hypertension, it interfers with noradrenaline biosynthesis and storage. It acts as competitive inhibitor in the dopa to dopamine conversion; it is converted into α – methylnoradrenaline, which displaces noradrenaline from storage sites. When given alone, sodium retension is common; therefore, it is best given with oral diuretics (NIH, 2014)



(xlii)

1. Vasodilators: these include oral Vasodilators, hydralazine and Mioxidil, which are used for long-term outpatient therapy of hypertension, and the parenteral vasodilators, Nitroprusside and diazoxide, which are useful in the treatment of hypertensive emergencies.

   Hydralazine (xliii) (Apresoline), it is a weak base, useful for the treatment of mild to moderate hypertension. It is partly metabolized by acetylation.



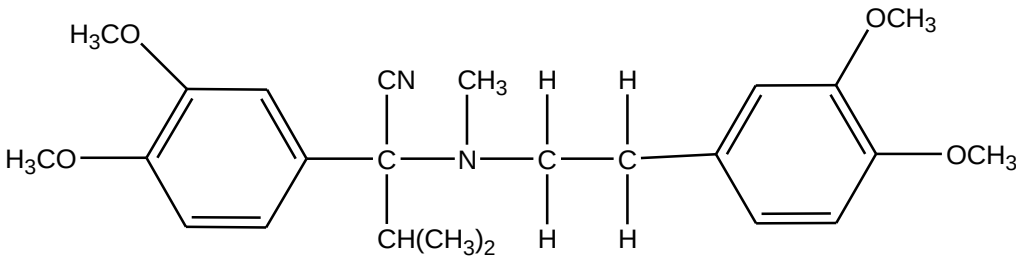
(xliii)

2. Angiotension-Converting enzyme (ACE) Inhibitors: They block the conversion of inactive angiotensin I to the potent Vasoconstrictors angiotensin II. The reduced angiotensin II concentration also lowers aldosterone concentration, which in turn, limits sodium retension, eg Captopril (Capoten), Enalapril (Casotec).

   Captopril contains a sulphydryl moiety and Mercapto proline. It is administered orally, absorbed rapidly with bioavailablity of 75% (NIH, 2014).

3. Calcium Channel Blockers: these are chemical heterogeneous group of compounds having a primary action on potential dependent Ca^{2+} channels. The advantages of Ca^{2+} channel antagonists in hypertension include improvement in blood supply to vital organs, diuretic

activity, coronary vasodilation reduce in heart size and vascular cytoprotention eg Verapamil (xliv) (Calan, Securon).



(xliv)

4. Various artertial and venous Vasodilators are useful for managing hypertension in special situations. Hydralazine is a well known cause of clinically apparent liver injury, usually presenting with autoimmune features. (NIH, 2014).

1.7.1 Role of medicinal plants in the treatment of hypertension

Herbs and herbal drugs have been an integral part of human life. These have been used for food and as medicinal treatment since the beginning of civilation. Herbal medicines have made many contributions to commercial drugs preparations. Examples among many include: Reserpine from *Rauwolfia serpentina*, Ephedrine from *Ephedra sanica*

Hypertension has a tendency to be recurring and long-lasting. Prescription drugs provide inconsistent cure and maintenance of blood pressure levels. Whenever the patients stop the medication, there is a risk relapse or rebound effect and the need in the medical treatment will arise again. Herbal treatment of hypertension provides an alternative to prescription drugs and medication. Drugs are quite effective in the treatment of hypertension, but they are known to cause side effects. Even though some herbal remedies are known to cause side effects like bleeding with *Ginkgo biloba,* hypertension insomnia with *Ephedra* herbal formulas have been used for centuries

to support the systems of the body. They provide nutrients and phytochemicals that can have extraordinary but safe actions on the body systems. They work not by blocking chemical reactions as drugs do, but by balancing body metabolism. The natural cures help in removing the symptoms of the hypertension and also in eradicating the problems from the root level (Barton, 2008).

1.8 Bacterial Infections

Infectious diseases are the major cause of death worldwide and in the tropical part of the world, they account for approximately 50% of death cases (Zampini *et al.,* 2009; WHO, 2005). These might be attributed to poverty and increasing incidence of multiple drug resistance. Microorganisms resistance to almost all anti-microbial agents has been reported, this might be attributed to an indiscriminate use of anti-microbial drugs commonly employed for the treatment of infectious diseases (Gbodossou, 2005). Apart from the development of resistance, some antibiotics have serious undesirable side effects which limit their application. Therefore, there is an urgent need to develop new anti-microbial agents that are highly effective with less toxicity from natural sources (Maurer-Grams *et al.,* 1996).

Long before the discovery of the existence of microbes, the idea that certain plants had healing potentials, are currently characterize as antimicrobial principles. Since antiquity, humans have used plants to treat common infectious diseases. For example, the use of bearberry *Arctostaphylos uvaursi* and craneberry juice (*Vaccinium macrocarpon)* to treat urinary tract infections has been reported. It was also reported that species such as lemon balm *(Melissa officinalis),* garlic (*Allium sativum),* and tea tree *(Melaleuca alternifolia)*have broad-spectrum antimicrobial activities. The essential oils from these plants have been shown to exhibit greater activities than their extracts in the treatment of respiratory system, urinary tract, gastrointestinal, and biliary system infections as well as skin diseases.The essential oils from *Melaleuca alternifolia,* is used as a common therapeutic tool to treat acne and other infectious troubles of the skin (Mendonca-Filho, 2006).

Antimicrobial resistance is one of the biggest challenges facing global public health. Although antimicrobial drugs have saved many lives and eased the suffering of many million people from infectious diseases. Poverty, ignorance, poor sanitation, hunger and malnutrition, inadequate access to drugs, poor and inadequate health care systems, civil conflicts and bad governance in developing countries have adversely limited the benefits of these drugs in controlling infectious diseases (WHO, 1996). The emergence of multidrug-resistant isolates in tuberculosis, acute respiratory tract infections and diarrhea often referred to as the diseases of poverty, has had its greatest toll in developing countries. The epidemics of HIV/AIDS,with over 30 million cases in developing countries,have greatly enlarged the population of immune compromised patients. The disease has left these patients at great risk of numerous infections and even greater risk of acquiring highly resistant organisms during long periods of hospitalization (WHO, 2005).

1.9 Statement of the Research Problem

Hypertension involves mainly two types, essential and secondary. The pathogenesis of essential hypertension is multi factorial and highly complex which can be caused by increase in sympathetic nervous system activity, increase in production of sodium-retaining hormones and vasoconstrictors, deficiencies of vasodilators such as prostacycline and nitric oxide, inappropriate or increased rennin secretion resulting in increased production of angiotensin II and genetic predisposition.

Pathogenesis of secondary hypertension can be caused by chronic kidney disease, renovascular disease, Cushing's syndrome, pheochromocytoma, drugs such as nonsteroid anti-inflammatory drugs and oral contraceptives. These factors can cause increase in preload, increased in contractility, functional constriction and structural hypertrophy, which result in increase in cardiac output and peripheral vascular resistance which can lead to hypertension (Kearney *et al*., 2005)

High blood pressure (BP) or hypertension is a medical condition in which the blood pressure is chronically elevated. Hypertension is the most important modifiable risk factor for coronary heart disease (CHD), stroke, congestive heart failure (CHF), end-stage renal disease and peripheral vascular disease.

High blood pressure affects about 65 million adult Americans. Worldwide, hypertension is seen in about 1 billion people and the prevalence has been estimated to increase by more than 29% by the year 2025. This condition is associated with increased obesity and aging population. Due to the associated morbidity, mortality and economical burden to the society, hypertension remains as a significant public health challenge (Kearney *et al*., 2005).

As hypertension rarely causes specific symptoms, it is undetected until an individual's blood pressure is measured by a physician or until it had caused complications such as stroke or heart attack. The primary goal of treatment is to lower the blood pressure to a normal level through appropriate combination of drugs that achieves this goal.

Since time immomerial, people have used plants in treating various diseases with a little side effect. Their use has been safe and they are cost effective also, one well documented use of plant products is their use as antihypertensive and hypotensive agents. Therefore, there is ever increasing need for safe antihypertensive and hypotensive agents (Agarwal and Sharma, 2012)

In Hausa ethnomedicine of Northern Nigeria, some medicinal plants are used frequently for treating hypertension and this includes: *Stereospermum kunthianum* (Bignoniaceae). The bark is valued both by Hausas and Fulanis as a remedy for diarrheoa and dysentery. It is also used for veneral disease, a decoction boiled with natron, or, as in Sokoto, the bark mixed with a white variety of Guinea-corn to which red natron is added in boiling, being used for gonorrhea. The root, along with other roots, including that of the palm *Hyphaene thebaica* is a remedy for the disease called 'rana' with

symptoms of haematuria (Dalziel, 1999). As part of our efforts to screen some ethnomedicinal plants of Northern Nigeria for antihypertensive and antimicrobial activities, the stem bark of *Stereopermum kunthianum* was investigated.

1.10 Justification of the Study

In recent years, it has been shown that the approved and regulated chemical armamentum available today is failling to provide effective health care for even one-third of the human population (Cordell *et al*., 2001). In addition, a number of significant global disease states. Including, cancer, tuberculosis, malaria and certain viral, fungal and bacterial infections, are showing significant pattern of resistant to the known therapeutic agents (Henry, 2000). Therefore, there is need to develop alternative therapeutic agents from various sources such as medicinal plants.

S. Kunthinum has been used in management of painful conditions and diseases related to bacterial infections. However there is no reported scientific work on this plant. The work could possibly provide clues in terms of chemical markers and chemotaxonomic relationship within the genus and also the Bignoniaceae family.

1.11 Aim of the study

The aim of the study is to isolate some of the bioactive compounds present in the stem bark of *S. Kunthianum* and to provide scientific rationale for the ethnomedicinal use of the plant in the management of hypertension and microbial infections.

1.12 Objectives of the study

The primary objectives of this study were to identify, isolate and characterise bioactive compounds from the stem bark of *S. Kunthinum* using standard phytochemical methods. Secondly to provide biological rationale for the ethnomedical use of the plant in the management of hypertension and

infections. And using standard isolates to determine antimicrobial properties to validate the claimed ethnomedicinal uses of the plant.

1.13 Specific objectives of the study

To Collect and identify the plant.

To extract the plant material with organic solvents.

To evaluate the phytochemical constituents of the plant

To determine antimicrobial activity of the crude methanol and the fractions.

To determine antihyertensive property of the fractions

To isolate and characterise some of the bioactive compounds present in the plant

To determine antimicrobial activity of the isolated compounds

1.14 Hypothesis of the Study

The stem bark of *S. Kunthianum* (Bignoniaceae) contain bioactive compounds with antihypertensive and antimicrobial activities that can be isolated and tested using laboratory models.

CHAPTER TWO

2.0 Literature Review

2.1 Taxonomy of *Stereospermum kunthianum*

2.1.1 Description of *Stereospermum kunthianum*

It is a slender tree that grow up to 15m high, of wooded Savannah and less (5-6m) when in drier Sahel region, present throughout the region and widespread across Africa to the Red sea and Southwards to Malawi, the Congo basin and Angola. The trunk is rarely straight, and the branches too are crooked. The infloerscence is a drooping panicle of pink or purplish flowers which are borne when the tree is wintering. The tree in flower is a very showy and well worth cultivated as an ornamental. The seeds however have a very poor germination and propagation by suckers is recommended. The scaly bark confers some degree of resistance to fire damage. The wood is whitish tinged with yellow or pink and is fairly hard (Burkill, 1985).

Streeospermum kunthianum belong to the family Bignoniaceae with 120 genera and 650 species. It is called Pink Jakaranda with local names such as:Hausa : Sansami, Jiri, Dan Sarkin itatuwa, Turken Doki and Hatsin Tunkiya. Yoruba called it ayada.

It is a small or medium sized tree up to 15(20) m. or small shrub. The Bark grey to whitish, smooth or flaking in plaques. Leaves 18–45 cm. long, 2–5-jugate; petiole 2.7–9(11) cm. long; leaflet-lamina 5–13(16) x 2–6(8.5) cm., ovate, obovate or elliptic, apex obtuse or acute or even apiculate, base cuneate or rounded and slightly asymmetric, margins entire or rarely toothed; superior surface glabrous, sometimes puberulous on the nerves, inferior surface minutely tomentose to velvety or glabrous, frequently with small glands on each side of the midrib near the base; petiolules very short or up to 1 cm. in the lateral leaflets even reaching 4.5 cm. in the terminal leaflet. Inflorescence a lax, terminal, many-flowered panicle 15–48 cm. long, pubescent to tomentose or glabrescent; flowers appearing before the leaves; pedicels 4–12 mm. long; bracts 3–4 mm. long, oblong. Calyx 5–8(10)mm. long campanulate, slightly 5-lobed, or almost truncate tomentose outside mainly on the lower half. Corolla funnel shaped, with a pinkish tube 30–40 mm. long, showing red streaks on the throat and along the lower corolla lobes, puberulous outside, glabrous inside except in a band opposite to staminode; corolla lobes 20 mm. in diam. more or less circular, lighter coloured than the tube. Stamen-filaments 6–15 mm. long; anthers 2 mm. long; staminode 1.5 mm. long. Disk 1.5mm. long. Ovary 5–6mm. long, glabrous, reddish-brown. Seeds 7 x 20–30mm. including the wing. (Keay, 1989).

2.1.2 Ecological distribution

Natural Habitat

Stereospermum kunthianum is found in dry areas of deciduous forest, woodland bush, rocky outcrops, termite mounds and margin of evergreen forests. The specie is well spread all over the Sahel region and is often found near streams.

Geographic distribution

The geographical distribution of *Stereospermum kunthianum* cut across countries of the Sudan-guinea Savanna up to an altitude of 500 - 2400m, with the mean annual rainfall 450 - 900mm and mean annual temperature up to 40^0C (Keay, 1989).

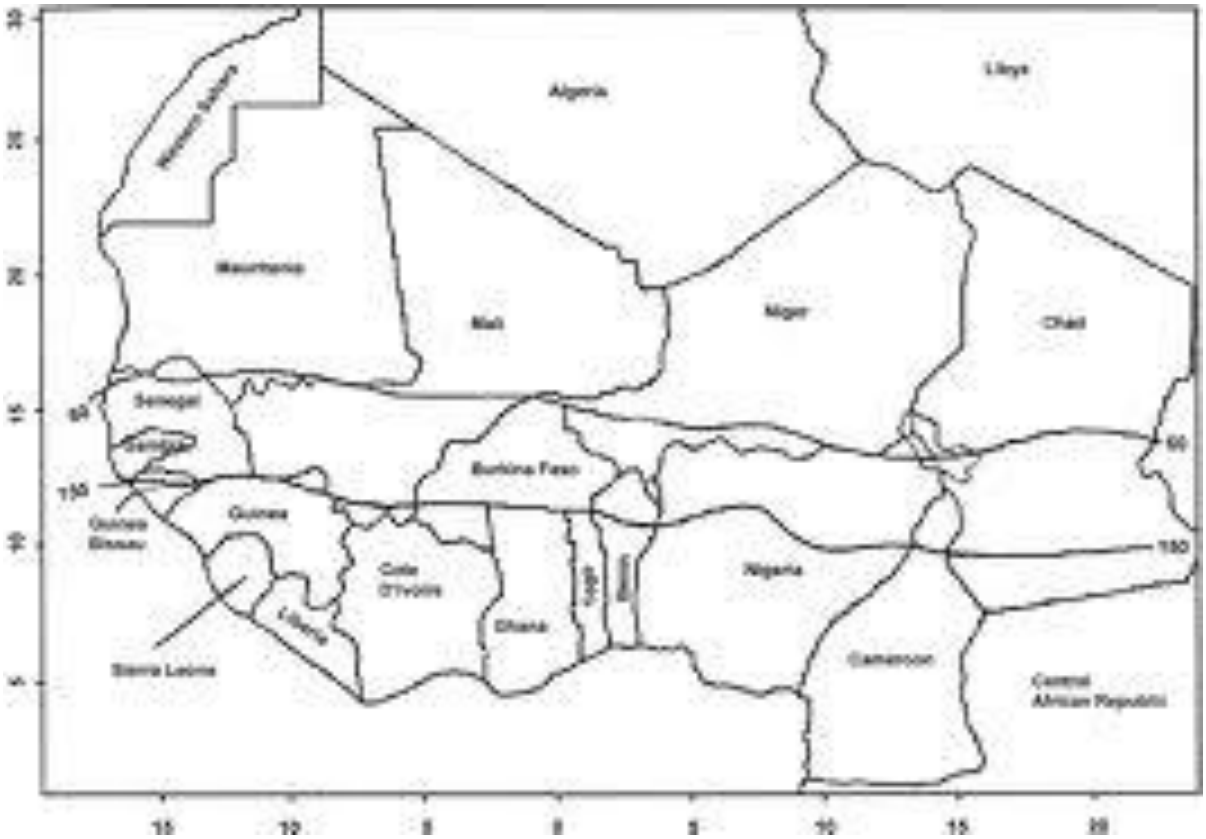
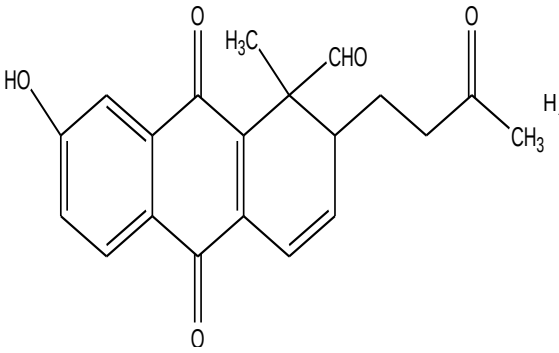


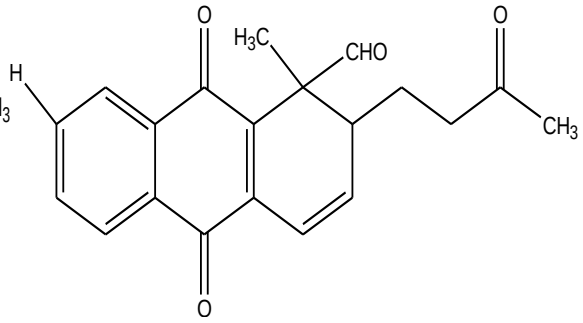
Fig 2. 1: Map of Sudan – Guinea Savannah showing geographical distribution of *Stereospermum kunthianum*

2.1.3 Chemical constituents of *Stereospermum kunthianum* and other genus in the family bignoniaceae

The bioassay - guided fractionation of root bark of extract of *Stereospermum kunthianum* led to the isolation of four novel naphthoquinones (Sterekunthals A(xlv) and B(xlvi), pyranokunthones A(xlvii) and B(xlviii)) together with the known naphthoquinone pinnatal (xlvix) (Onegi *et al.*, 2002).



(xlv)



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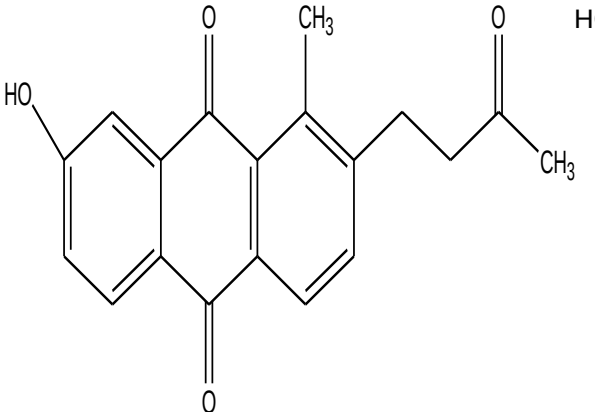

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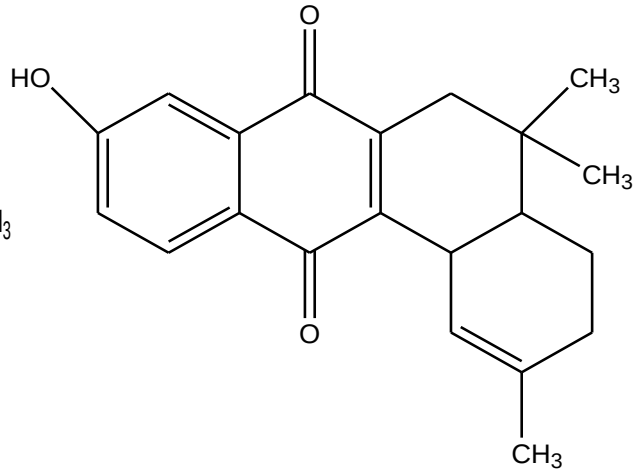

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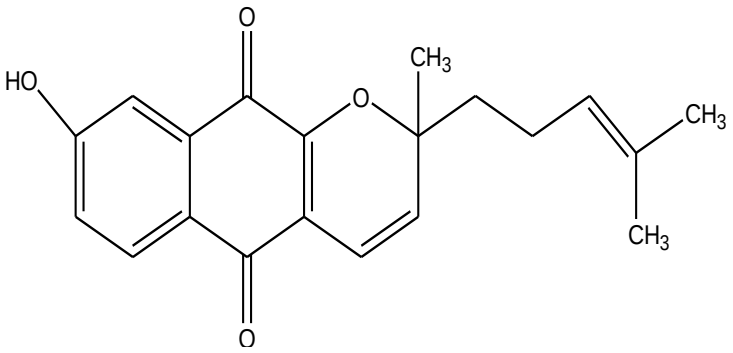
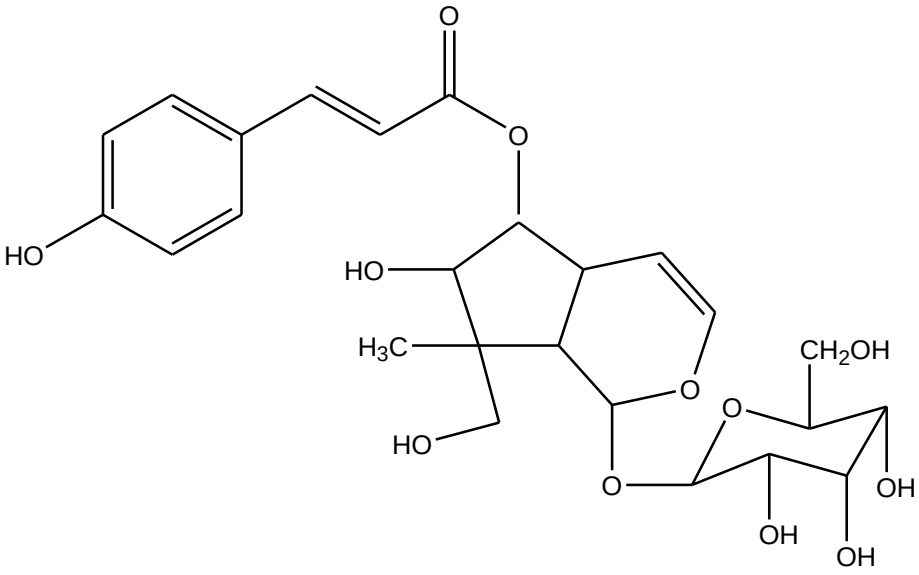
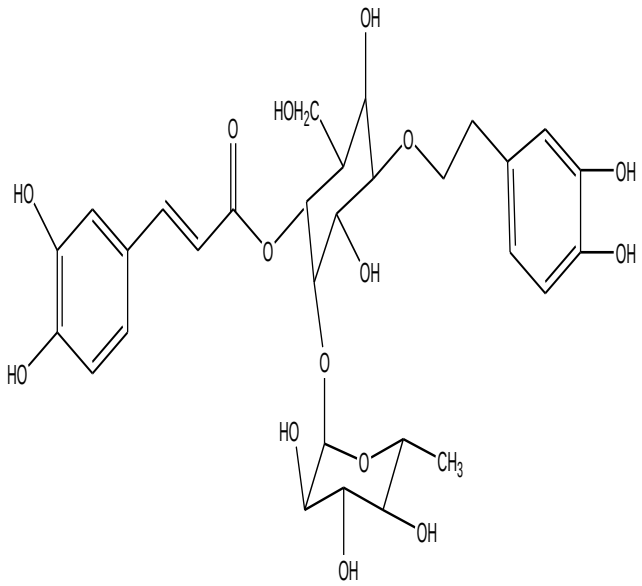


(xlix)

One iridoid (l) and 2 phenylpropanoid (li and lii) were isolated from the stem bark of *S. kunthianum* together with mixtures of β-sitoserol and β- sitosterol glucoside (Falodun *et al*., 2009).



(l)



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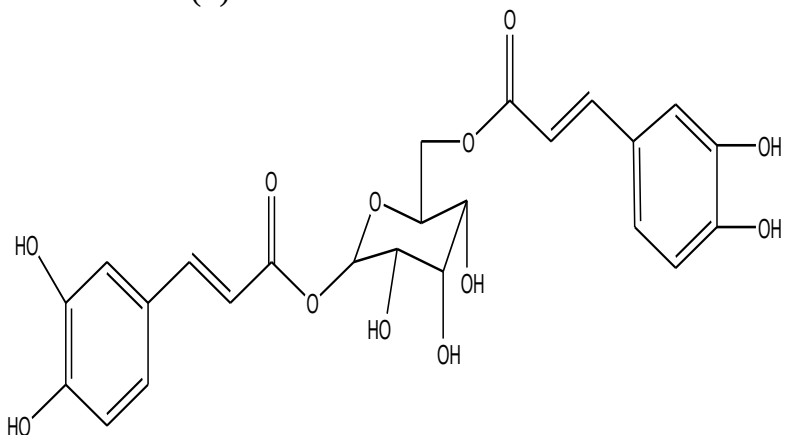
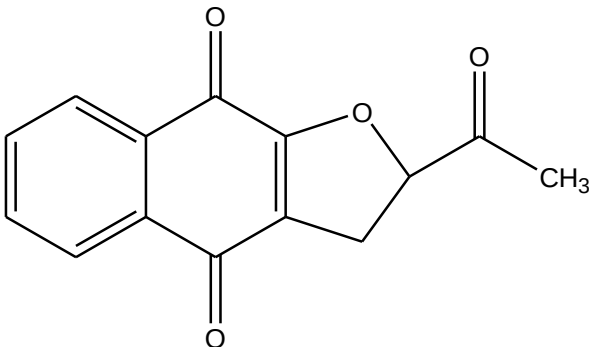


(lii)

The bark of *Kigelia pinnata* is used in traditional medicine; it contains naphthoquinoids and iridoids and has been investigated for antibacterial activity. Also, examination of the non-polar constituents of the root bark of *Kigelia pinna* resulted in isolation of five minor constituents which consisted of two naphaquinones (liii) and three new aromatic monoterpenes (liv and lv) (Akunyili and Housghton, 1993).



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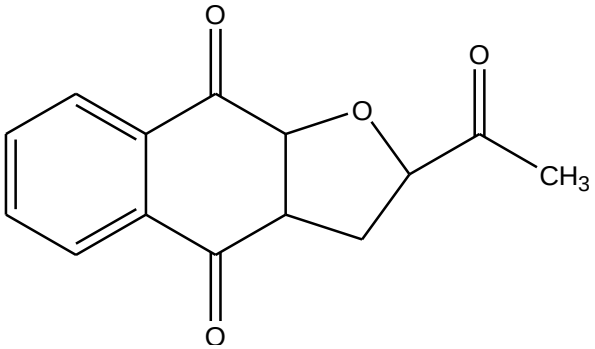

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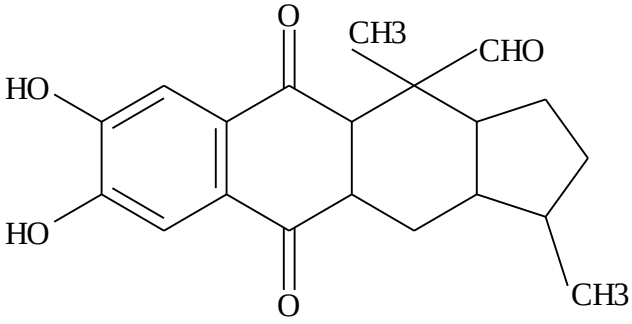

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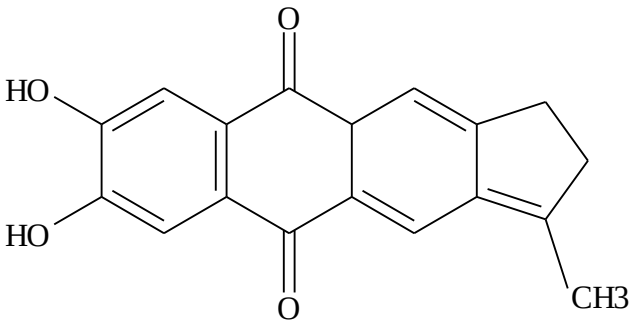


(lvi)

Phytochemical analysis of the root bark of *Newbouldia laevis* revealed the presence of four Pyrazole alkaloids. These were characterized using various spectroscopic data as Withasomnine, and the novel 4' – hydroxywithasomnine, newbouldine and 4' – hydroxynewbouldine (Saburi, 1994).

Tecoma stan is a shrub with bright yellow funnel-shaped flowers, it is found in British greenhouse; it contains monoterpene alkaloids. *Mussata hyacinthiana*, contains iridoids and iridoid glycoside, saponins, phenylpropanoids, tannins and quinines, alkaloids are rare (Trease and Evans, 1997).

2.1.4 Ethnomedicinal uses of *Stereospermum kunthianum*

Locally, *Stereospermum kunthianum* is known to be used**: -**

(a) In Burkina Faso, as toxic plant by the local people and the source of bad feelings in household (Dalziel, 1999). However, despite the toxicity of *Stereospermum kunthianum*, it is widely used as a medicinal remedy of many ailments in Africa.

(b) In treating bronchitis (roots and leaves) (Watt and Brayer, 1962).

(c) As a remedy for cough. The pod of *Stereospermum kunthianum* is used by the Ha people in Tanganyika when chewed with salt.

(d) A decoction of the plant is used in treating venereal diseases; gonorrhea; diarrhea and dysentery (Dalziel, 1999).

(e) *Stereospermum kunthianum* is used in the treatment of ulcers, leprosy, skin eruptions and pneumonia. The roots and leaves are used for respiratory ailments and gastritis (Dalziel, 1999).

(f) The root including that of the palm *Hypaene thebaica,* is a remedy for the disease called 'rana' with symptoms of haematuria, the root bark is value as remedy for certain tribes in Uganda to treat fever (Onegi *et al.,* 2002).

(g) The root-bark is used in inducing labour. The leave and the stem bark are used in the treatment of hypertension in Northern Nigeria.

2.1.5 Pharmacological properties of *Stereospermum kunthinum*

The compounds (xlv) and (xlvi) found in *Stereospernum kunthianum* showed antiplasmodial activity *invitro* against endotheliod cell line EcV-304; its activity against Plasmodium falciparum represents them as interesting lead compounds for drugs against malaria (Onegi *et al*., 2002)

A study carried out on the ethanolic leaves extract of *Stereospermum kunthianum* revealed that the extract have pharmacological activity against diarrhea. (Hanwa *et al., 2007),* also the study on the aqueous extract of the stem bark of the plant indicated that the extract possessed anti diarrheal property (Ching *et al.,* 2008). Anticonvulsant activity was carried out in rodents using the aqueous stem back. The results of a study conducted on the ethanol fraction of *Stereospermum kunthianum* revealed that the stem bark possessed analgesic activity evident in the two analgesic models, which is suggestive of the presence of both centrally and peripherally mediated mechanisms. In the acetic acid-induced abdominal constriction test, the fractions of Stereospermum kunthianum stem bark dose -dependently and significantly reduced the abdominal writhing (Ching et *al*., 2009).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials/Reagents/Equipments and analytical procedure

3.1.1 Chemical/reagents

All organic solvent used are of general purpose grade and were distilled twice before used. All equipment and capacity are quoted accordingly and operated to standard procedures. The silica gel for column chromatography is of mesh size (60 – 120) column size and that of thin layer chromatography is 0.25mm thickness. Thiopentone sodium (LD International: Batch No: 1030), Acetylcholine (Sigma chemicals Co. U.S.A.), Adrenaline (Sigma chemical Co. U.S.A.), Atropine (Halewood chemicals England B/N. 69524), Heparin sodium (Biological ELTD Batch No. 340)..

3.1.2 Collection and preparation of plant material

The plant *Stereospermum kunthianum* Cham (Bignoniaceae) was collected in the month of March, 2012 from Hanwa Village. The plant was authenticated by a taxonomist Umar Gallah at herbarium unit, Department of Biological Sciences, A.B.U., Zaria. By comparing with the existing Herbarium Specimen, (voucher No. 1381). The fresh plant material was carefully cut, air-dried and made into powder using mortar and pestle and subsequently referred to as powdered plant material of stem bark.

3.1.3 Extraction of plant material

The powdered Stem bark (2.00Kg) of the plant was extracted with methanol using cold maceration method. Solvent used was removed at reduced pressure to afford a black oily material (337.56g) that was there after referred to as the methanolic extract coded ME. The methanol extract (200g)

was treated successively with hexane, ethylacetate and acetone to afford hexane (6.71g), ethylacetate (5.25g), and acetone (33.67g) fractions.

3.2 Preliminary phytochemical screening

The crude methanol extract of *S. kunthianum* was subjected to phytochemical screening using standard protocols as summarized below.

3.2.1 Test for steroids and triterpenes

(a) Liebermann – Burchard Test

A little portion of the extract was dissolved in 1ml chloroform and cooled to 0^0C then one drop of concentrated sulphuric acid was added to the cooled mixture followed by addition of 2-3 drops of acetic anhydride. The solution was observed at the upper layer for blue, green, red or orange colour that changes with time. (Silva *et al*., 1998).

(b) Salkowski Test

A little portion of the extract was dissolved in 1ml of chloroform and to it 1ml of conc. Sulphuric acid was added to the test tube to form two phases. Formation of red or yellow colour was taken as an indication for the presence of sterols (Silva *et al*., 1998).

3.2.2 Alkaloids

0.5g of the extract was stirred with 5ml of 1% aqeuos hydrochloric acid on a water bath and filterd. Three ml of the filtrate was divided into three. To the first portion few drops of freshly prepared Dragendoff reagent was added and observed for formation of orange to brownish precipitate. To the second portion 1 drop of Mayer reagent was added and observed for formation of white to yellowish or cream colour precipitate. To the third portion 1 drop of Wagner reagent was added to give a brown or reddish or reddish- brown precipitate (Silva *et al*., 1998).

3.2.3 Test for anthraquinones

(a) Free Anthraquinones

0.5g of the plant extract was shaken with 10ml benzene, the content was filtered and 5ml of 10% ammonia solution added to the filtrate. The mixture was shaken. Presences of a pink, red or violet colour in the ammoniacal layer (lower phase) indicate the presence of free anthraquinones ((Cannel, 2000).

(b) Combined Anthraquinones

The methanol extract was boiled with 10ml of aqueous sulphuric acid and filtered. The filtrate was shaken with 5ml benzene, the benzene layer was separated and to half its volume, 10% NH_4OH was added. A pink, red or violet colouration in the ammoniacal phase indicates the presence of combined anthraquinone derivatives *(Silva et al.,* 1998).

3.2.4 Test for coumarins

A small portion of the ME was dissolved in hot water, and filtered, after cooling, the solution was divided into two test tubes. One contains the reference; the aqueous solution in the second tube was made alkaline with 0.5ml of ammonia solution (10%). They were observed under the ultraviolet light for intense fluorescence.

3.2.5 Test for tannins

A small quantity of methanol extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was an indication for the presence of tannins (Silva *et al*.,1998).

3.2.6 Test for carbohydrate

(a) Molisch Test

A few drops of molisch's reagent was added to the extract dissolved in water, followed by addition of 1ml of concentrated sulphuric acid down the side of the tube. The mixture was allowed ot stand for 2mins, and then diluted to 5ml with water. Formation of a red- or dull violet colour at the interphase of the layer is taken as positive test. (Trease and Evans. 1983).

(b) Barfored Test

Abount 0.2g of the extract was dissloled in water,. Filtered and 1ml of the filtrate was mixed with 1ml of Barford"s reagent in a test tube and heated on a water bath for 2mins, a red precipitate of cupric oxide is taken as positive test (Brain and Turner, 1975).

(c) Reducing Sugars (Fehlings Test)

Abount 0.2g of the extract was dissoled in water and filtered. The filtrate was heated with 5ml equal volume of Fehling's solution A and B. Formation of a red precipitates indicates the presence of reducing sugars (Trease and Evans, 1983)

(d) Combined Reducing Sugar

Abount 0.2g of the extract was hydrolysed by boiling with 5ml of dil. HCl and reulting solution neutralised with NaOH solution. Two drops of Fehling's solution A and B was added and heated on a water bath for 2mins. Formation of a reddish-brown precipitate of cupric oxide indicates the presence of reducing sugars (Trease and Evans,1983).

3.2.7 Test for flavonoids

Shinoda Test

Abount 0.5g of the extract was dissoled in ethanol, warmed and filtered. 3 pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. An orange, pink or

red to purple colour will appear when flavonones, flavonols, the corresponding 2,3-dihydro derivatives and Xanthones are present (Silva *et al*., 1998).

Ferric Chloride Test

Abount 0.5g of the extract was boiled and filtered. To 2ml of the filtrate, two drops of Ferric Chloride solution was added, a green, blue or violet colouration indicates the presence of phenolic hydroxyl group. (Trease and Evans,1983).

Sodium Hydroxide Test

2ml of the filtered extract was dissoled in 10% sodium hydroxide solution to give a yellow colour, a change in colour from yellow to colourless on addition of dilute HCl indicate the presence of Flavonoids.(Trease and Evans,1983).

3. 3 Column Chromatography Procedure

3.3.1 Thin layer chromatography

Thin layer chromatography was carried out on TLC precoated aluminium sheet silica gel Pf_{254} with layer thickness of 0.25mm.

Technique: One way ascending technique was used

Spotting and development: The spots were applied manually using capillary tubes and the plates developed at room temperature using a shandom chromatotank.

Mobile phase: Details in text

Solvent systems: Various solvent systems were used depending on the material and they include:

a. Ethyl acetate: Chloroform (4:6)

b. Hexane : Ethyl acetate (7:3)

c. Hexane : Ethyl acetate (9:1)

d. Ethyl acetate : Chlorofom (8:2)

e. Chloroform : Methanol (7:3)

g. Ethyl acetate: Chloroform : Methanol: Water (15:8.4:1)

Detection: Spots on TLC plates were visualized under UV light (254 and 366 nm) and spraying with 10% sulphuric acid, followed by heating at 110˚C for 5-10 min.

3.3.2 Column Chromatography

The following column conditions were employed in running the column chromatography.

(a) Technique - Gradient elution

(b) Column - Glass column with sintered disc at the bottom of various dimensions

(c) Stationary phase - Silica gel, 60 – 120 mesh size

(d) Column packing - Wet slurry method.

(e) Sample loading - The sample was applied using dry load method (Cannell, 1998), the sample was dissolve in small amount of suitable organic solvent, it was then mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the column.

(f) Solvent for elution - Various solvents systems were used depending on the materials. Elution was carried out using one or mixture of the following solvents:

Hexane

Petroleum ether

Chloroform

Ethylacetate

Methanol

3.4 Gel filtration chromatography

Gel filtration was performed using Sephadex LH-20 (Sigma)

Packing method*:* The gel filter was suspended in methnol and allowed to swell for 24 hours prior to use. It was then poured to the column and allowed to settle tightly.

Sample application*:* The sample was dissolved in small volume of the eluent and applied on top of the column.

Solvent*:* Methanol.

3.4.1 Preparative thin layer chromatography (PTLC)

PTLC was carried out using Fluka silica gel precoated glass plates 20 x 20 cm with layer thickness of 0.25mm. A thin line about 1.5cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of solvent to give an approximate concentration of 20mg/ml. It was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using chloroform: ethyl acetate (6:4) as solvent system. The developed plate was air dried in a fume cupboard and the position of the band of interest was marked with pencil and scraped off the backing of the plate on to a foil. The scrapped sorbent was size reduced using pestle and mortar, transferred to a sintered glass funnel and washed repeatedly with acetone and the solution obtained was evaporated to give the compound (Gibbons and Gray, 1998).

3.4.2 Melting point (m.p.) determination

The melting points of the isolated compounds were determined using Gallenkamp melting point apparatus. Results were uncorrected.

3.4.3 Spectral analysis

Infrared (IR) absorption spectra were recorded using an infrared spectrophotometer Shimadzu 8400S.

NMR spectra were obtained on a Bruker AVANCE (400 and 600 MHz for ^{1}H and ^{13}C) spectrometer, using the residual solvent peaks as internal standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants (J value) are given in Hertz. HNBC spectra were optimized for a log range J_{H-C} of 7Hz (d_6=0.07s) and NOESY experiment was carried out with a mixing time of 0.4s. The NMR solvents used for these measurements are deutrated chloroform and deutrated methanol.

3.5 Chromatographic Separation

3.5.1 Thin Layer Chromatography of n - hexane Fraction

The hexane fraction (0.5g) was dissolved in few drops of hexane, the resulting solution was subjected to thin layer chromatography using precoated TLC aluminum plate. The solvents system used was hexane: ethylacetate (9:1). The plate was allowed to dry, and then visualized under:

(i). visible light

(ii) Ultraviolet light

(iii) Spraying with freshly prepared sulphuric Acid.

3.5.2 Column chromatography of n-hexane fraction

N - hexane fraction (5g) was chromatographed over silica gel (60-120 mesh) packed column of dimension (100 x 4cm), the column was eluted continuously using hexane, then hexane ethylacetate mixture as solvent system. A total of one hundred and eight fractions (100ml each) were collected. The fractions were pooled together based on their TLC profiles to give fifteen major fractions and the column was finally washed with ethylacetate to give the sixteenth fraction. Fractions 22 – 27 gave six spots using hexane: ethyl acetate (9: 1) with a greenish white substance

that was subjected to purification by soaking in hexane over night. The hexane soluble portion was gently decanted, and on evaporation, a white substance which consist of one major spot and two minor spots. This was subjected to a preparative thin layer chromatography using hexane ethylacetate (9:1) to afford a single homogeneous spot. After filtration , a white amorphous powder was obtained and the TLC was carried out using different solvent systems hexane : ethyl acetate 9 :1, 8 : 2 and 7 : 3 and also in chloroform : ethyl acetate 7 :3. This was coded as HW1 and the melting point and the solubility were subsequently recorded.

3.6 Thin layer chromatography of acetone fraction

The acetone extract of *S. kunthianum* was subjected to thin layer chromatography using precoated TLC aluminum plate. The solvent systems used were hexane: ethylacetate (7:3) and ethylacetate: chloroform: methanol: water (15: 8: 4: 1).The developed plates were visualized under visible light, ultraviolet light and by spraying with sulphuric acid.

3.6.1 Column chromatography of acetone fraction

Acetone fraction (10g) was chromatographed over silica gel (60 – 120 mesh) packed column of dimension (100 x 4cm), the column was eluted continuously using petroleum ether, mixtures of petroleum ether and chloroform, chloroform and ethylacetate, and ethylacetate and methanol. One hundred and ninety fractions, (100ml each) were collected. The fractions were pooled together based on their TLC profile to give twenty five fractions and the column was finally washed with methanol to give the twenty sixth fractions. Fraction 9 which showed one major spot and four minor spots in chloroform: methanol (9:1) was subjected to repeat (3 times) gel filtration using sephadex LH20 and eluted with methanol. sixty fractions ($A_1 - A_{60}$), 2ml each were collected.The fractions were pooled together based on their TLC profiles to give one major spot and two minor spots using chloroform : methanol (9 :1) and final purification with preparative thin layer

chromatography (PTLC) using chloroform: ethyl acetate : methanol : water (8 : 15 :4 :1) afford 50mg of compound HNW3. A light brown amorphous solid. This was subjected to physico-chemical tests and the results recorded.

Pooled fractions with eluent number 14 from the column chromatography of the acetone fraction on TLC using chloroform : ethylacetate (8:2) with close R_f values were Purified using repeated gel filtration over sephadex LH-20 eluted with methanol, led to the isolation of brownish-yellow solid compound coded HNW2 (20mg) which revealed a single homogenous spot using chloroform: methanol (8:2), chloroform : methanol : acetic acid (3:2:0.5) and chloroform: ethyl acetate : methanol : water (8 : 15 :4 :1) as the TLC solvent systems. HNW2 was subjected to physio-chemical tests and the results recorded.

3.7 Antihypertensive Screening

Animals

Cats (1.33-1.72Kg) were obtained from the Animal House of the Departrment of Pharmacology and Therapeutics Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. Animals were approved for use by the Animal Facility Centre (AFC) committee after reviewing the protocol. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimise the number of animals used and their suffering.

3.7.1 Effect of acetone fraction on cat blood pressure

Male cats weighing between 1.33 – 1.72kg were used for the experiment. The animals were anaesthesized using thiopentone sodium (40mg/kg) intraperitoneally and later injected with heparin to prevent clotting during dissection. The femoral vein was exposed and cannulated for drug administration. The carotid artery was also exposed and canulated with a tube inserted that connect to transducer and microdynamometer for blood pressure recordings. The trachea was exposed to assist in respiration. The paper speed was set and maintained at 95mm/min. The microdynamometer (Ugo basile 7050) was set at sensitivity of 3.9.

0.9% Normal saline (0.2ml) was injected through the femoral vein and subsequently Acetylcholine (0.75µg/kg), Acetone fraction (3.4mg/kg – 6.79mg/kg) was administered and the responses recorded. Acetone fraction (1.5mg/kg) was administered in the presence of Atropine (0.75µg/kg – 1.5µg/kg) and the responses were also recorded. Adrenaline (1µg/kg) was administered alone and in the presences of acetone fraction (1.5mg/kg – 6.79mg/kg) and adrenaline (1µg/kg) and acetone fraction (1.4mg/kg-13.75mg/kg)) was also administered and the responses recorded (Enye *et al*., 2013).

3.8 Antimicrobial Studies

3.8.1 Test microorganisms

The test microorganisms were clinical isolates obtained from the Medical Microbiology Department, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. These organisms included; *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi, Klebsiella pneumoniea*, and *Candida krusei*. For the methanol extract, acetone, ethylacetate,

hexane fractions and the isolated compound HNW3. All the isolates were checked for purity and maintained on slants of nutrient agar at 4^{o}C for preservation before used.

3.8.2 *Preliminary* antimicrobial studies

The paper discs diffusion method was used to determine the antimicrobial activities of the stem bark methanol extract, acetone, ethyl acetetae and hexane fractions as well as that of the isolated compound HNW3. A quantity (0.2g) of the extracts and the fractions was dissolved in 10ml each of the solvent medium in which it was extracted. Nutrient agar was used as the medium for the microorganisms. The medium was prepared according to manufacturer's instructions and 20ml of the sterilized medium was poured into sterile Petri dishes. The plates containing the medium were allowed to solidify and later seeded with the test organisms by the spread plate method and left to dry for half an hour (Cowan, 1999; Ntiejumokwu *et al.,* 1991). Filter paper discs were punched and sterilized at 160^{o}c for 30mins. 1ml of the extract was used to sock 20 discs and the sterilized discs were soaked in different concentration of (150mg/ml, 100mg/ml, 75mg/ml, and 50mg/ml) of the stem bark methanol extract and the fractions. The plates were incubated at 37^{0}C for 24hrs, after which it was inspected for the zones of inhibition of growth. Standard antibiotic Cirprofloxacin (10µg) was used as reference standard to determine the sensitivity of the isolates. Discs were directly placed onto the bacterial culture. The plates were allowed to stand for 2hr at room temperature for extract and fractions to diffuse into the agar and then incubated at 37^{o}C over night. At the end of incubation, the zones of inhibition were measured and recorded in millimeters using a transparent ruler (Kunle *et al.,* 2003; Ogbulie, *et al.,* 2007).

The antimicrobial activities of compound isolated HNW3 from the acetone fraction of *Stereospermum kunthianum* was determined using stock concentration of 50µg/ml. HNW3 (0.005mg) was weighed and dissolved in 10ml of DMSO to obtain a concentration of 50µg/ml. This

was the initial concentration of the HNW3 used to check for the antimicrobial activities of the compound.

The test organisms from growth on nutrient agar incubated at 37^{o}C for 18hr for bacteria, while for *Fungi* sabraud dextrose broth was used and was incubated for 48 hours. After incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the Gram negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient agar at 45^{o}C and poured into sterile petridish. Similarly 1ml of the diluted fungal suspension was poured into sterile saboraud dextrose agar plates and the excess sucked up with Pasteur pipette. The wells were filled respectively with equal volume of 0.1ml of 0.005mg/ml of the HNW3 making 50µg/ml. Standard disc (5µg/disc) of erythromycin and (100units/disc) fluconazole for bacteria and for fungi respectively were placed on the agar and saboraud dextrose agar plates and serve as positive control. One hour was allowed for the extract to diffuse into the agar after which the plates were incubated overnight at 37^{o}C for 24hours and 25^{0}C for 48hours for fungi and bacteria respectively. At the end of incubation period, diameter of inhibition zone was measured and recorded. The inhibition zones with diameter of 12mm and above were consider as an indication of antimicrobial activity (NCCL, 1993)

3.8.3 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the stem bark methanol extract and the fractions against the test microorganisms were determined using broth dilution technique (Sidney *et al.,* 1978; Vollokova *et al.,* 2001). Nutrient broth was prepared according to manufacturer's instruction and 10mls of the broth was dispensed into screw capped tubes for further use.

Each of the extract and the fractions were separately dissolved in sterile distilled water and 2ml of the sterile Mueller Hinton broth was transferred into a set of 5 tubes and 2ml of each concentration of 150mg/ml, 100mg/ml, 75mg/ml and 50mg/ml was added to the test tubes. The test organisms

were inoculated into the labeled tubes except the control; the tubes were incubated at 37^{o}C for 18hr. The lowest concentration of the extract and the fractions inhibiting the visible growth of each microorganism represented the minimum inhibitory concentration (Osadebe and Ukueze, 2004).

The minimum inhibitory concentration of the HNW3 against the test microorganisms were determined using broth dilution technique (Sidney *et al.*, 1978; Vollokova *et al.*, 2001). Nutrient broth was prepared according to manufacturer's instruction; 10mls of the broth was dispensed into screw capped tubes for further use.

Normal saline was prepared, 10ml was dispersed into sterile test tubes and the test organisms was inoculated and incubated at 37^{o}C for 6hr. Dilution of the test organisms was carried out in the normal saline (0.85% NaCl) and adjusted to match a turbidity of 0.5 (10^{8} cells/ml) McFarland standard.

Two fold serial dilution of the HNW3 in the sterilized broth was made, to obtain the concentrations of 50, 25, 12.5, 6.25 and 3.125µg/ml. The initial concentration was obtained by dissolving 0.005mg of HNW3. 0.1ml of the test microbes in the normal saline was then inculated into different concentrations of HNW3. Incubation was made at 37^{0}C for 24 hrs. for bacteria and 25^{0}C for 48h. for fungi, after which the test tubes of the broth was observed for turbidity (growth). The lowest concentration of the compound in the broth which showed no turbidity was recorded as the minimum inhibition concentration (Osadebe and Ukueze, 2004).

3.8.4 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was determined using broth dilution technique (Sidney *et al.*, 1978; Vollokova *et al.*, 2001). Nutrient agar base used in this experiment was prepared, sterilized at 121^{o}c for 15min and was poured into sterile Petri dishes, left to cool and solidify. The content of the tubes used in determining the MIC that showed no visible growth were then sub cultured onto freshly prepare Mueller Hinton agar and incubated at 37^{o}C for 24hrs, after which the

colony growth was inspected. The plate with lowest concentration of the extract or the fraction at which the organisms did not recover and grow was taken as the minimum bactericidal concentration (Ndukwe et *al.*, 2001; Meenakshi *et al.*, 2001).

The minimum bactericidal concentration was determined using the method described by Rotimi *et al.*, (1988) by assaying test tubes content from MIC determination. A loopful of the content of each tube was inoculated by streaking on solidified agar plate and then incubated at 37^0C for 24 hr for bacteria and 25^0C for 48h. for fungi, for bacteria and for fungus respectively after which the plates were observed for organisms growth. The concentration of the sub-culture with no growth was considered as MBC/MFC (NCCL 1993).

CHAPTER FOUR

4.0 Results

4.1 The Yield of the Extract and the Fractions

The yield of the extract and the fractions are shown in table 4.1. The highest yield were obtained from methanol extract and acetone fraction.

Table 4.1: Percentage yield of the extract and the fractions

Extract/fractions	Yield (g)	% Yield
Methanol	200	16.88
Hexane	6.71	1.98
Ethylacetate	5.25	1.56
Acetone	33.67	16.88

4.2 Preliminary Phytochemical Constituents of the Stem Bark Extract of *Stereospermum kunthianum*

The phytochemical screening of the *Stereospermum kunthianum revealed* the presences of flavonoids, tannins, saponins, steroids, coumarins and carbohydrates while alkaloids are absent. (Table 4.2)

Table 4.2: Preliminary phytochemical screening of stem bark of methanol extract of *Stereospermum kunthian*

Compounds/Group	Test	Observation	Inference
Steroids/triterpene	Liebermann-Burchard test	Greenish coloration	+
	Salkowski test	Red colour	+
Flavonoids	Sodium Hydroxide test	Yellow colour	+
	Shinoda test	Green colouration	+
	Ferric chloride test	Blue/Black colour	+
Coumarins	Extract + NH_3	Intence fluorescence	+
Alkaloids	Dragendroff's test		-
	Mayer's test		-
	Wagner's test		-
Saponins	Frothing test	Frothing Persist	+
Carbohydrates			
General	Molisch'test	Red colouration	+
Monosaccharide	Barfoed's test	Red ppt.	+
Reducing Sugars	Fehling's test	Red ppt.	+
Tannins	Ferric chroride	Blue/black ppt	+

Key: - **+** **=** **Present**
- **=** **Absent**

Table 4.3: Column chromatography of n-Hexane fraction of *Stereospermum kunthianum*

Eluent No.	Fraction No.	Eluenting Solvent	Spot
1	1 – 3	Hex 100%	-
2	4 – 14	Hex : EtOAc 97.5: 2.5	3
3	15 – 21	Hex : EtOAc 95: 5	4
4	22 – 27	Hex : EtOAc 92.5:7.5	6
5	28 – 36	Hex : EtOAc 90: 10	2
6	37 – 40	Hex : EtOAc 87.5:12.5	3
7	41 – 48	Hex : EtOAc 85: 15	6
8	49 – 62	Hex : EtOAc 82.5:17.5	-
9	63 – 69	Hex : EtOAc 80: 20	3
10	70 – 76	Hex : EtOAc 77.5:22.5	2
11	77 – 82	Hex : EtOAc 70 :30	2
12	83 – 87	Hex : EtOAc 65: 35	3
13	88 – 92	Hex : EtOAc 60 : 40	2
14	93 – 96	Hex : EtOAc 50: 50	3
15	97 – 100	Hex : EtOAc 40: 60	2
16	101 – 104	Hex : EtOAc 30: 70	2
17	105 – 108	Hex : EtOAc 20: 80	2

Note: Hex = Hexane, EtOAc = Ethyl acetate

4.3 Analysis of HW1

4.3. 1 Solubility of HW1

HW1 was found to be soluble in chloroform.

4.3.2 Melting point of HW1

The sample was found to have a melting point range of 137^0 C - 139^0 C.

4.3.3 Chemical tests

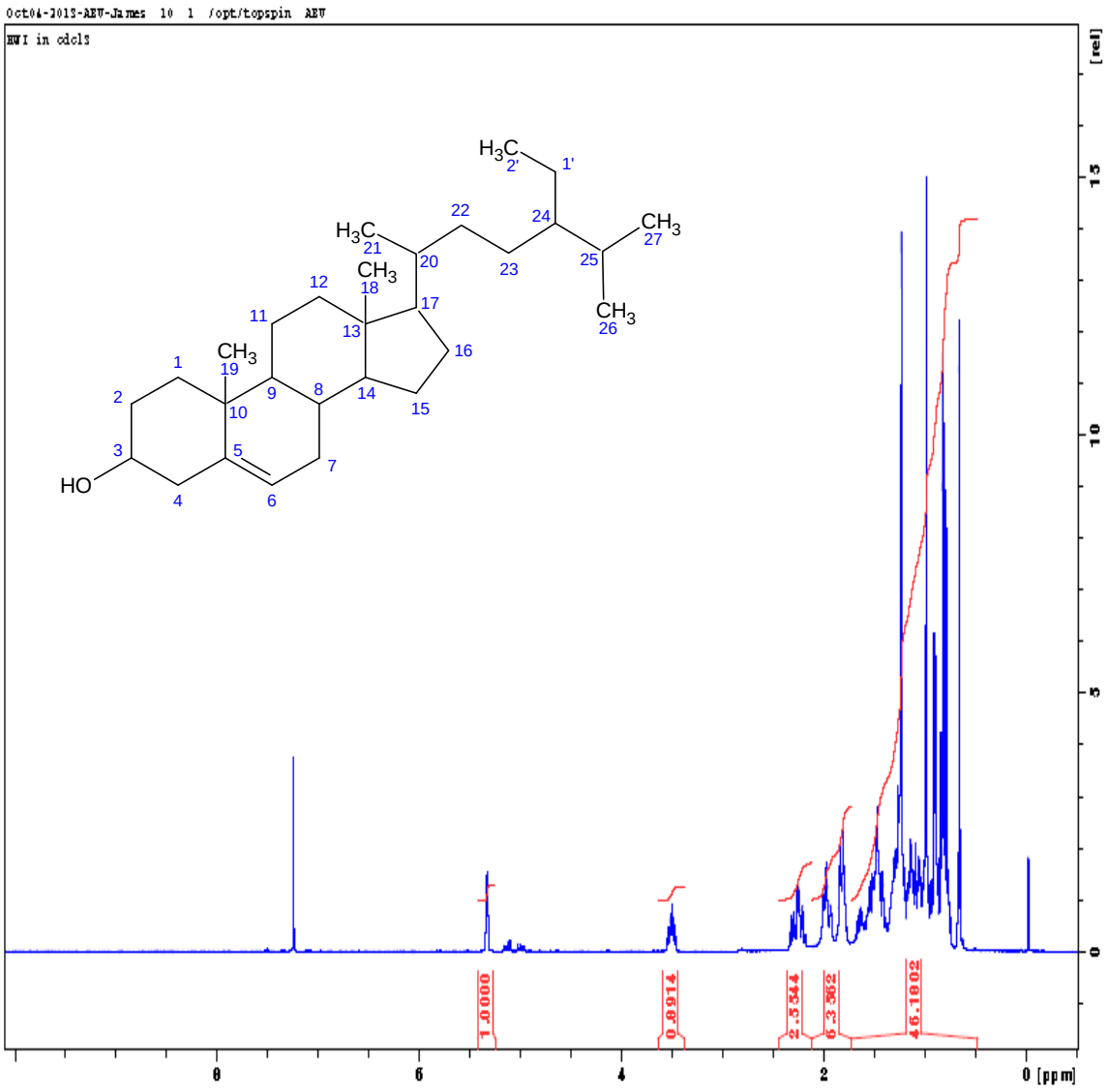
Test for Steroids: HW1 gave reddish colour at the upper chloroform layer, for Salkowski test. While violet, blue and finally green colour was observed for the liebermann-Burchard test.

4.3.4 Spectral analysis of HW1

4.3.5 Proton magnetic resonace of HW1

Proton Magnetic Resonance (^{1}HNMR) of HW1

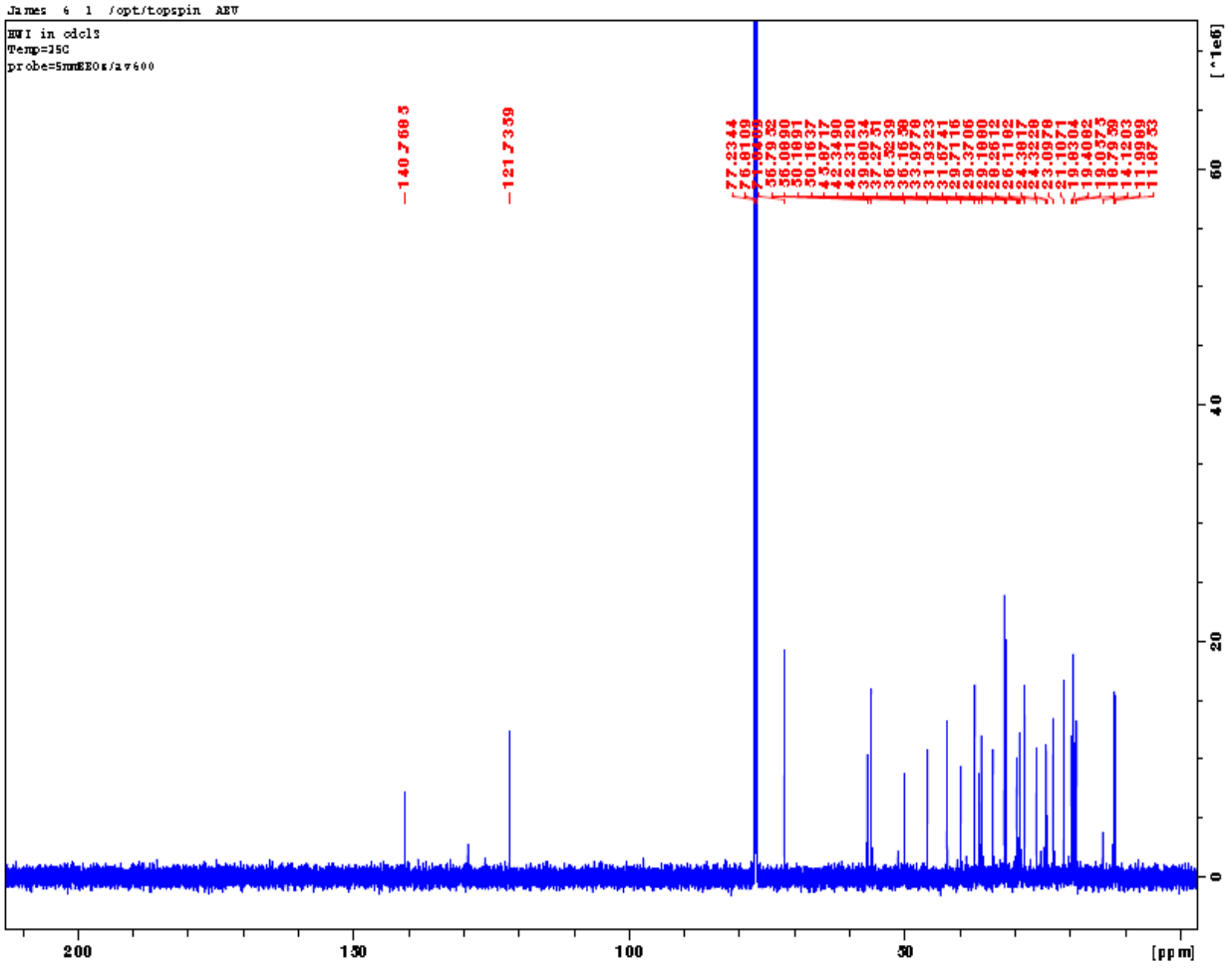
The ^{1}H NMR spectrum of HW1 (Figure 4.1) revealed the presence of resonances at δ 0.80, δ 0.81, δ 0.91, δ 0.94, δ 1.01, and δ 1.36ppm, also at far downfield chemical shifts was observed at δ 5.36ppm. The spectrum also showed a proton which appeared as a triplet of double of doublets at δ 3.54ppm. Whereas other resonances were observed at δ 1.57, δ 1.28, δ 1.85, δ 2.01 and δ 3.28ppm.



Figrure 4.1: Proton Nuclear Magnetic Resonance (NMR) Spectrum of HW1

Carbon -13 Magnetic Resonance of HW1

The ^{13}C NMR spectrum of HW1 (Figure 4.2) indicated signals at δ (ppm) 72.0 for C_3 (β – hydroxyl group) and δ 142.10 and 122.1 for C_5 = C_6 respectively. While 20.3 and 20.5ppm are also observed. Others appear at δ 24.8, 19.7, 19.6 and 12.3. The spectrum also showed resonance within the range of δ 20 - 57 ppm. In all a total of twenty nine carbon atoms were shown from the spectrum of HW1.



Figrure 4.2: Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of HW1

DEPT Carbon-13 NMR Spectrum of HW1

DEPT Carbon – 13 spectrum (figure 4.3) revealed the presence of six methyl carbons at positions 18, 19, 21, 26, 27 and 29; eleven methylene carbons ranges from 20-42ppm, Nine methine and four quartenary carbon atoms.

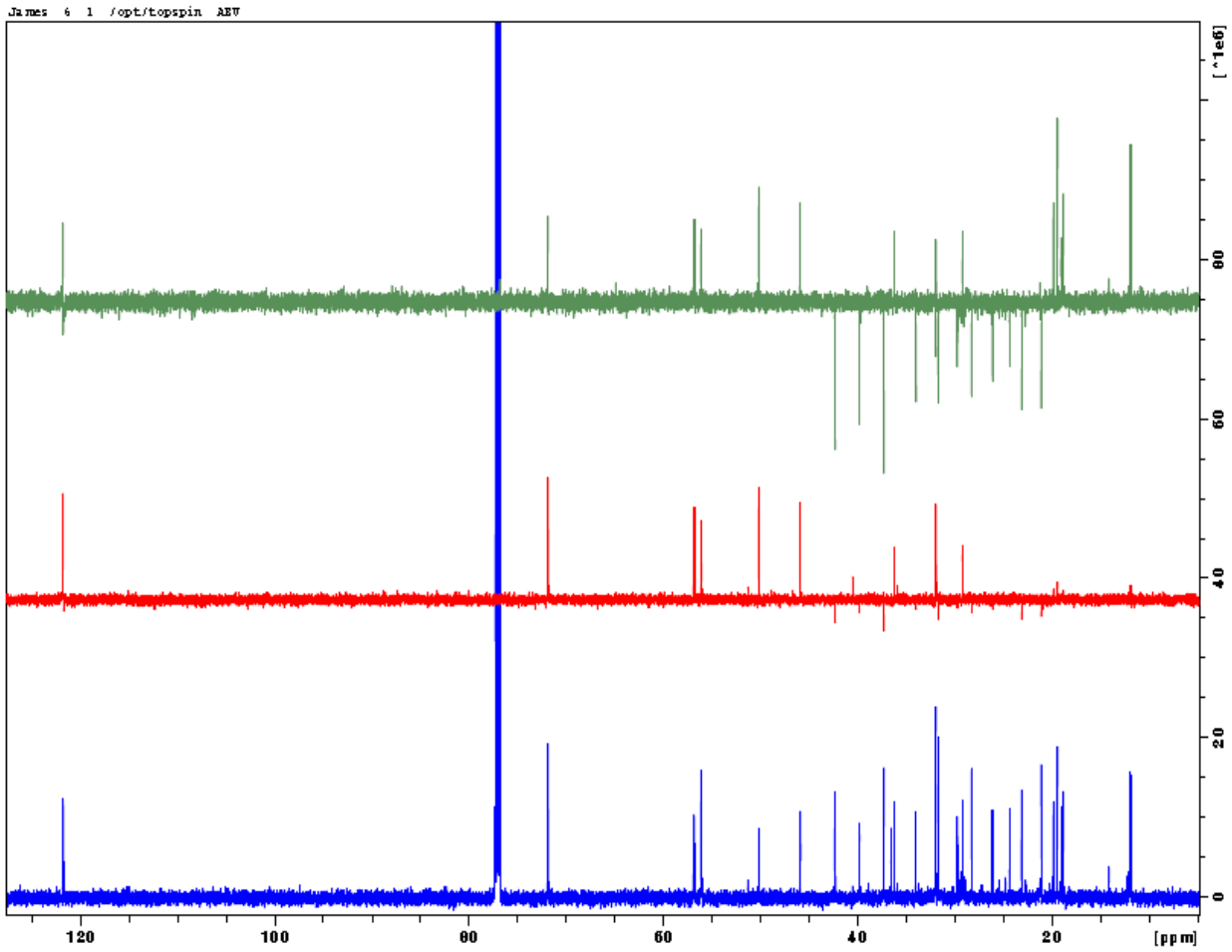


Figure 4.3: DEPT Carbon-13 NMR Spectrum of HW1

Correlation Spectroscopy of HW1 (COSY)

The COSY spectrum of HW1 (figure 4.4) showed correlation between carbinol proton at 3.55ppm correlate with the proton at 1.45 and 2.25ppm respectively. Another correlation can observed between the olefinic proton at 5.38ppm and the proton at 2.00pm.

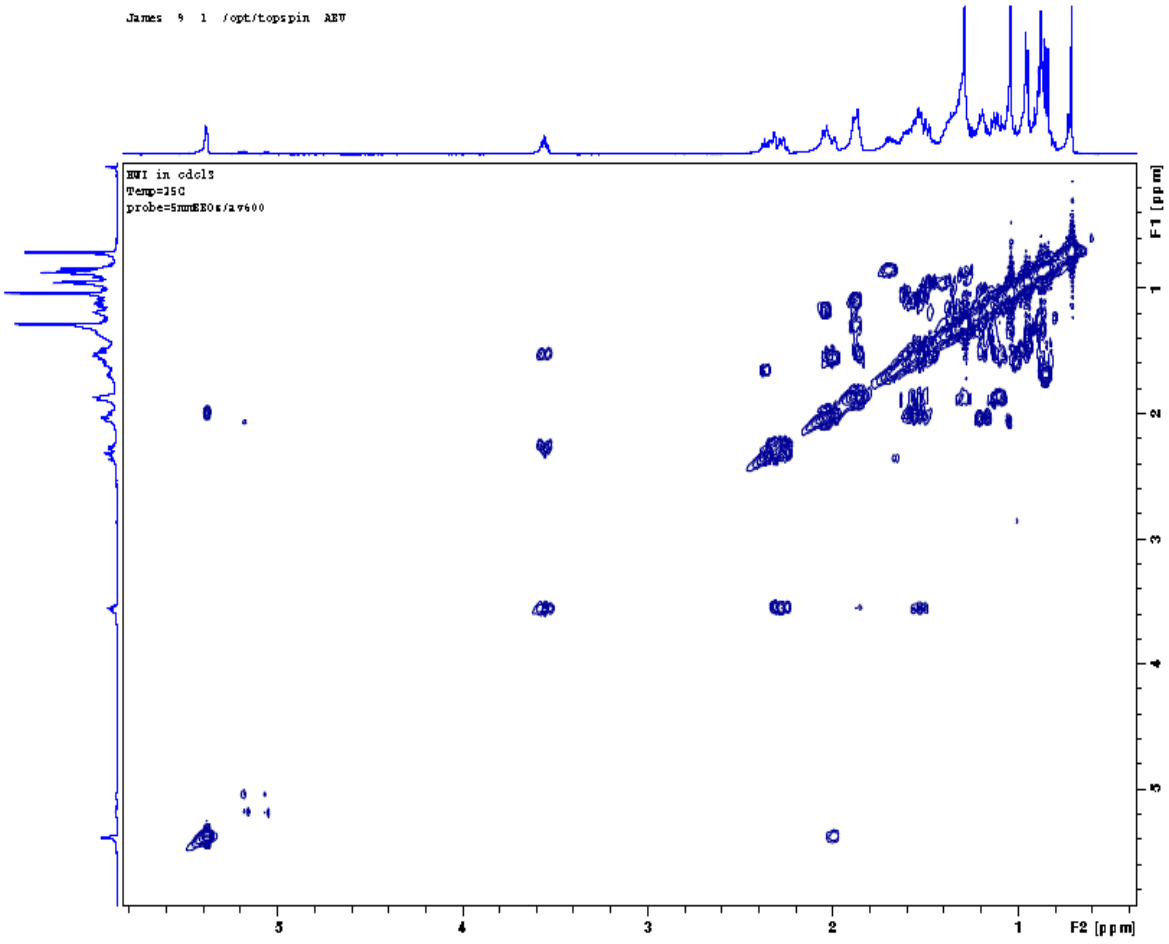


Figure 4.4: H-H COSY of HW1

Homo Nuclear Single Quantum Correlation of HW1 (HSQC)

The result of HSQC (figure 4.5) was used to assign protons on their respective carbons. The proton at position 5.38ppm is on carbon 121.7ppm, while the proton on position 3.54ppm is on carbon at position 71.8ppm.

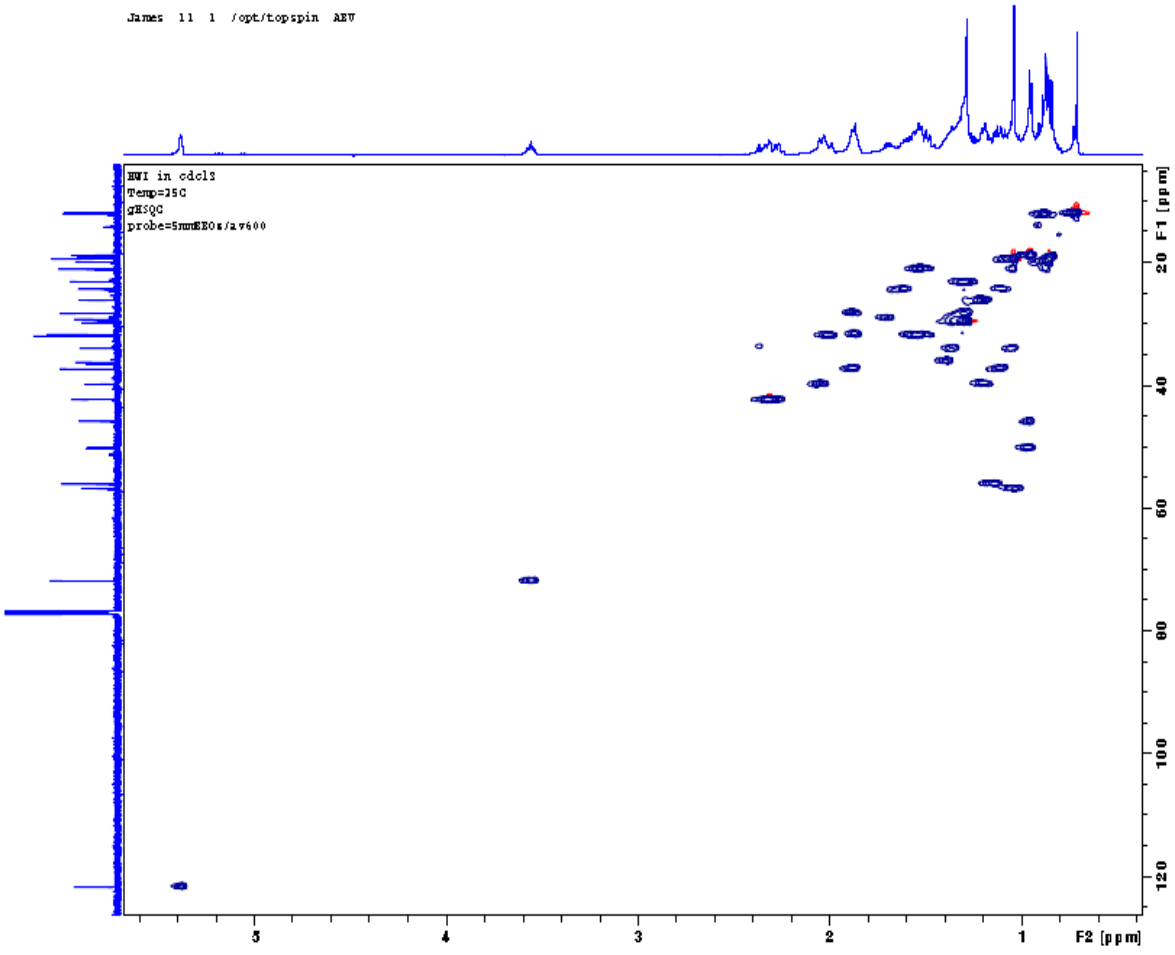


Figure 4.5: HSQC Spectrum of HW1

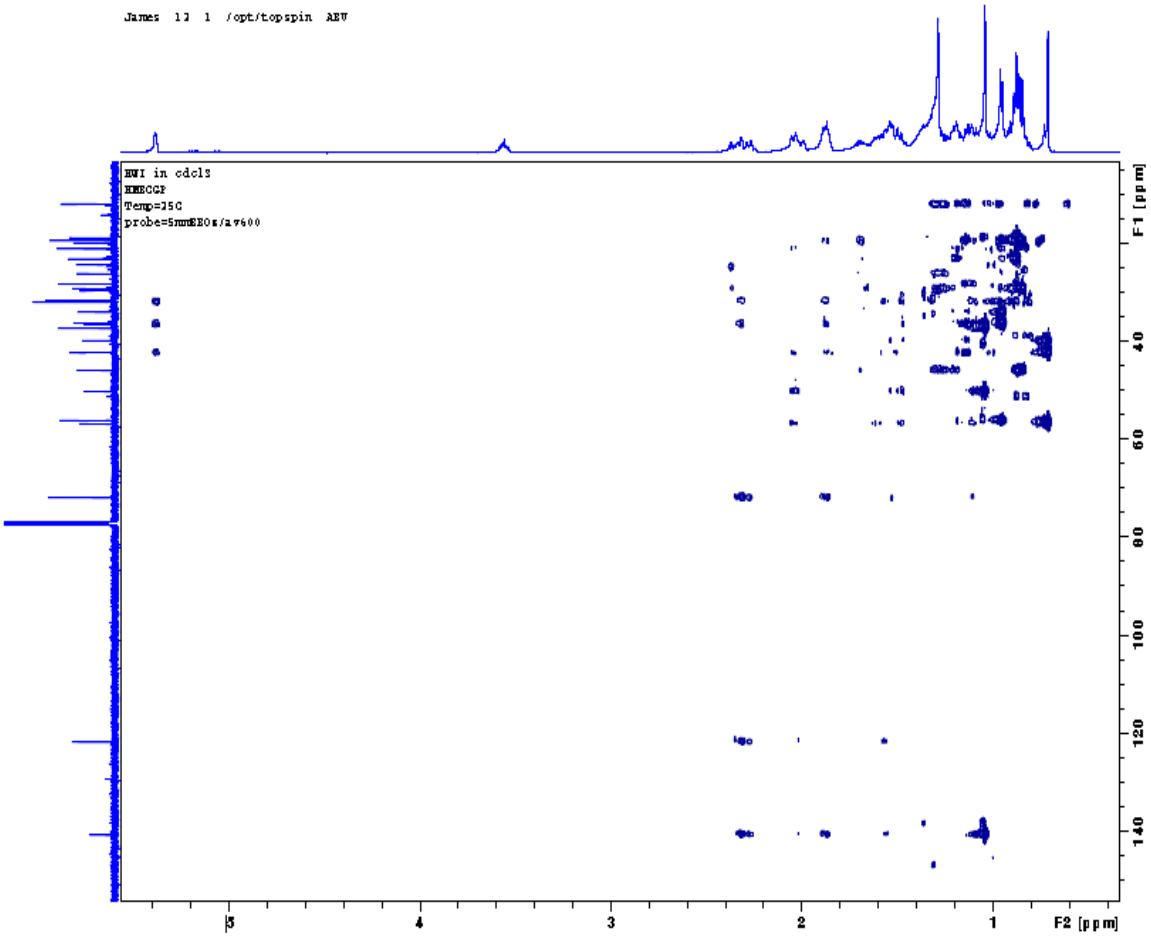


Figure 4.6: **HMBC Spectrum of HW1**

In the NEOSY spectra of HW1, the methyl proton of (δ_H 0.74, H-18) showed space coupling with methylene proton at (δ_H 2.06, H-12). While methyl proton of (δ_H 1.06, H-19) showed space coupling with methylene proton at (δ_H 1.90, H-1).

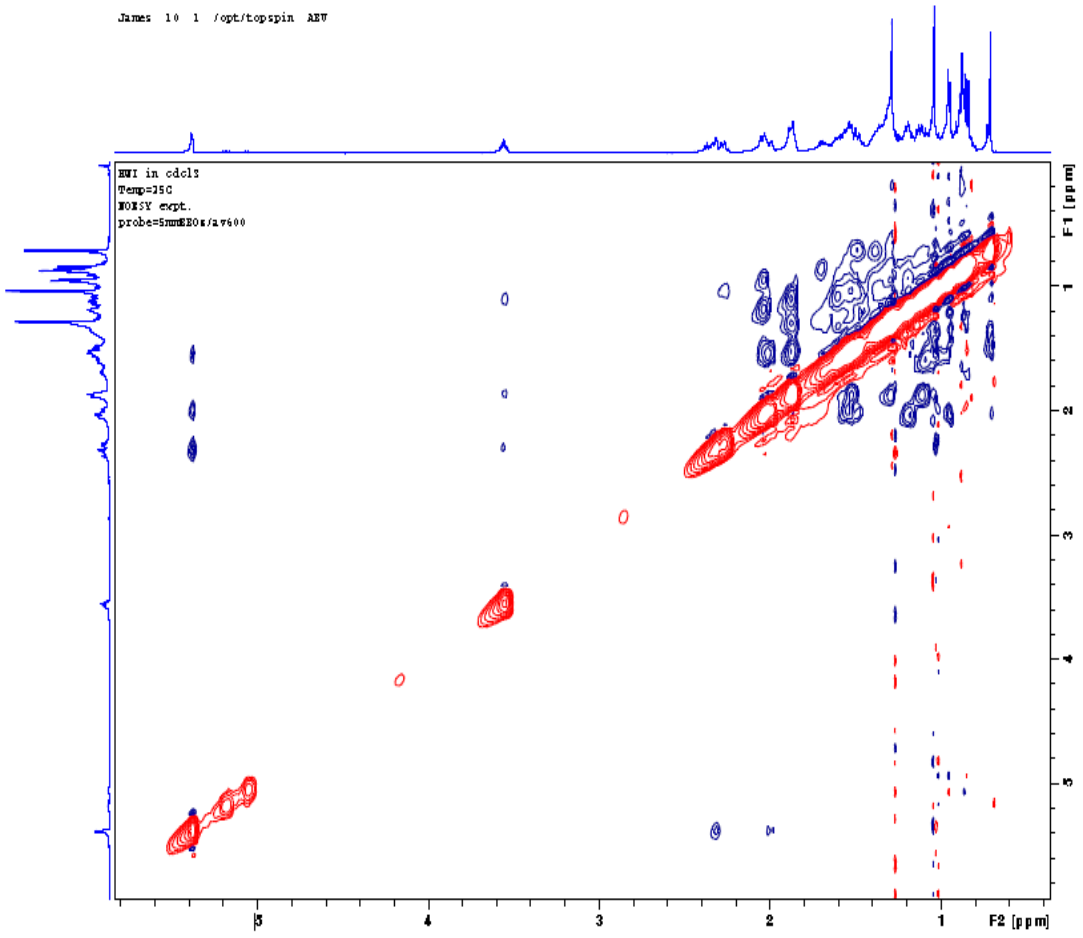


Figure 4.7: NOESY Spectrum of HW1

The chemical structure of HW1 was identified by comparing its spectrum data with those reported by Jamal *et al* (2009); Pateh *et al*. (2009); Habib *et al*. (2007) and Conolly and Hill (1994).

Table 4.4 : ^{1}H NMR (600 MHz) and ^{13}C NMR (125 MHz) data of compound HW1 in $CDCl_3$

Position	Group	δC (ppm)		Group	δH (ppm)	
		HW1	Ref.		HW1	Ref.
1	CH_2	32.8	32.4			
2	CH_2	35.8	36.1			
3	CH	72.0	71.8	CH	3.50	3.25
4	CH_2	42.0	42.3	CH_2		
5	C	140.0	142.7			
6	CH	121.9	122.7	CH	5.38	5.31
7	CH_2	30.2	31.6			
8	CH	29.6	28.2	CH		
9	CH	40.5	42.3			
10	C	39.6	39.7			
11	CH_2	21.8	20.9			
12	CH_2	31.8	31.8			
13	C	41.2	40.4			
14	CH	46.5	45.8			
15	CH_2	21.4	21.5			
16	CH_2	21.2	21.2	CH_2		
17	CH	50.0	50.1			
18	CH_3	20.3	20.5	CH_3	1.01	
19	CH_3	20.5	20.2	CH_3	1.36	
20	CH	29.8	29.6			
21	CH_3	19.8	18.7	CH_3	0.91	
22	CH_2	33.7	33.7			
23	CH_2	28.9	29.1			
24	CH	42.8	42.3			
25	CH	30.6	30.5	CH	1.57	
26	CH_3	19.6	19.8	CH_3	0.81	1.01
27	CH_3	19.7	19.3	CH_3	0.80	0.98
28	CH_2	23.4	24.2		0.74	
29	CH_3	11.3	11.8	CH_3	0.94	0.96

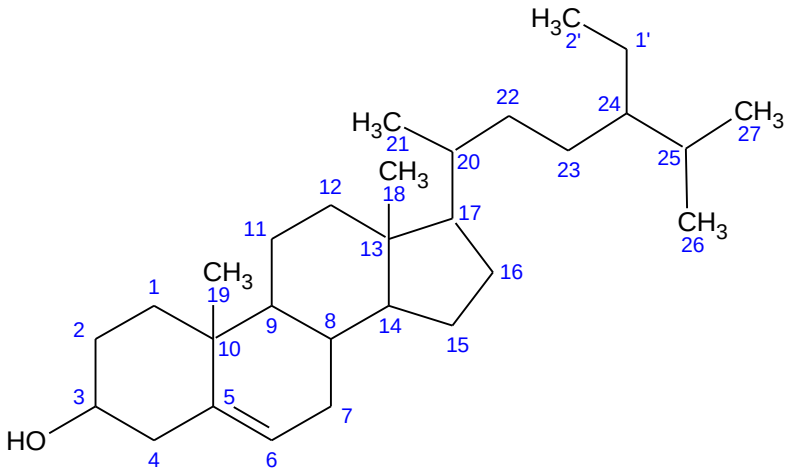
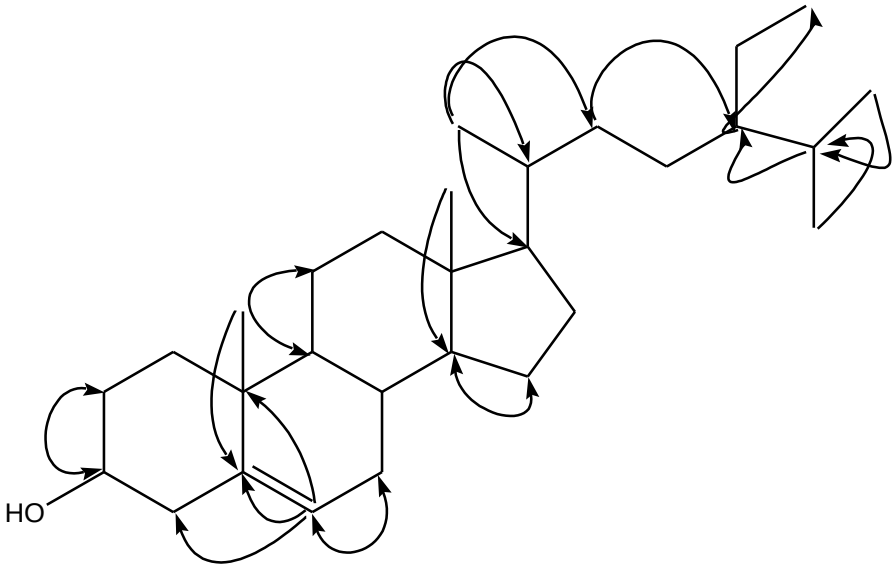


Figure 4.8 : Proposed chemical structure of HW1 (β – Sitosterol)



COSY = ↔

HMBC = →

Table 4.5: Column chromatography of Acetone Fraction of *Stereospermum kunthianum*

Eluent No.	Fraction No.	Eluting Solvent	No. of Spots
1	1 – 4	Pet. Ether 100%	-
2	5 – 6	Pet. Ether: $CHCl_3$ (9:1)	-
3	7 – 10	Pet. Ether: $CHCl_3$ (8:2)	-
4	11 – 13	Pet. Ether: $CHCl_3$ (7:3)	-
5	14 – 16	Pet. Ether: $CHCl_3$ (6:4)	-
6	17 – 20	Pet. Ether: $CHCl_3$ (5:5)	-
7	21 – 23	Pet. Ether: $CHCl_3$ (4: 6)	-
8	24 – 29	Pet. Ether: $CHCl_3$ (3:7)	-
9	**30 – 43**	**Pet. Ether: $CHCl_3$ (2:8)**	**5**
10	44 – 45	Pet. Ether: $CHCl_3$ (2:8)	1
11	46 – 53	Pet. Ether: $CHCl_3$ (1:9)	1
12	54 – 59	$CHCl_3$ 100%	-
13	60 – 79	$CHCl_3$: EtO_{AC} (9:1)	3
14	**80 – 87**	**$CHCl_3$: EtO_{AC} (8:2)**	**3**
15	88 – 91	$CHCl_3$: EtO_{AC} (7:3)	2
16	92 – 95	$CHCl_3$: EtO_{AC} (6:4)	2
17	96 – 103	$CHCl_3$: EtO_{AC} (5:5)	2
18	104 – 113	$CHCl_3$: EtO_{AC} (4:6)	2
19	114 – 122	$CHCl_3$: EtO_{AC} (3:7)	2
20	123 – 139	$CHCl_3$: EtO_{AC} (2:8)	3
21	140 – 150	$CHCl_3$: EtO_{AC} (1:9)	5
22	151 – 161	EtO_{AC} 100%	5
23	162 – 180	EtO_{AC} : MeOH(9:1)	5
24	181 – 190	EtO_{AC} : Me OH(8:2)	5

4.4 Analysis of HNW2

4.4.1 Solubility of HNW2

HNW2 was found to be soluble in methanol and acetone.

4.4.2 Melting point of HNW2

The melting point of compound HNW2 was found to be 244 - 247^{0}C.

4.4.3 Chemical test

HNW2 produced red colouration when subjected to Shinoda test, brown precpitate when treated with ferric chloride solution and dissolve in concentrated sulphuric acid to give red colour solution

4.4.4 Spectral analysis of HNW2

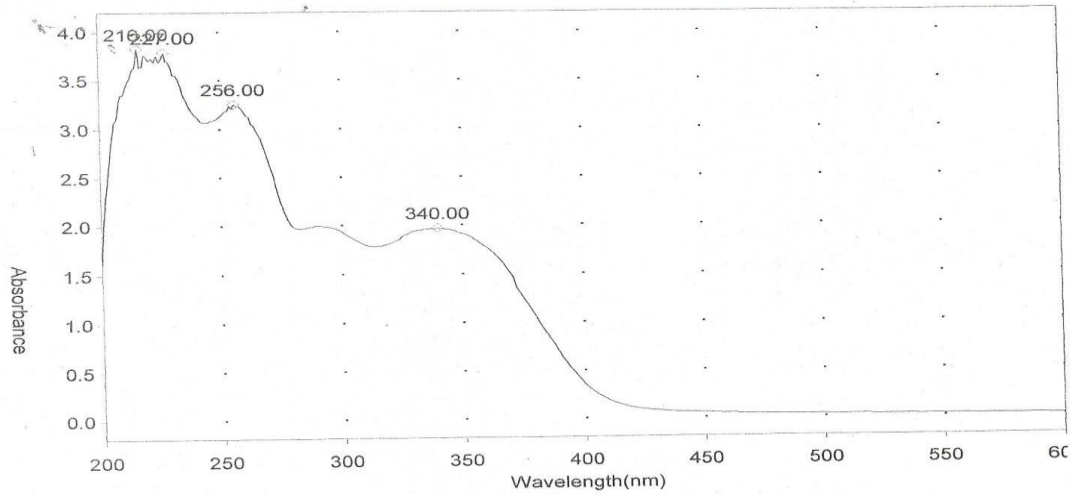
4.4.5 Ultra Violet Spectra of HNW2

HNW2 revealed two absorptions bands of 256.00nm and another band at 340.00nm.

THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00

Operator Name	Umar U.Usman	Date of Report	10/13/2015
Department	Pharm Chemistry	Time of Report	1:44:14PM
Organisation	Ahmadu Bello University Zaria		
Information	(None Entered)		

Scan Graph



Results Table - scan001,Sample001,Cycle01

nm	A
216.00	3.830
227.00	3.790
256.00	3.253
340.00	1.967
Sensitivity	Auto

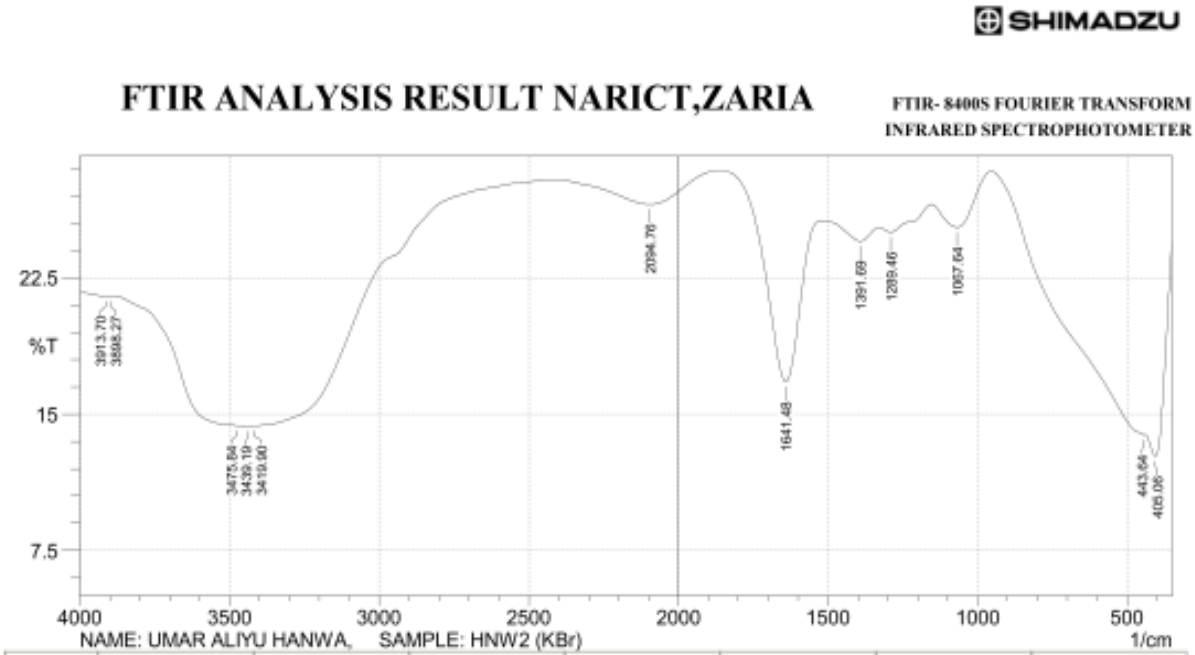
Peak Pick Method
Find 8 Peaks Above -3.0000 A
Start Wavelength 200.00 nm
Stop Wavelength 600.00 nm
Sort By Wavelength

Page 1, Scan Graph

Fig: 4.9: Ultra Violet spectrum of HNW2

4.4.6 IR Spectrum of HNW2

The IR spectrum of HNW2 revealed signals at 3409cm^{-1} for OH, 2927 cm^{-1} for CH_2 and 1639 cm^{-1} for CO Stretchings.



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	405.06	12.68	5.28	439.78	349.13	73.46	7.03
2	443.64	13.86	0.12	953.83	440.75	361.5	2.39
3	1067.64	25.27	2.05	1153.47	954.8	115.09	3.49
4	1289.46	25	0.53	1329	1154.43	103.48	1.01
5	1391.69	24.51	0.85	1524.78	1329.96	117.03	1.17
6	1641.48	16.77	9.8	1859.44	1525.74	211.69	21.85
7	2094.76	26.53	1.64	2402.42	1860.4	305.1	6.59
8	3419.9	14.35	0.01	3420.87	2403.38	670.1	4.48
9	3439.19	14.34	0.01	3451.73	3421.83	25.21	0
10	3475.84	14.37	0	3493.2	3472.95	17.06	0
11	3898.27	21.47	0.01	3905.02	3891.52	9.02	0
12	3913.7	21.47	0.03	4000.5	3905.98	62.94	0.02

Comment;
NAME: UMAR ALIYU HANWA, SAMPLE: HNW2
(KBr)

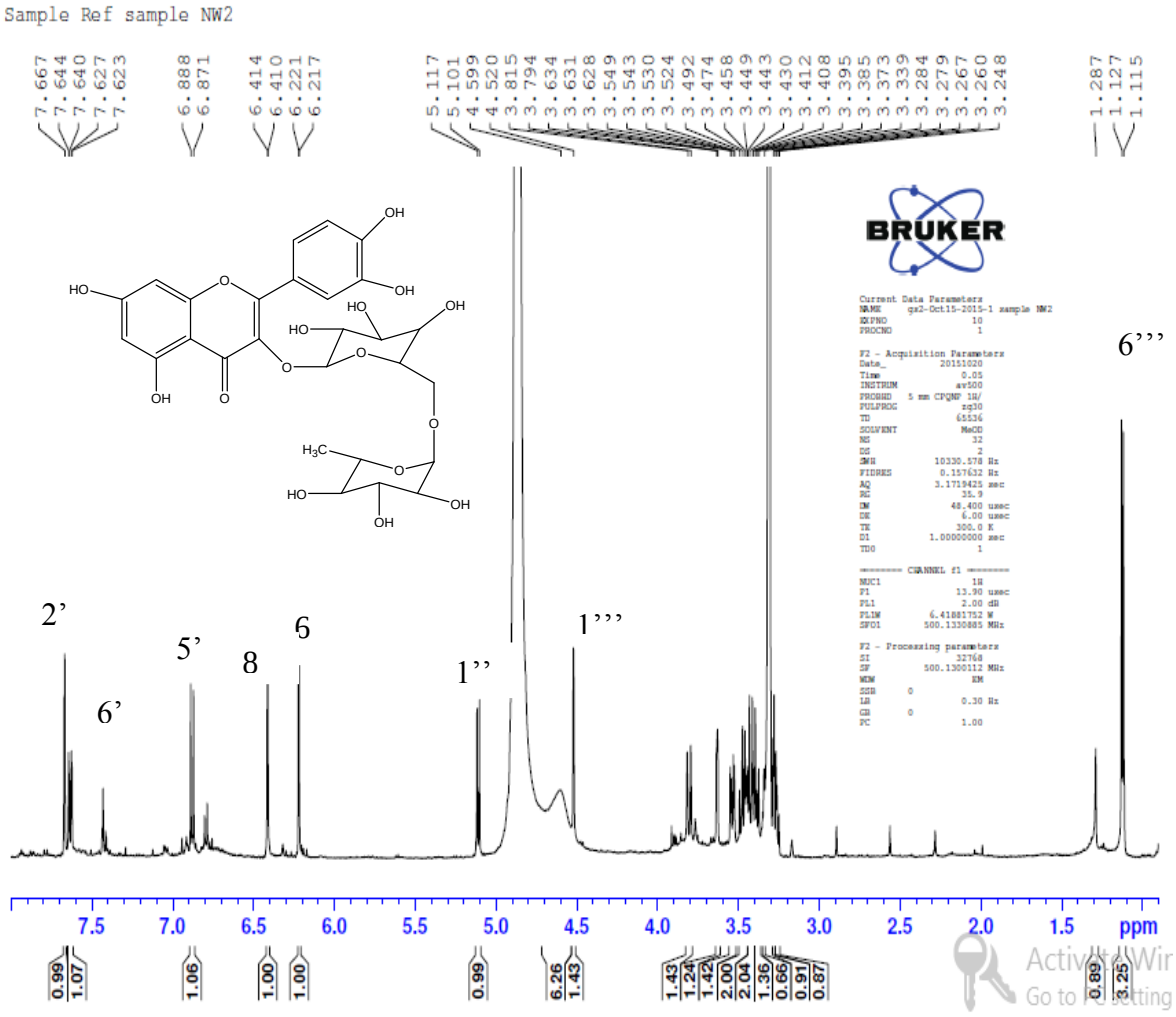
User; Administrator

No. of Scans;
Date/Time; 10/14/2015 11:00:42 AM
Resolution;

Fig 4.10: Infrared spectroscopic analysis of compound HNW2

4.4.7 Proton nuclear magnetic resonace of HNW2

The [1]HNMR spectrum of HNW2 in MeOD revealed the presence of the following signals. The sugar region has the following signals at: δ3.25, 3.33, 3.43, 3.44, 3.47, 3.52, 3.55, 3.63, 3.80, 4.52, and 5.12 with the δ1.12 for CH_3. The aglycone has the following chemical shift, δ 6.22, 6.41, 6.88, 7.63, and 7.64 (figure 4.11 – 4.13)



Figrure 4.11: Proton Nuclear Magnetic Resonance (NMR) Spectrum of HNW2

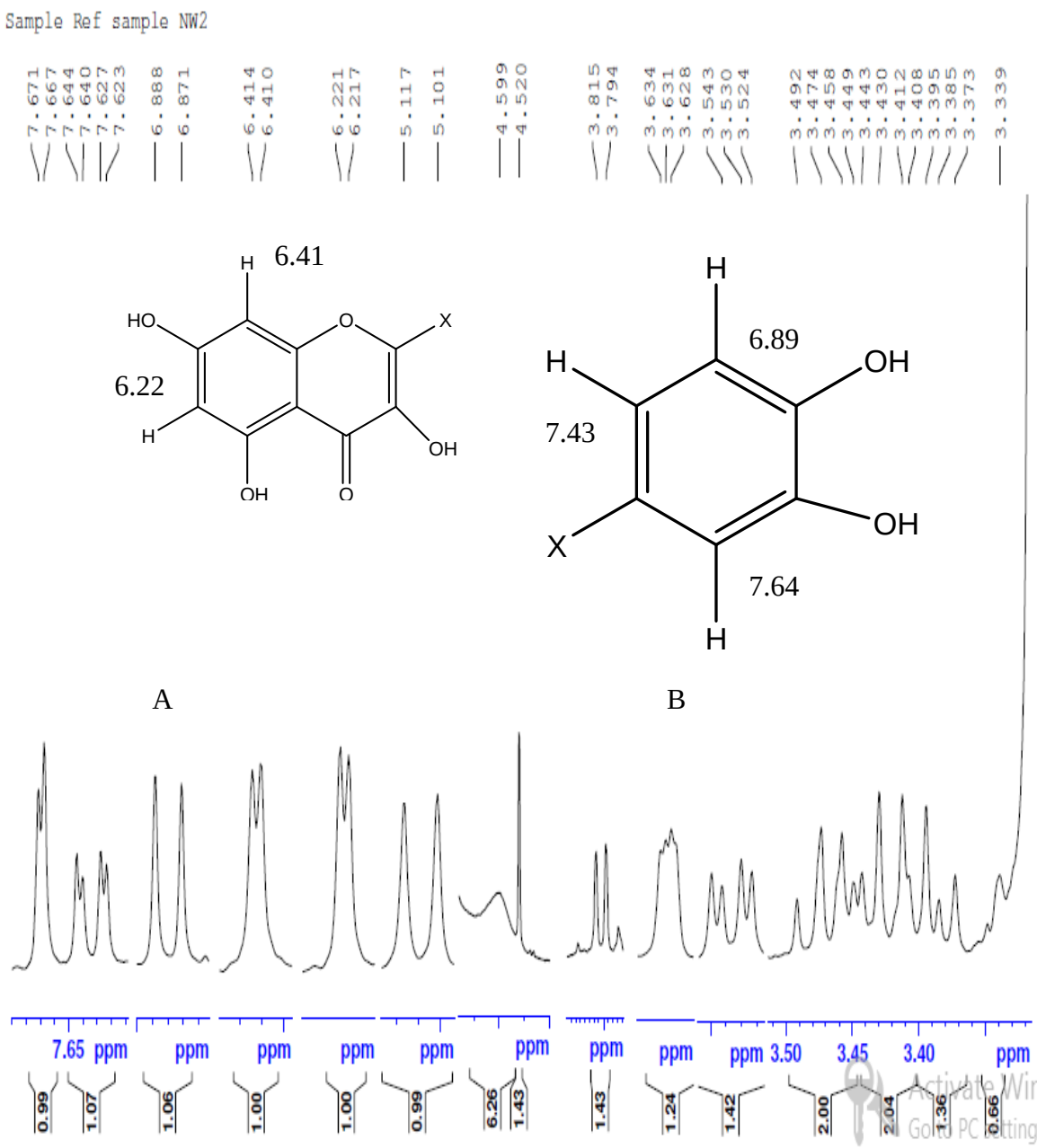
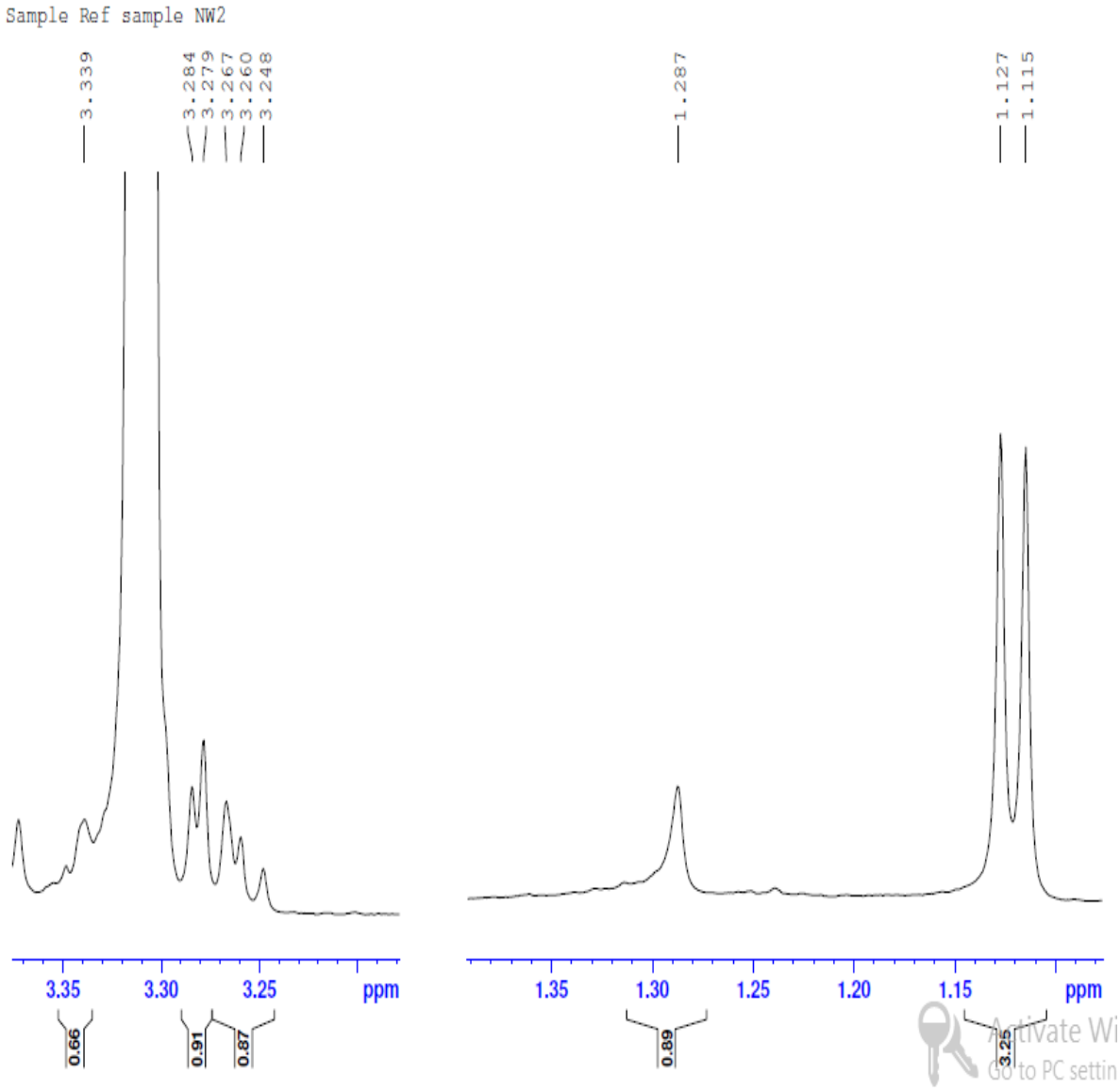


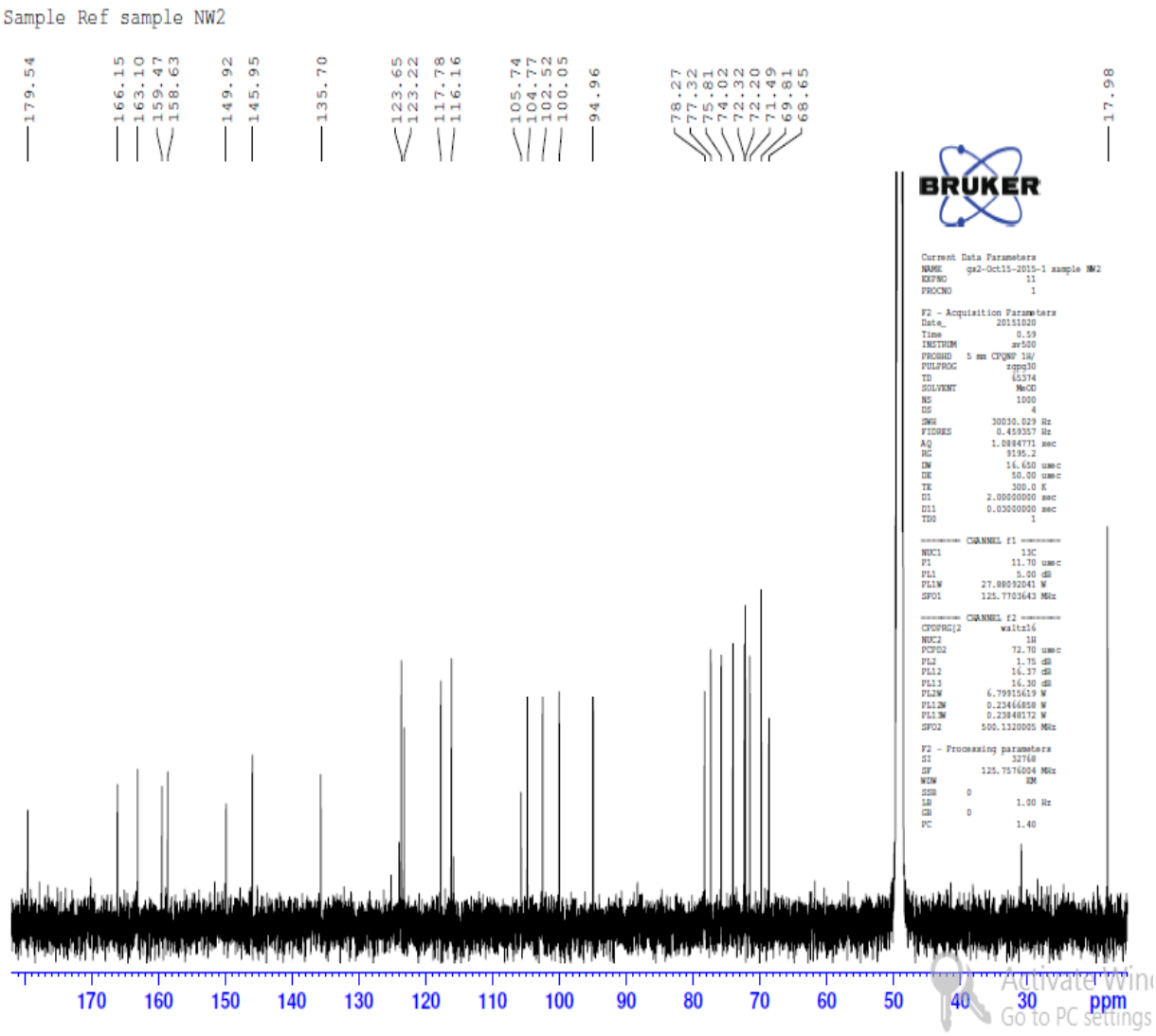
Figure 4.12: Proton Nuclear Magnetic Resonance (NMR) Spectrum of HNW2 (Expanded)



Figrure 4.13: Proton Nuclear Magnetic Resonance (NMR) Spectrum of HNW2 (Expanded)

Carbon -13 Magnetic resonance of HNW2

The ^{13}C – NMR (δppm, 500MHz and MeOD) experiment revealed the presence of 27 carbon atoms; 179.54(q), 166.15(q), 163.10(q), 159.47(q), 158.63(q), 149.92(q), 145.95(q), 135.70(q), 123.65(q), 123.22(CH), 177.78(CH), 116.16(CH), 105.74(q), 104.77(CH), 102.52(CH), 100.05(CH), 94.96(CH), 78.27(CH), 77.32(CH), 75.81(CH), 74.02(CH), 72.32(CH),, 72.20(CH) 71.49(CH),, 69.81(CH), 68.65(CH_2) and 17.98(CH_3) as showed in figure 4.14.



Figrur 4.14: Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of HNW2

DEPT of HNW2 Spectrum

Figure (4.15) shows the nature of carbon atoms together with their multiplicities as methyl with 1 carbon atom; methylene 1; methine 15 and quartenary 10 carbon atoms.

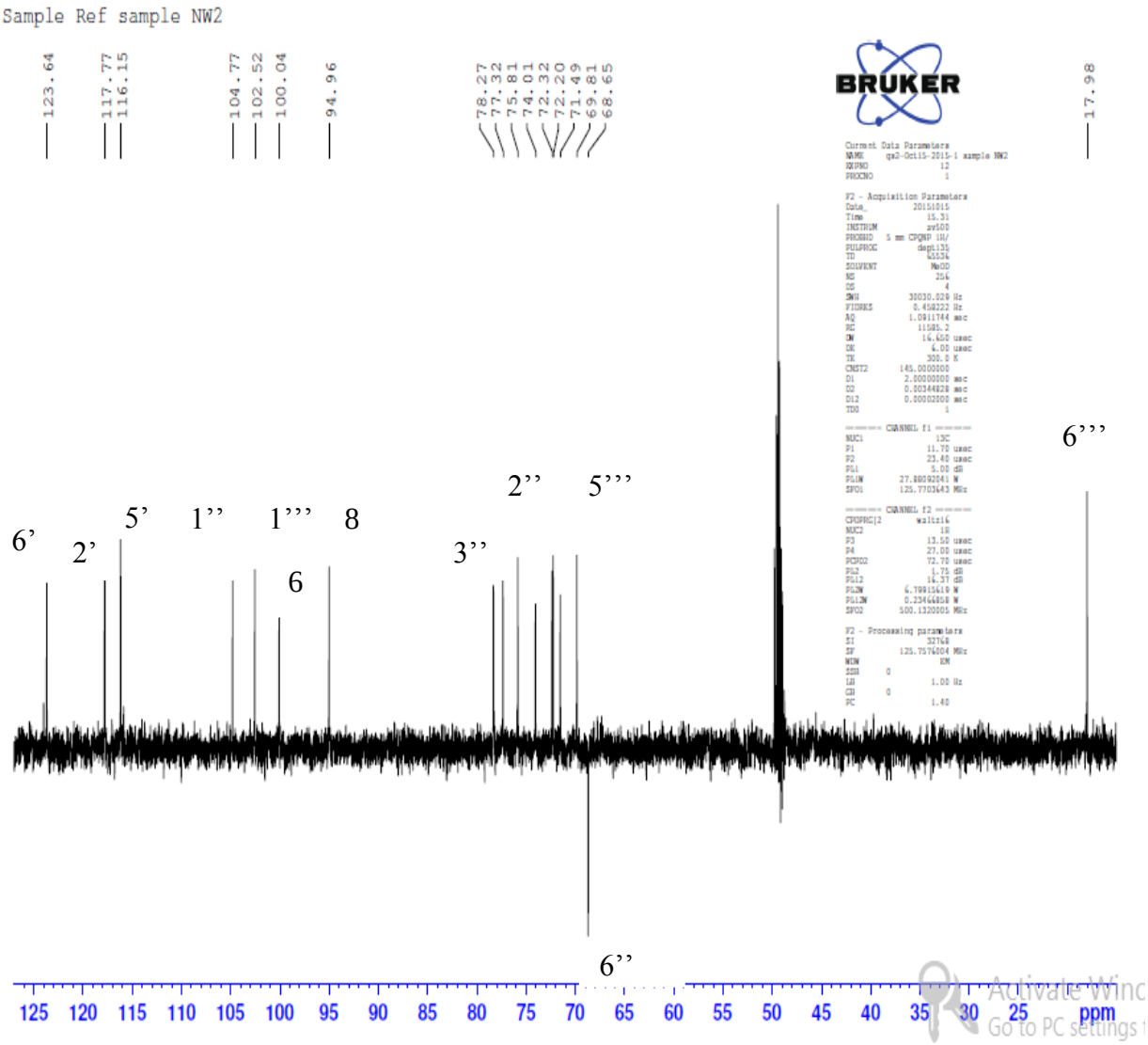


Figure 4.15: DEPT Carbon-13 NMR Spectrum of HW2

^{1}H - ^{1}H COSY of HNW2

Same chemical environment of the protons and the nature of aromatic substitution are shown ^{1}H – ^{1}H Correlation Spectroscopy from figure (4.16 – 4.19). The spectrum revealed correlation between H-6 (6.22) and H-8 (6.41); H-1□ □ □(4.52) and H-2□ □ □(3.27); H-1□ □ (5.12) and H-2□ □ (3.44); H-2□ □ □(3.27) and H-3□ □ □(3.33) and H-4□ □ □(3.39) and H-3□ □ □(3.33).

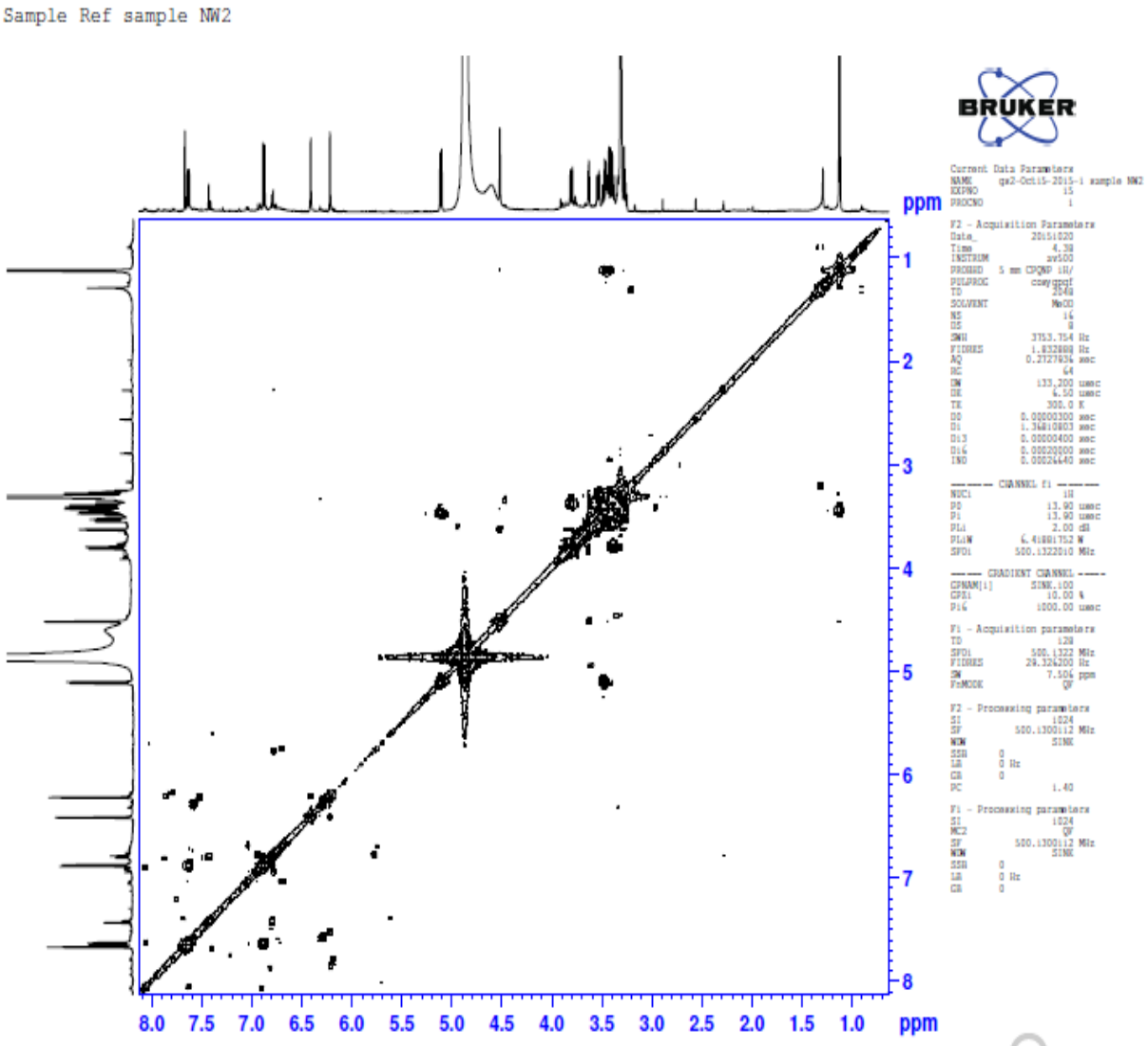


Figure 4.16: H-H COSY of HNW2

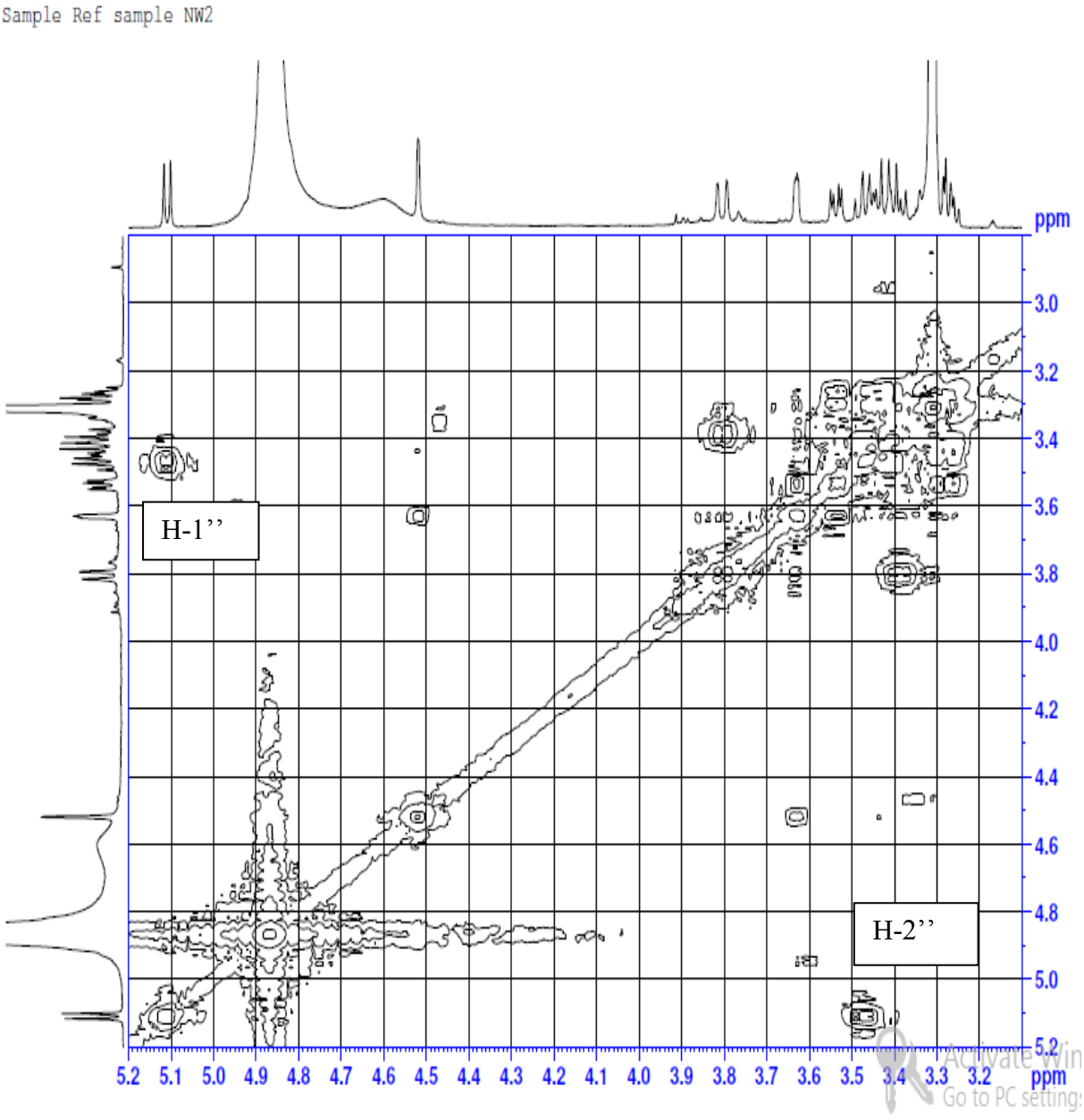


Figure 4.17: H-H COSY of HNW2 (Expanded)

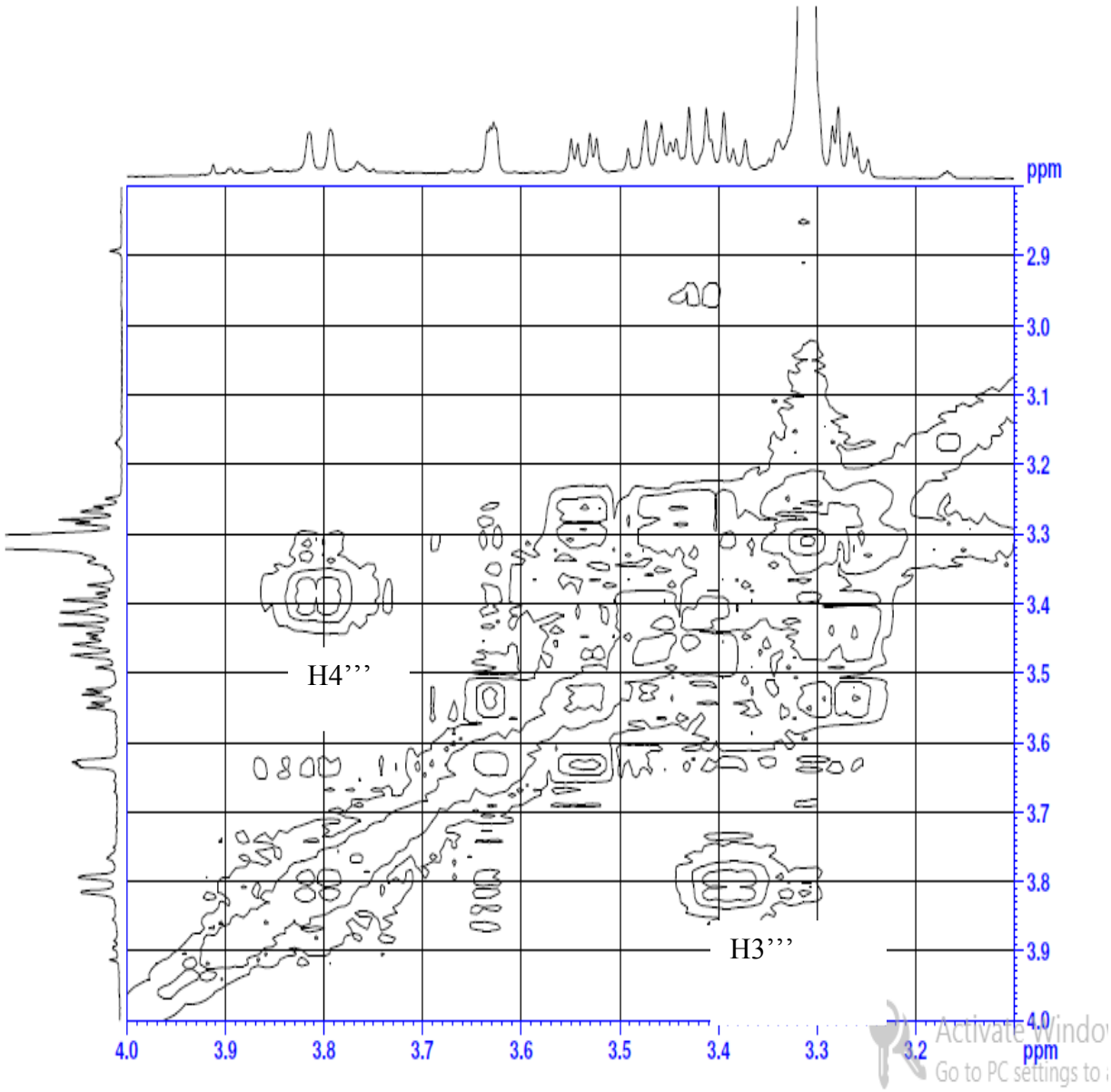


Figure 4.18: H-H COSY of HNW2 (Expanded)

Sample Ref sample NW2

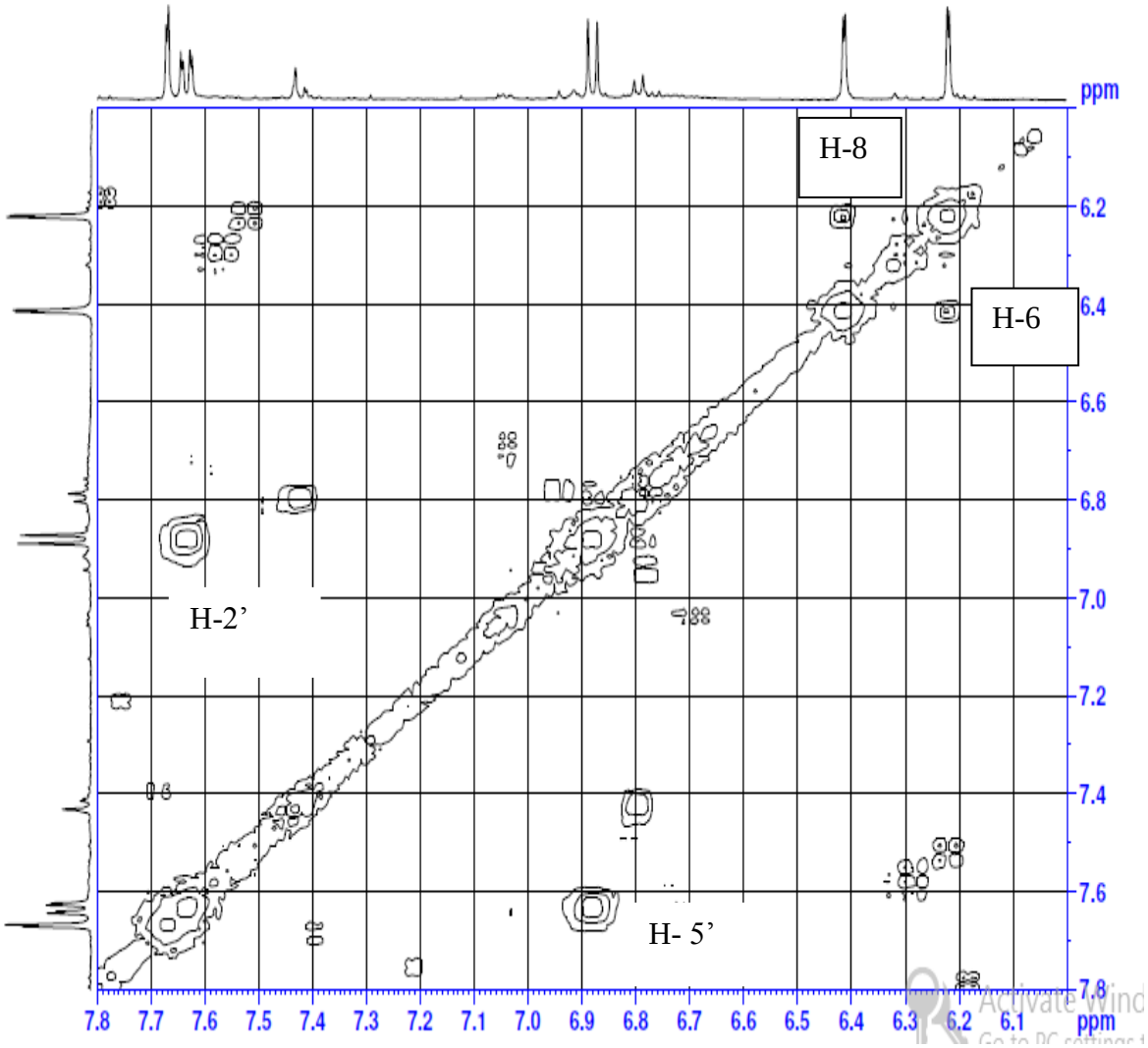


Figure 4.19: H-H COSY of HNW2 (Expanded)

Homo Nuclear Single Quantum Correlation of HNW2 (HSQC)

Protons are assigned to their respective carbons from the data extracted from the Heteronuclear Single Quantum Correlation in figure (4.20 – 4.23).

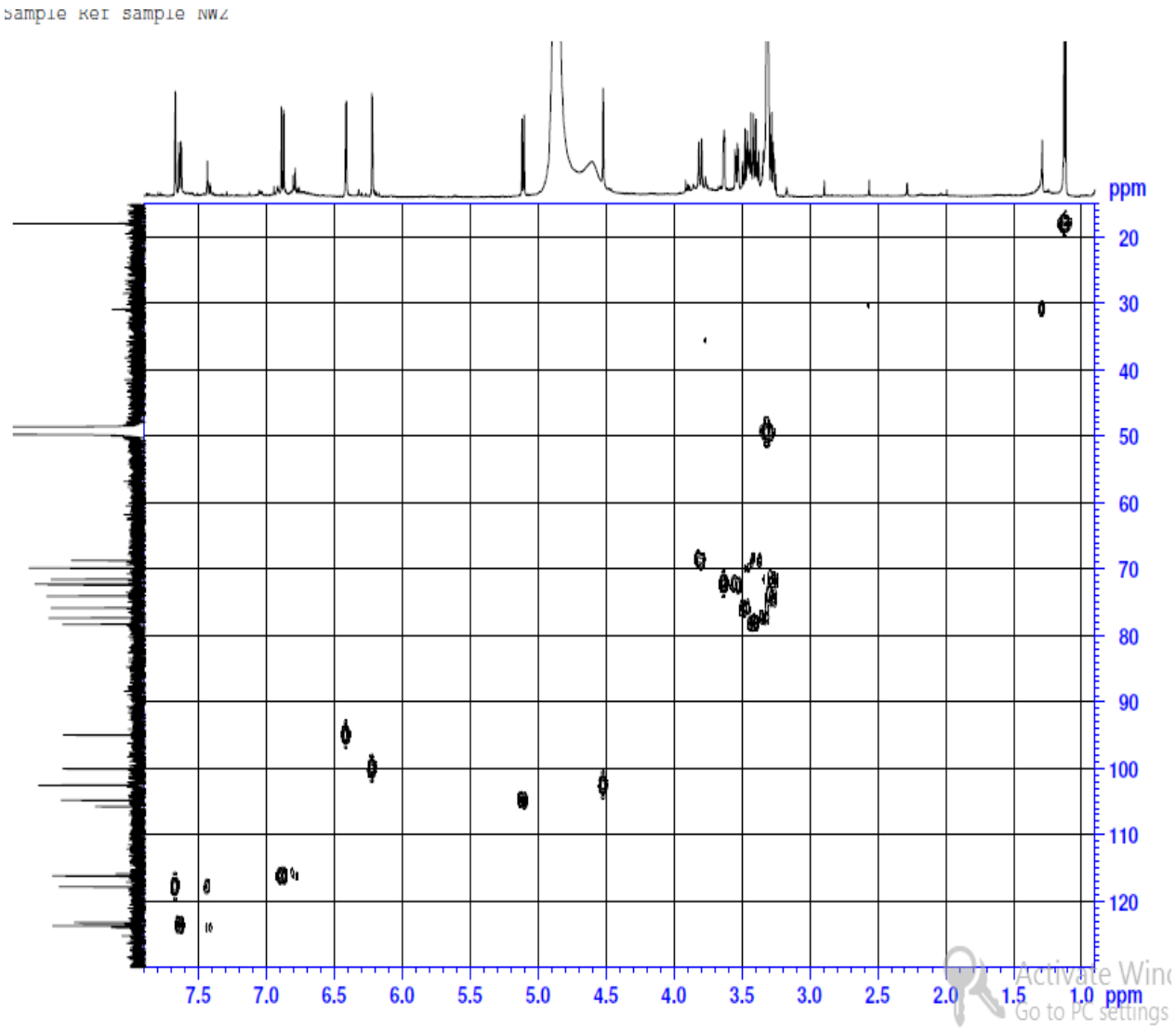


Figure 4.20: HSQC spectrum of HNW2

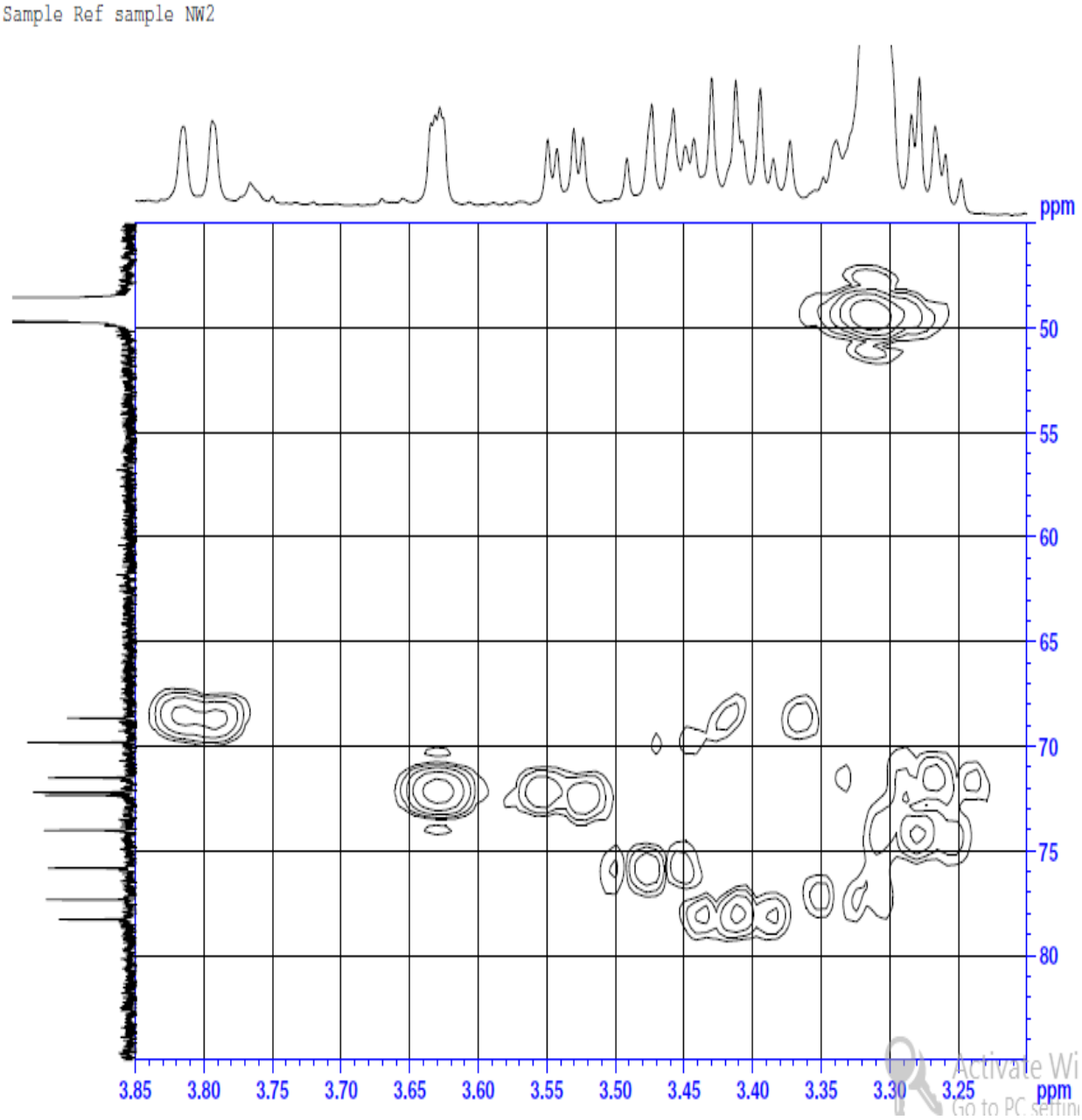


Figure 4.21: HSQC spectrum of HNW2 (Expanded)

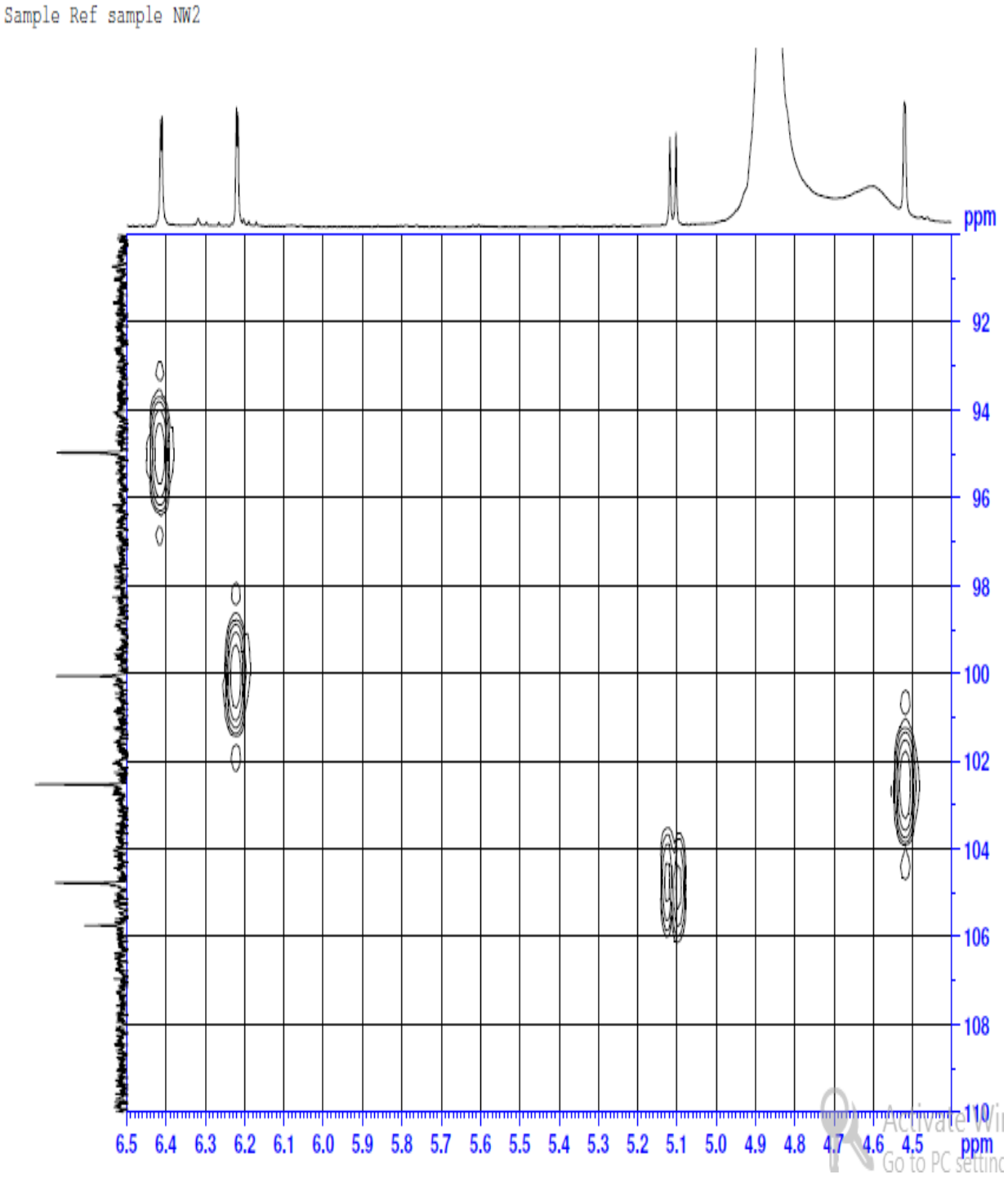


Figure 4.22: HSQC spectrum of HNW2 (Expanded)

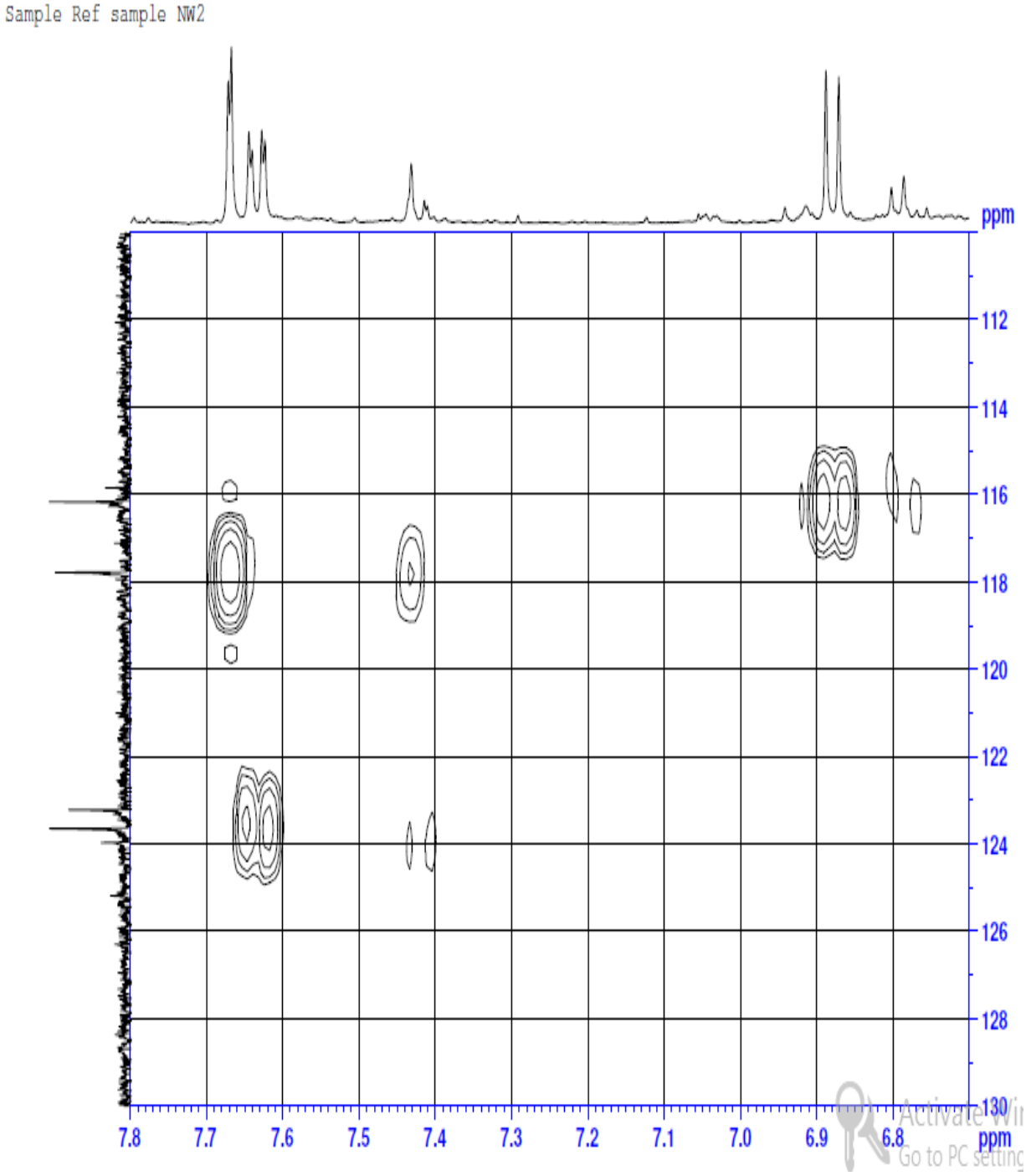


Figure 4.23: HSQC spectrum of HNW2 (Expanded)

Hetero Nuclear Multiple Bond Correlation of HNW2 (HMBC)

Long range connectivity between various fragments of the molecules was exhibited by Heteronuclear Multiple Bond Correlation data from figure (4. 24 – 4.29).

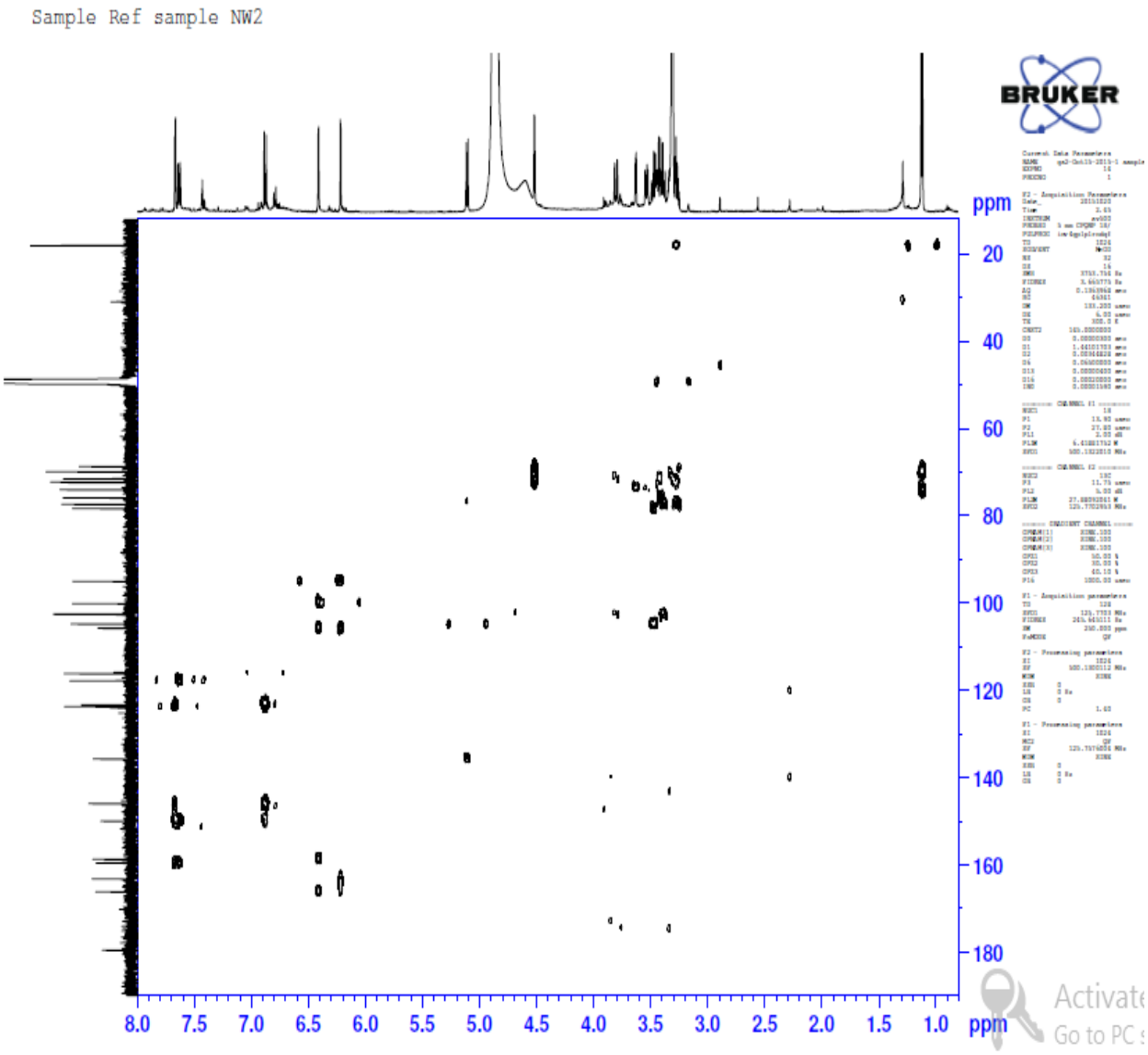


Figure 4.24: **HMBC spectrum of HNW2**

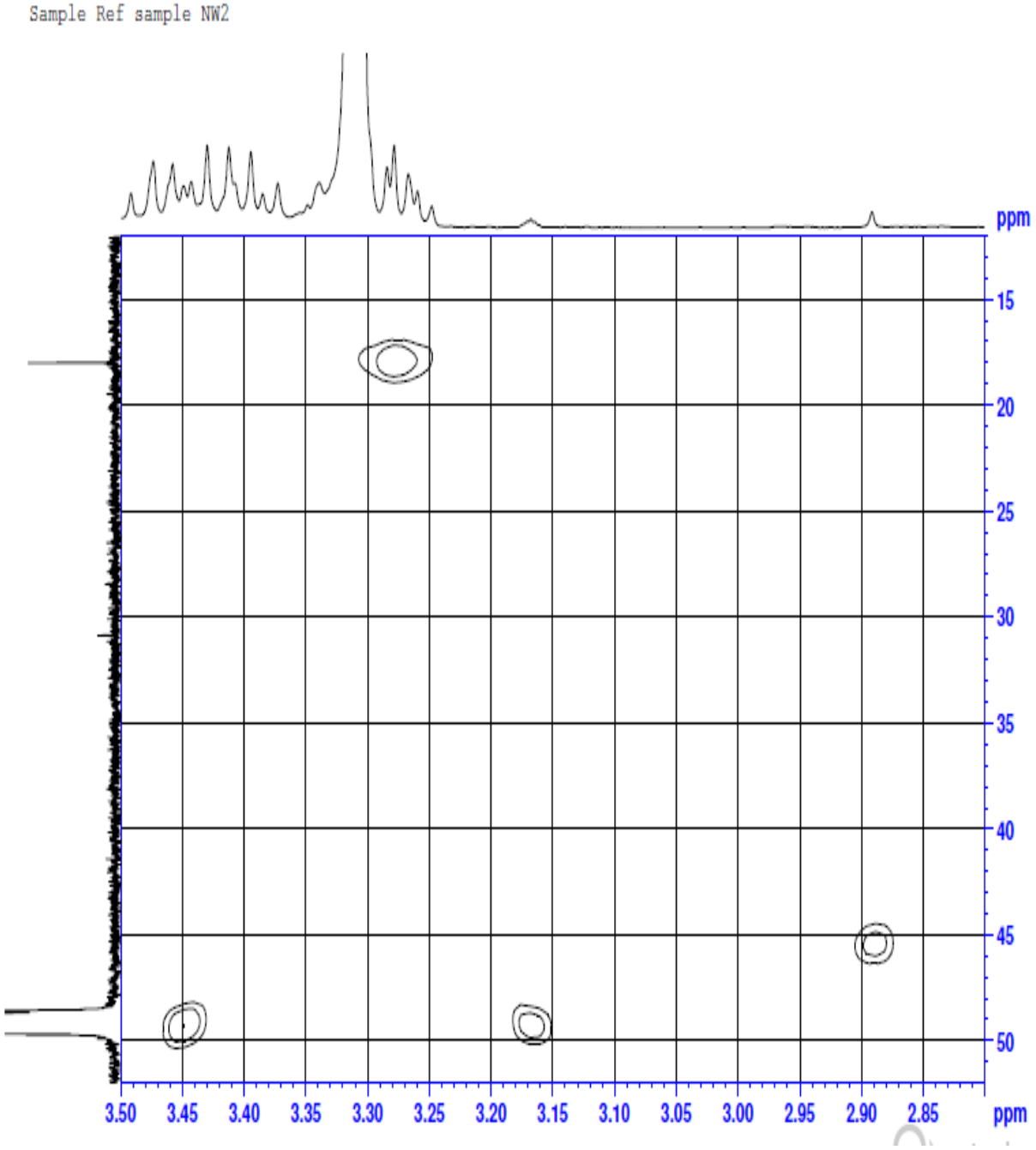


Figure 4.25: **HMBC spectrum of HNW2 (Expanded)**

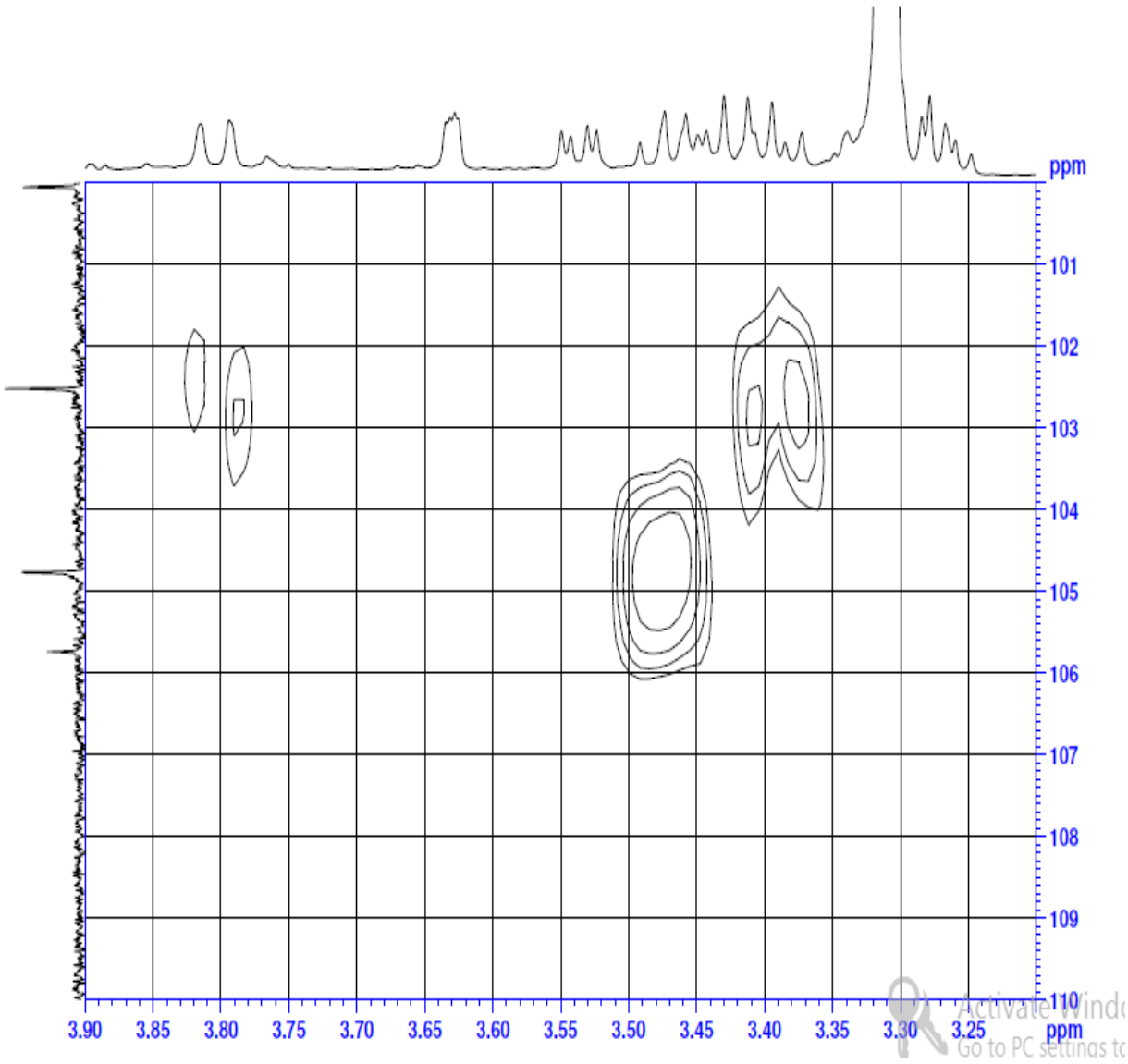


Figure 4.26: **HMBC spectrum of HNW2 (Expanded)**

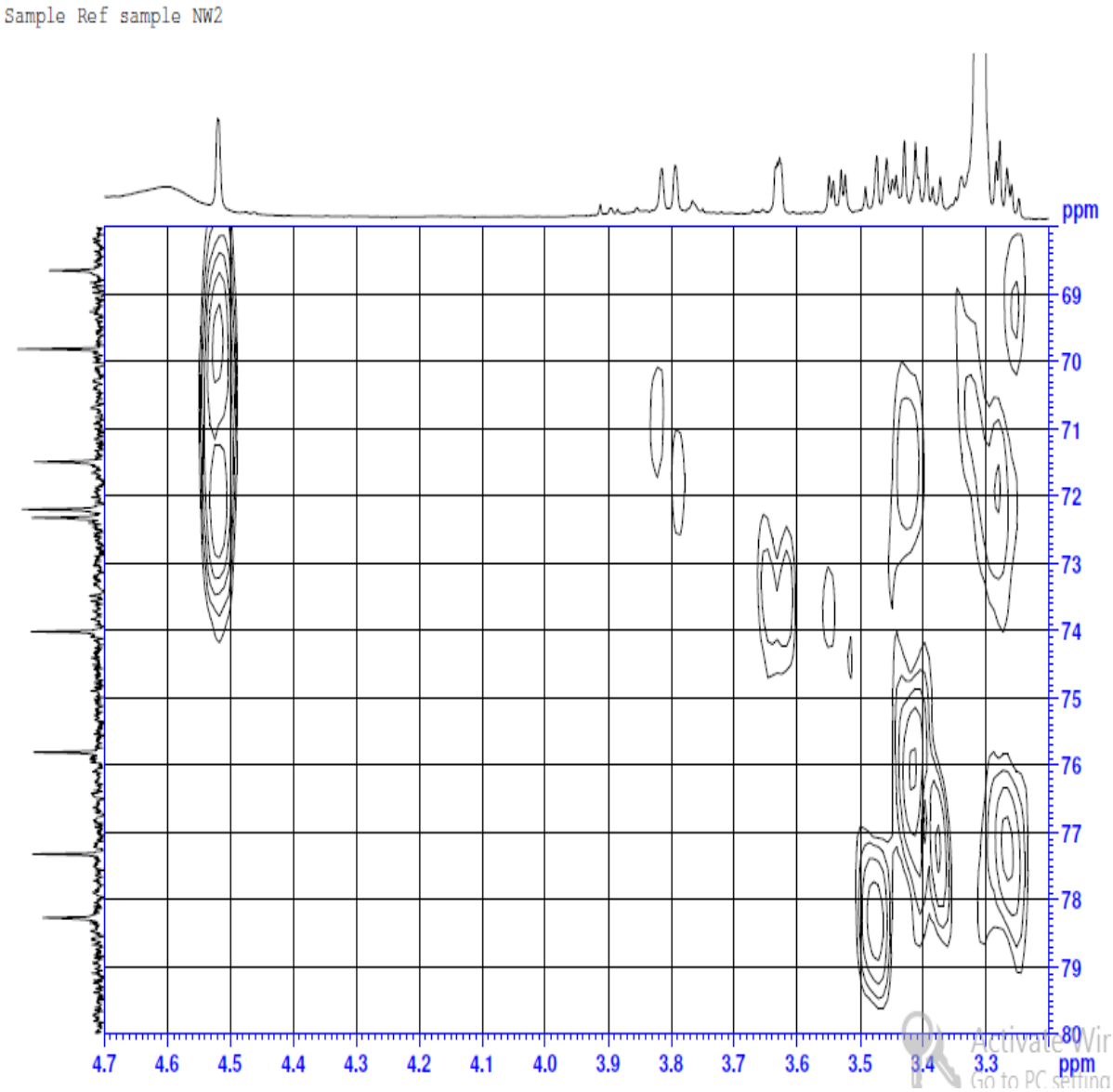


Figure 4.27: **HMBC spectrum of HNW2 (Expanded)**

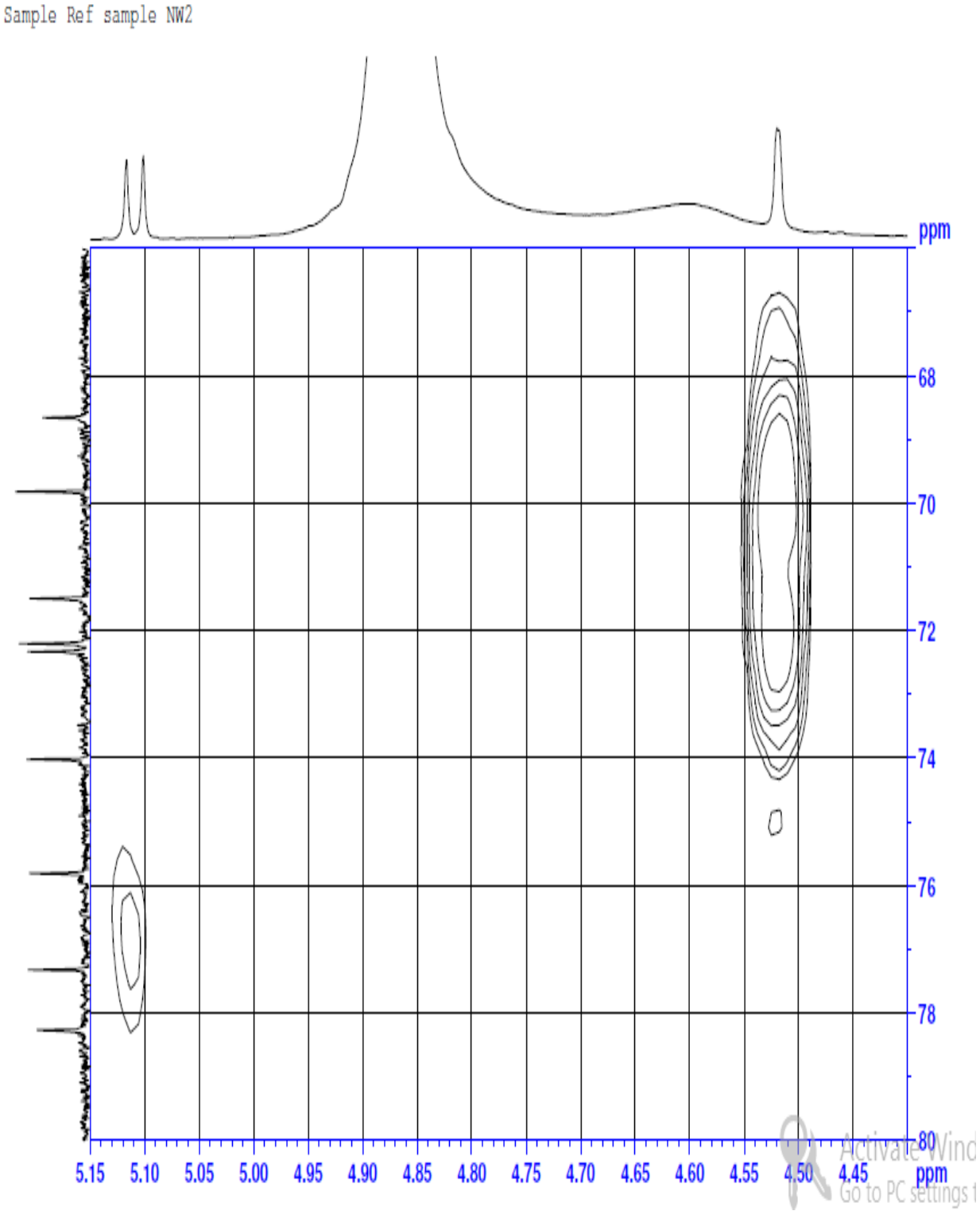


Figure 4.28: **HMBC spectrum of HNW2 (Expanded)**

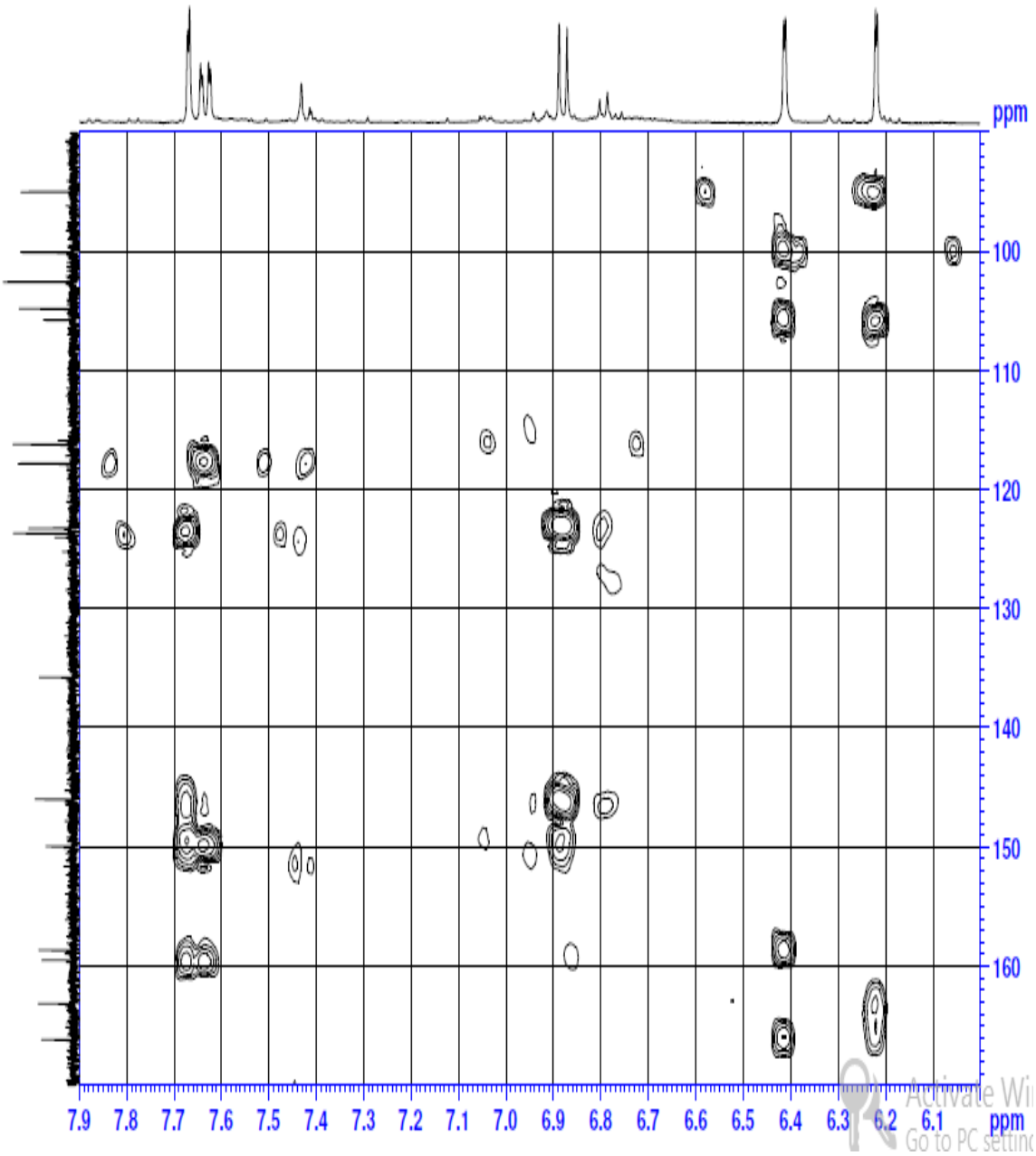


Figure 4.29: **HMBC spectrum of HNW2 (Expanded)**

Nuclear Overhauser Effect of HNW2 (NOESY)

Some of the correlations shown by the Nuclear Over-Hauser Effect Spectroscopy spectrum of HNW2 are those between from figure 4.30.

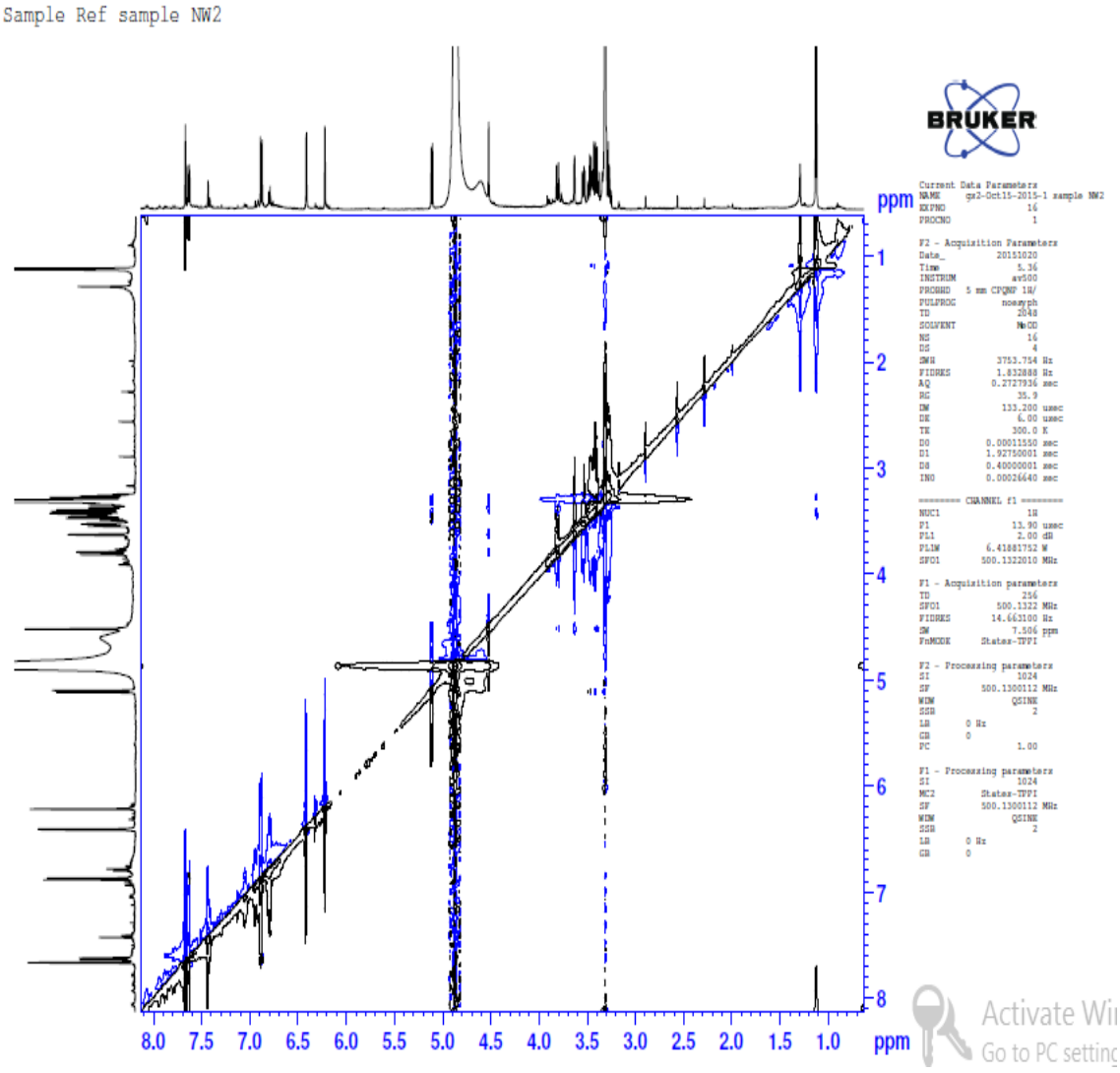


Figure 4.30: NOESY Spectrum of HNW2

Table 4.6: Comparison of 1D NMR data of HNW2 with literature report (Wu *et al*., 2007 and Marzieh *et al*., 2008)

Position	HNW2 δ ^{1}H, J – value (Hz)	Literature δ ^{1}H, J – value (Hz)
2		
3		
4		
5		12.56 (1H, s,5-OH)
6	6.22 (1H,d, J= 2.0Hz)	6.20 (1H, d, J= 2.0)
7		10.82 (1H, s, 7-OH)
8	6.41 (1H,d,J= 2.4Hz)	6.47(1H, d, J= 2.0)
9		
10		
1□		
2□	7.64 (1H, d, J= 2.0Hz)	7.84(1H, d, J= 2.0)
3□		
4□		
5□	6.89 (1H, d, J= 8.4Hz)	6.92(1H,d, J= 8.4)
6□	7.43 (1H, dd, J= 2.0, 8.4Hz)	7.87(1H, d, J= 8.4,2.0)
1□ □	5.12 (1H, d, J= 7.6Hz)	5.28(1H,d, J= 7.0)
2□ □	3.44	3.23(1H, m)
3□ □	3.43	3.21(1H, m)
4□ □	3.39	3.32(1H, m)
5□ □	3.33	2.96(1H,m)
6□ □	3.80	3.66(1H, d, J= 9.8),3.22(1H, m)
1□ □ □	4.52 (1H, d, J= 1.2Hz)	4.36(1H, s)
2□ □ □	3.27	2.96(1H, m)
3□ □ □	3.33	3.23(1H, m)
4□ □ □	3.39	3.07(1H, t, J= 9.5)
5□ □ □	3.44 (1H, m)	3.2(1H, m)
6□ □ □	1.12 (3H, d, J= 6.4Hz)	0.99(3H, d J=6.0)

Table 4.7: ^{1}H NMR (500MHz) and ^{13}C NMR (125 MHz) data of compound HNW2 in MeOD

Position	δ^{13}C HNW2	HNW2 δ ^{1}H, J – value (Hz)	DEPT	δ^{13}C Literature	Literature δ ^{1}H, J – value (Hz)
2	158.63		C	156.4	
3	135.70		C	133.6	
4	179.54		C	177.4	
5	163.10		C	161.2	12.56 (1H, s,5-OH)
6	100.05	6.22(1H,d, J= 2.0Hz)	CH	98.7	6.20 (1H, d, J= 2.0)
7	166.15		C	164.2	10.82 (1H, s, 7-OH)
8	94.96	6.41(1H,d,J= 2.4Hz)	CH	93.1	6.47(1H, d, J= 2.0)
9	159.47		C	156.6	
10	105.74		C	103.0	
1□	123.22		C	121.2	
2□	117.78	7.64(1H, d, J= 2.0Hz)	CH	115.2	7.84(1H, d, J= 2.0)
3□	145.95		C	144.8	
4□	149.92		C	148.4	
5□	116.16	6.89(1H,d, J= 8.4Hz)	CH	116.3	6.92(1H,d, J= 8.4)
6□	123.65	7.43(1H,dd, J= 2.0, 8.4Hz)	CH	121.6	7.87(1H, d, J= 8.4,2.0)
1□ □	104.77	5.12(1H, d, J= 7.6Hz)	CH	101.2	5.28(1H,d, J= 7.0)
2□ □	75.81	3.44	CH	74.1	3.23(1H, m)
3□ □	78.27	3.43	CH	76.4	3.21(1H, m)
4□ □	71.49	3.39	CH	70.6	3.32(1H, m)
5□ □	77.32	3.33	CH	75.9	2.96(1H,m)
6□ □	68.65	3.80	CH2	67.0	3.66(1H, d, J= 9.8), 3.22(1H, m)
1□ □ □	102.52	4.52(1H,d, J= 1.2Hz)	CH	100.8	4.36(1H, s)
2□ □ □	72.20	3.27	CH	70.4	2.96(1H, m)
3□ □ □	72.32	3.33	CH	70.0	3.23(1H, m)
4□ □ □	74.02	3.39	CH	71.8	3.07(1H, t, J= 9.5)
5□ □ □	69.81	3.44(1H, m)	CH	68.3	3.2(1H, m)
6□ □ □	17.98	1.12(3H, d, J= 6.4Hz)	CH_3	17.8	0.99(3H, d J=6.0)

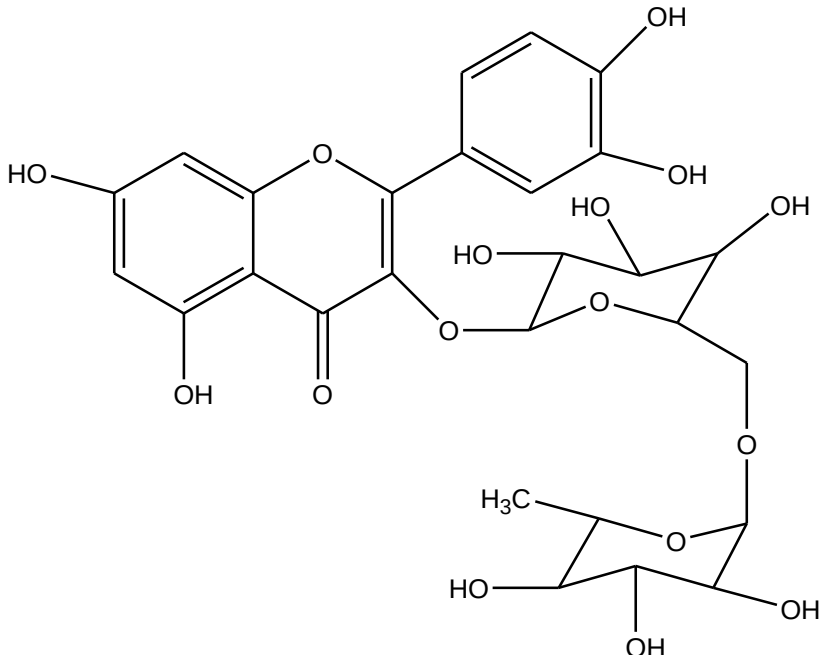


Figure 4.31 Proposed structure of HNW2

4.5 Analysis of HNW3

4.5.1 Melting point of HNW3

The melting point of compound HNW3 was determined to be 216 - 219^{0}C.

4.5.2 Solubility of HNW3

Compound HNW3 was found to be soluble in acetone and methanol.

4.5.3 Chemical tests

The HNW3 produced red coloration when subjected to Shinoda test, brown precipitates when treated with ferric chloride solution and dissolved in concentrated sulphuric acid to give red colour solution.

.

4.5.4 Spectral Analysis of HNW3

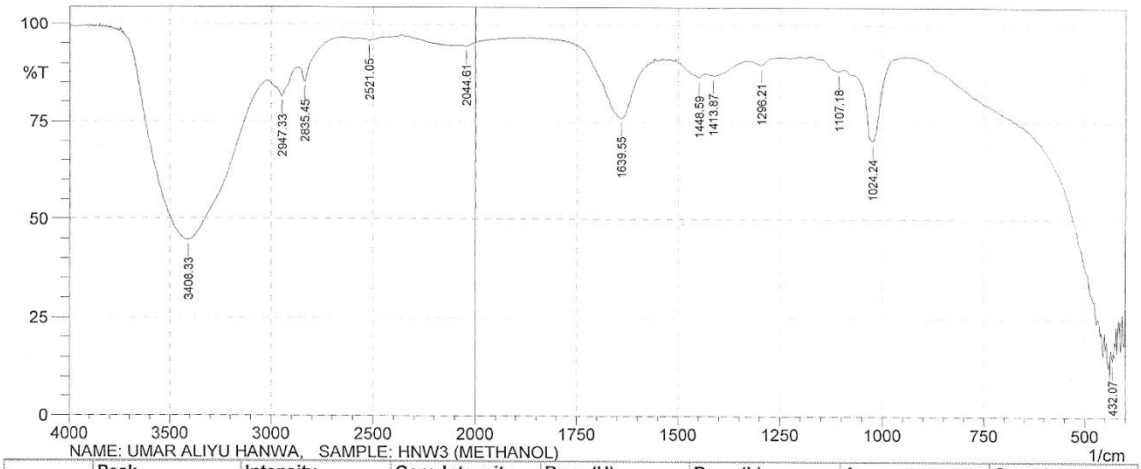
4.5.5 IR Spectrum of HNW3

The IR spectrum analysis (Fig. 4.32) below shows prominent absorptions at 3408cm $^{-1}$ signifying the presence of a hydroxyl group, 2947 cm $^{-1}$ (-CH_2) group and 1639 cm $^{-1}$ for carboanl group.

SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER



NAME: UMAR ALIYU HANWA, SAMPLE: HNW3 (METHANOL)

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	432.07	14.472	2.421	435.93	430.14	4.607	0.155
2	1024.24	70.247	2.715	1030.02	972.16	5.177	-0.276
3	1107.18	88.194	1.019	1147.68	1095.6	2.583	0.182
4	1296.21	89.735	1.471	1325.14	1269.2	2.42	0.18
5	1413.87	87.004	0.191	1417.73	1402.3	0.926	0.009
6	1448.59	86.465	0.923	1458.23	1435.09	1.407	0.056
7	1639.55	75.992	15.631	1734.06	1573.97	12.108	6.265
8	2044.61	94.504	0.045	2046.54	1990.6	1.244	0.031
9	2521.05	95.844	0.694	2563.48	2451.61	1.855	0.165
10	2835.45	85.175	5.018	2870.17	2667.64	6.997	0.292
11	2947.33	81.429	3.773	2978.19	2870.17	7.718	0.786
12	3408.33	44.745	0.018	3410.26	3402.54	2.693	0.001

Comment;
NAME: UMAR ALIYU HANWA, SAMPLE: HNW3 (METHANOL)

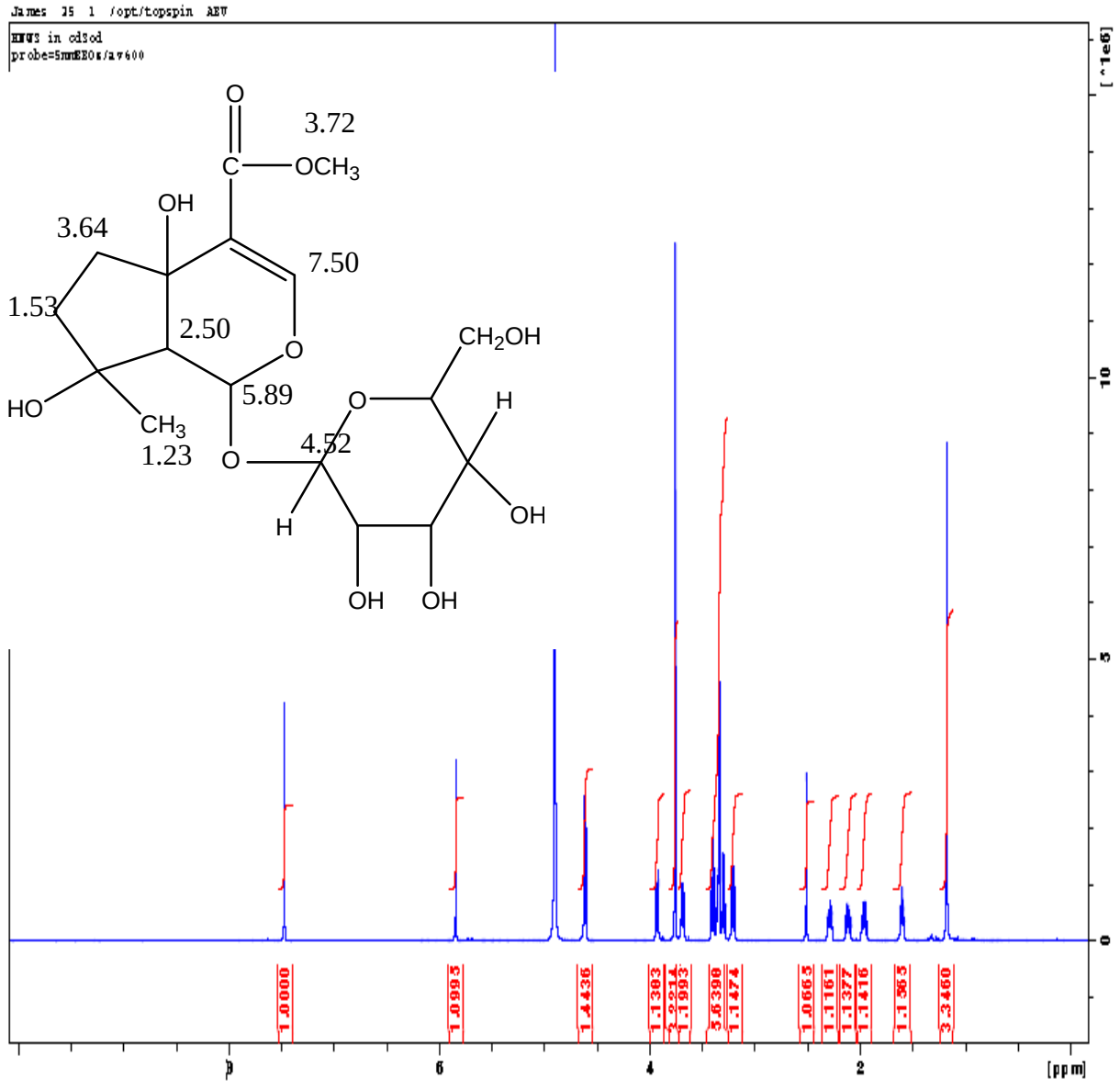
User; Administrator

No. of Scans;
Date/Time; 10/9/2013 9:43:04 AM
Resolution;

Figure 4.32: Infrared Spectroscopic Analysis of Compound HNW3

4.5.6 Proton nuclear magnetic resonace of HNW3

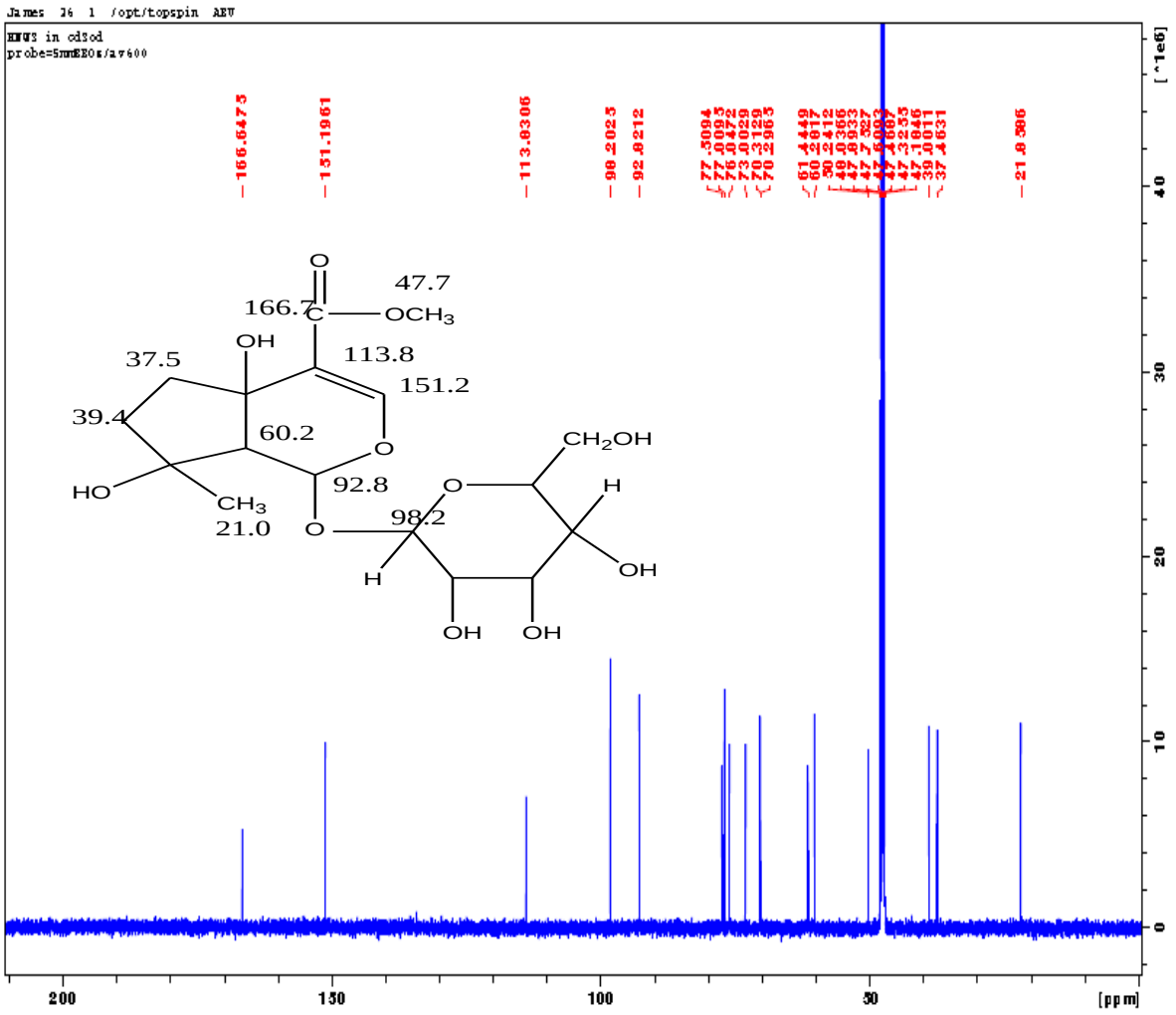
The ^{1}HNMR spectrum of compound HNW3 (Figure 4.33), revealed signals at δ 5.89ppm methine dioxy proton, at δ7.50ppm for oleifinic proton; at δ 4.52ppm for anomeric; at δ3.73 with three protons double doublet a methoxy proton and a single proton multiplet at δ1.23ppm a methyl proton.



Figrure 4.33: Proton Nuclear Magnetic Resonance (NMR) Spectrum of HNW3

CARBON -13 SPECTRUM OF HNW3

The ^{13}C-NMR Spectrum (fig. 4.34) revealed a total of 17 carbon signals in which 11 corresponds to the aglycon while 6 to the sugar moiety. At δ 92.8ppm (C-1), δ151.2ppm (C-3), δ166.7ppm (C-11), δ37.5ppm (C-7) , a methyl carbon at δ21.0ppm and anomeric carbon at δ98.2ppm (C-1′).



Figrure 4.34: Carbon-13 Nuclear Magnetic Resonance (NMR) Spectrum of HNW3

DEPT SPECTRUM OF HNW3

The DEPT spectrum of HNW3 shows a total of nine methine (CH); three methylene (CH_2); four quaternary and one methyl carbons (fig.4.35).

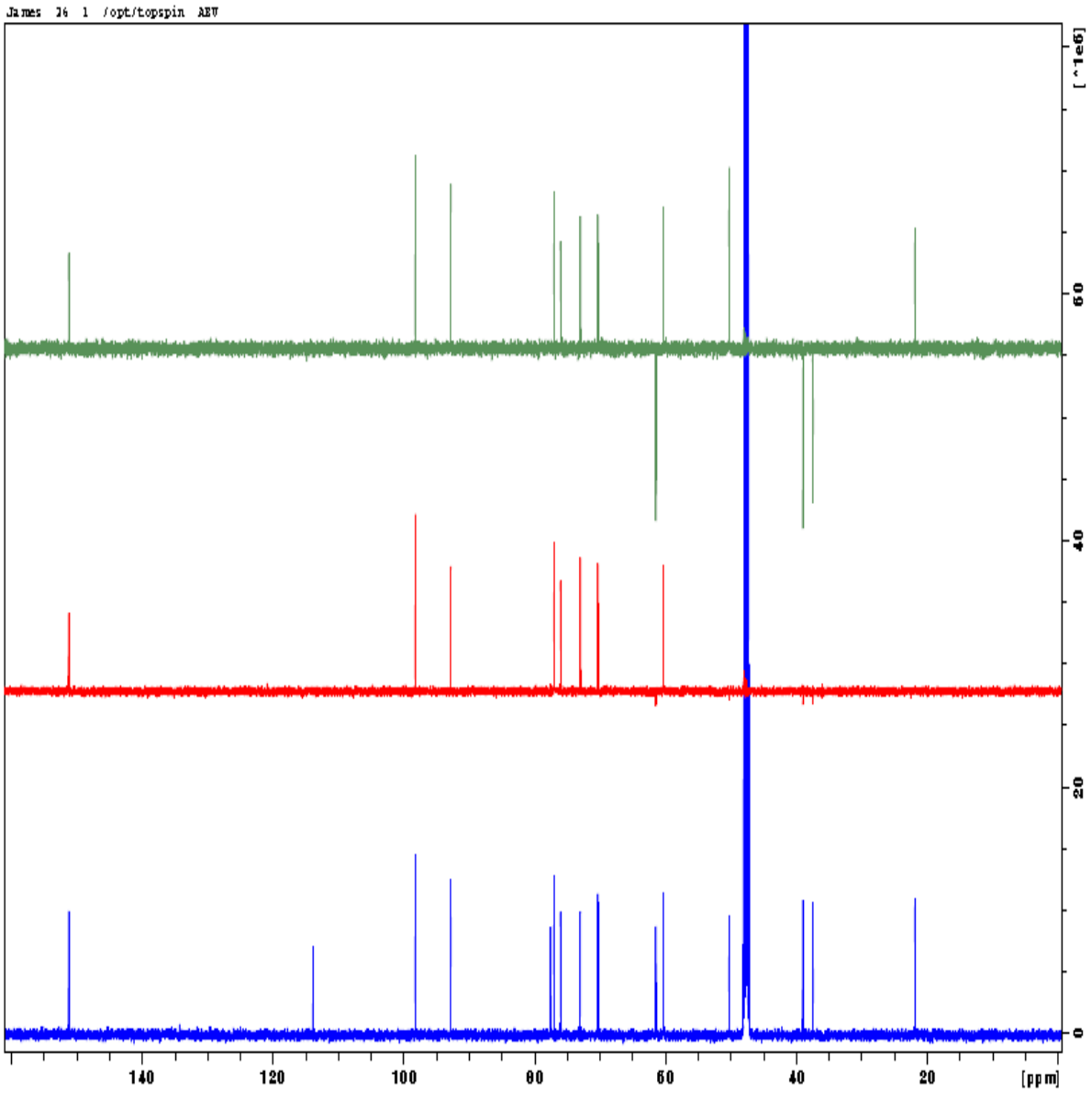


Figure 4.35: DEPT Carbon-13 NMR Spectrum of HNW3

H-H COSY of HNW3

Figure 4.36 revealed the H-H COSY of HNW3 spectrum with correlation at δ5.8ppm (H-1) and δ2.5 (H-9); δ4.5ppm (H-1) and δ3.2ppm (H-2) and δ2.5ppm (H-9) and δ1.2ppm (H-10).

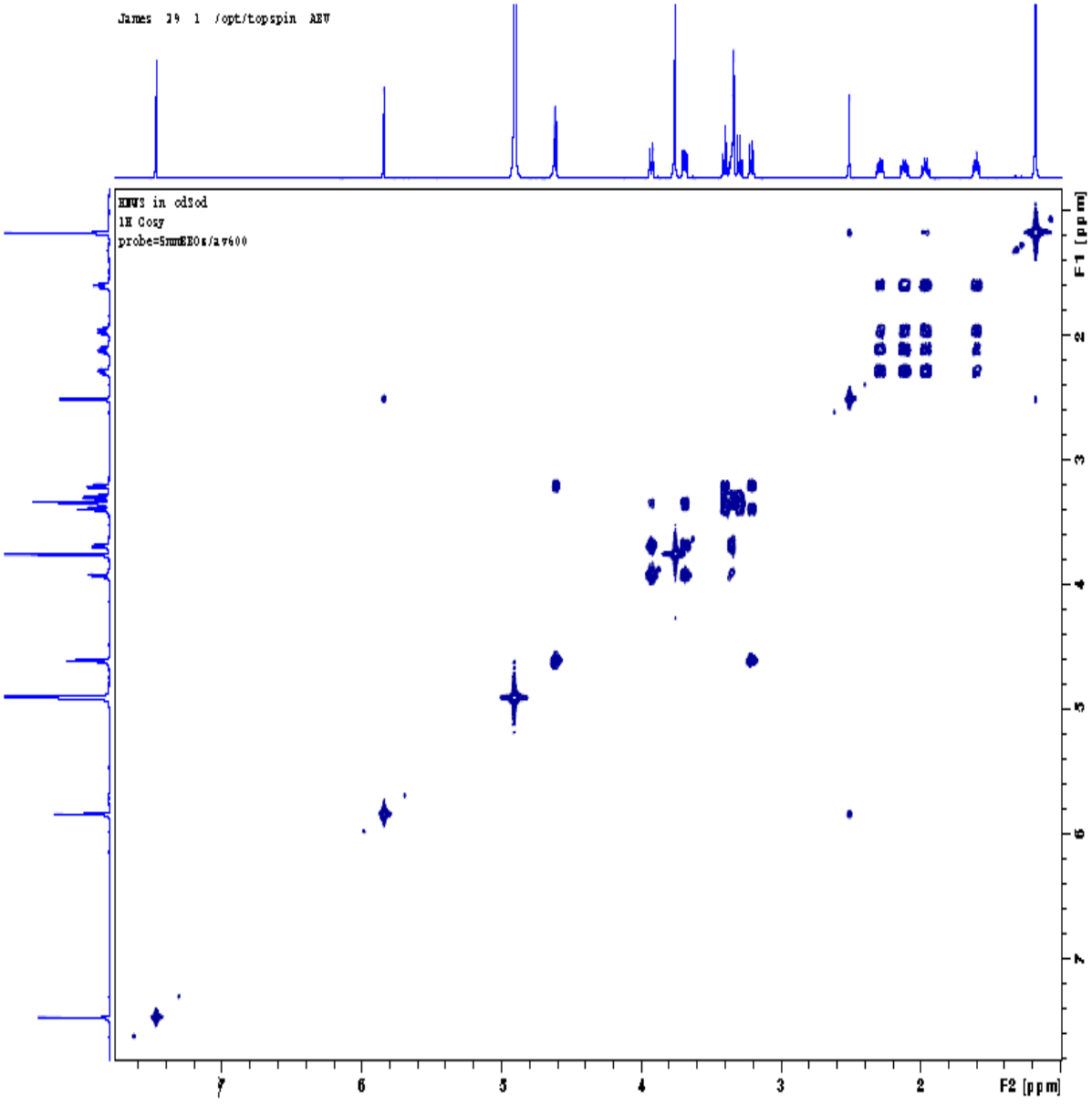


Figure 4.36: H-H COSY of HNW3

HSQC OF HNW3

The HSQC spectrum (fig. 4.37) revealed the correlation of δ_H 5.8ppm to δ_C 92.8212ppm; δ_H 4.6ppm to δ_C 98.2025; δ_H 3.68ppm to δ_C 50.2412ppm and δ_H 3.64ppm to δ_C 60.2817ppm.

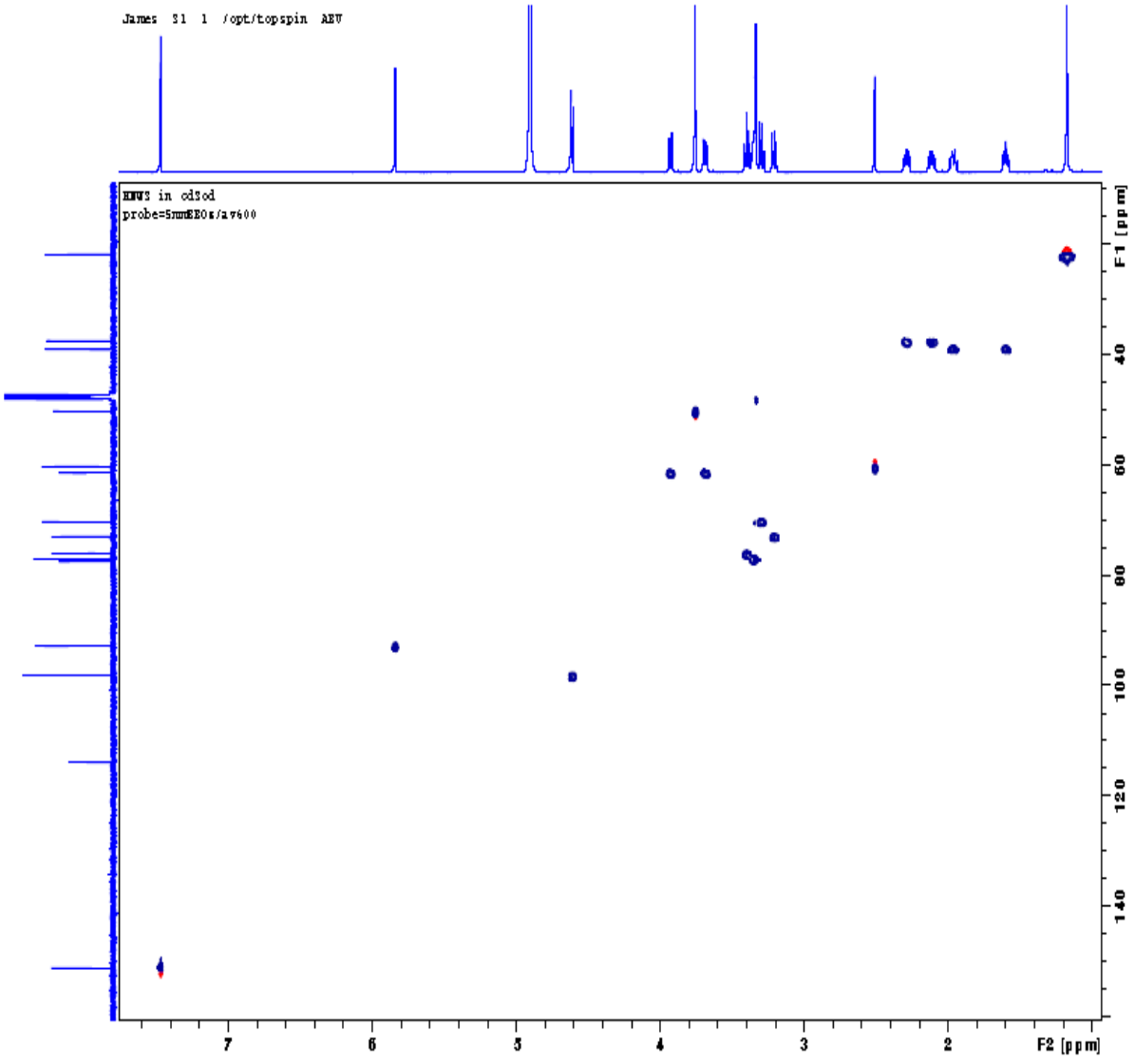


Figure 4.37: HSQC Spectrum of HNW3

HMBC OF HNW3

In the HMBC long range correlation was observed between proton at δ_H 7.4ppm with the carbons on δ_C 70.2965, 92.8212, and 113.8306; δ_H 5.8ppm correlate with δ_C 151.1961, 98.2025, 77.5094 and 70.2965ppm. While δ_H 4.6ppm correlate with δ_C 92.8212 and 77.5094ppm (Fig. 4.38)

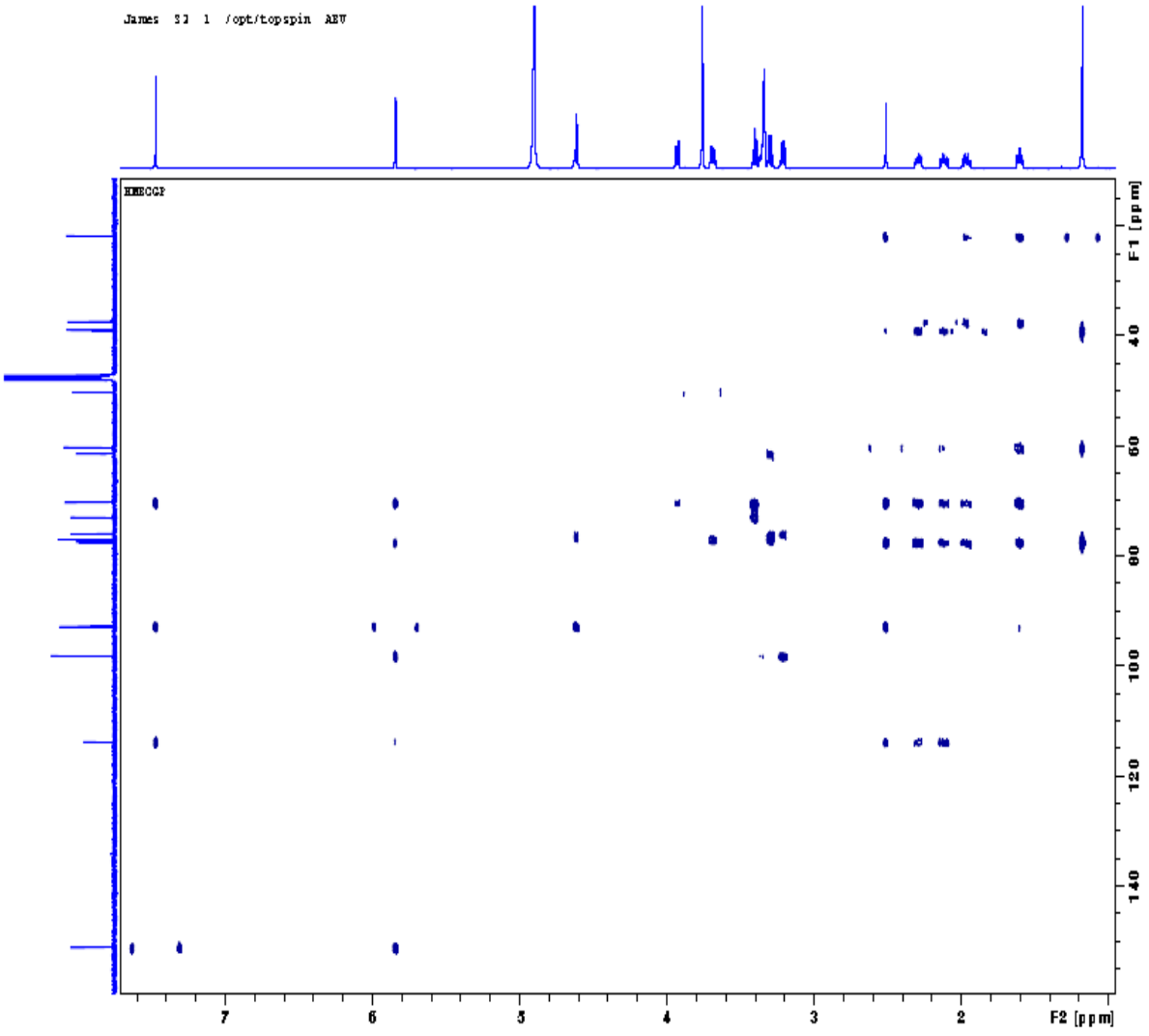


Figure 4.38: HMBC Spectrum of HNW3

NOESY SPECTRUM OF HNW3

The NOESY spectrum shows the correlation of δ_H 5.8ppm → δ_H 1.23ppm; δ_H 5.8ppm→ δ_H 2.45ppm and δ_H 5.8ppm → 4.60ppm (fig.4.39).

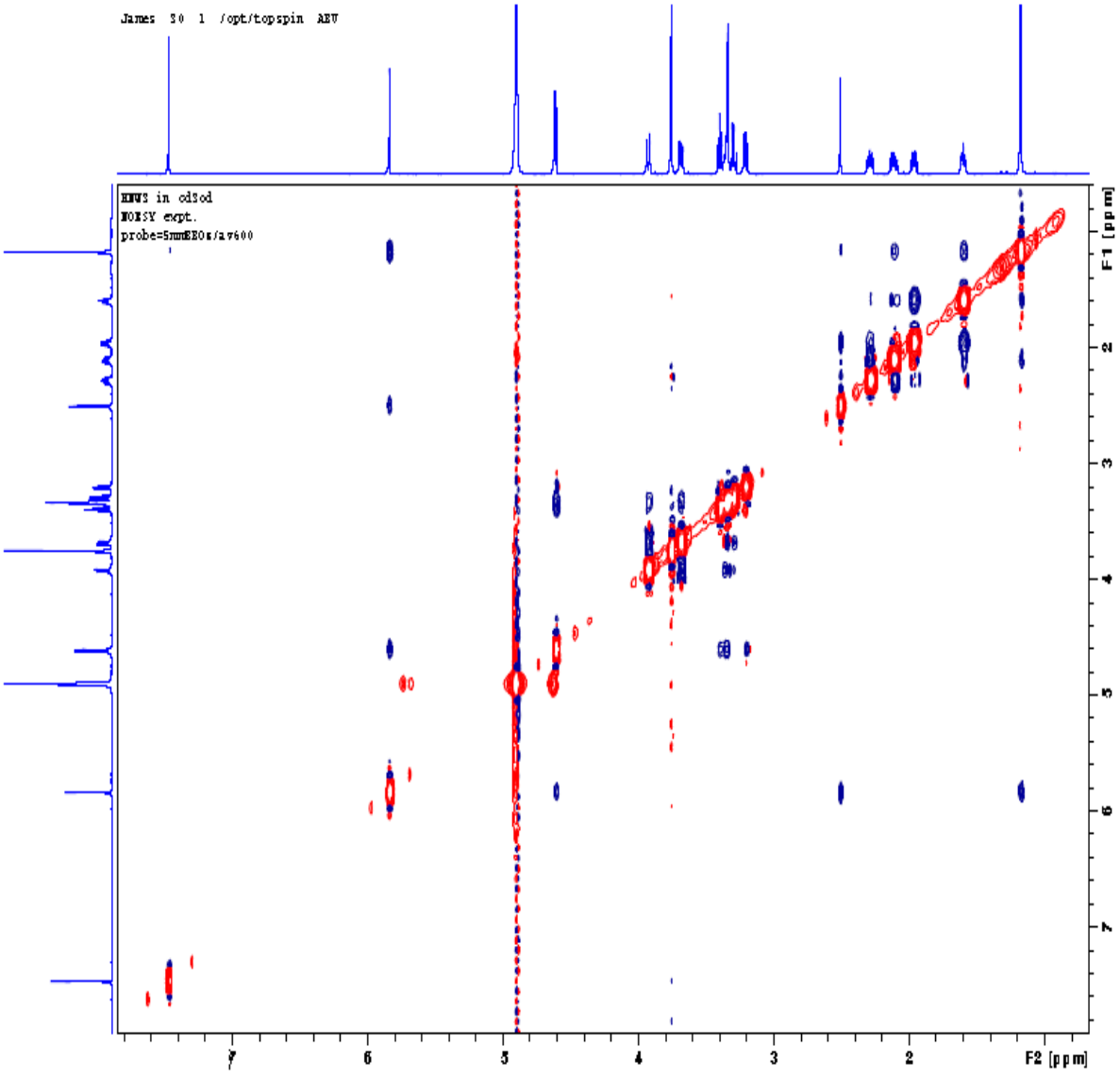


Figure 4.39: NOESY Spectrum of HNW3

Based on the results obtained from Infra-red Spectroscopic analysis, Proton (NMR) ^{13}C (NMR), COSY, DEPT, NOESY and HMBC of Compound HNW3 and comparison with data obtained from the works of (Tayfun *et al.,2*002; Zuhal, *et al.*, 2006). Compound HNW3 was suggested to have the same structural formula as the reference compound.

Table 4.8: ^{1}H NMR (600 MHz) and ^{13}C NMR (125 MHz) data of compound HNW3 in $CDCl_3$

Position	δ_C of HNW3	δ_H of HNW3	δ_C of Ref Cpd	δ_H of Ref Cpd
1	CH 92.8212	5.8378 (1H, d)	93.0	5.7 (1H, d)
3	CH 151.1961	7-4682 (1H, S)	151.4	7.4 (1H, S)
4	C 113.8306	-	114.0	-
5	C 70.3129	-	70.6	-
6	$CH_2$37.4631	1.9354 (1H,m) ,	37.6	2.0
7	$CH_2$39.0011	1.5971 (1H,m)	39.6	1.56 (1H,m)
8	C 77.5094	-	77.7	
9	CH 60.2817	2.5 062 (1H, brs)	60.5	2.79
10	$CH_3$21.8586	1.23 (3H, S)	22.0	1.14 (3H, S)
11	C 166.6475	-	166.8	-
Ome	50.2412	3.7232 (3H, S)	50.4	3.72
1□	CH 98.2025	4.5241 (1H, d)	98..4	4.5
2□	CH 73.0029	3.2190 (1H, dd)	73.2	3.17
3□	CH 76.0472	3.3366 (3H, m)	76.2	3.23
4□	CH 70.2965	3-4264 (3H, m)	70.6	3.32
5□	CH 77.0095	3.3551 (3H, m)	77.1	3.38
6□	$CH_2$61.4449	3.6480 (1H, dd)	61.5	3.65

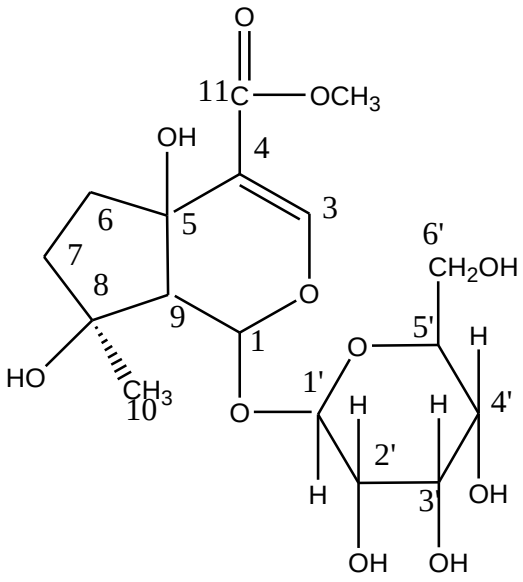


Figure 4.40: Proposed structure of HNW3 (Ipolamiide)

4.6 Results of the Pharmacological Studies

4.6.1 Effect of acetone fraction on cat blood pressure

Normal saline when administered produced no effect on the cat blood pressure (Figure 4.41a). Acetylcholine at concentration of 0.75µg/kg decreased the cat blood pressure (Figure 4.41b). The acetone fraction (3.4mg/kg – 6.79mg/kg) concentrations dependently decreased the cat blood pressure (Figures 4. 42c, d and e). The decreased in the blood pressure induced by the acetone fraction was not blocked by atropine (0.75µg/kg – 1.5µg/kg) (Figures 4.43f and g). Decrease in blood pressure by acetylcholine did not block the action of Atropine as observed in (Figure 4.44h). Adrenaline (1µg/kg) when administered alone, increased the cat blood pressure (Figure 4.45i), Adrenaline (1µg/kg) and acetone fraction (1.5mg/kg – 6.79mg/kg), there was a slight increase in the cat blood pressure (Figure 4.46j and k). But at a higher dose of 1µg/kg of adrenaline and 13.75mg/kg of acetone fraction, the fraction blocked the action of adrenaline (Figure 4.47 l and m).

(a)

(b)

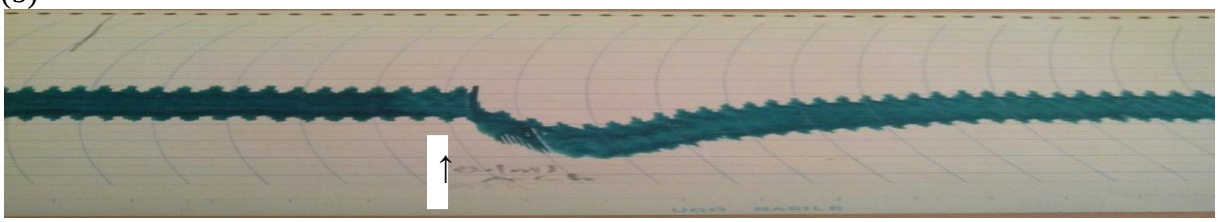
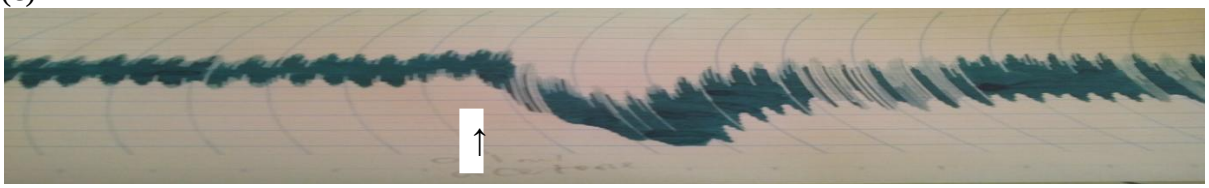


Figure 4.41a and b: Effect of 0.9% Normal Saline (a) and Acetylcholine (0.75µg/kg) (b) on cat blood pressure

(c)



(d)

(e)

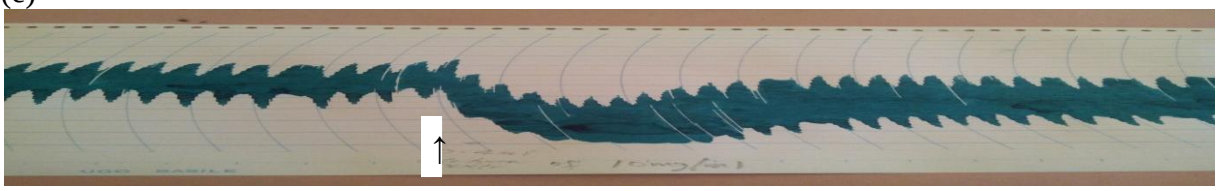


Figure 4.42c, d, e : Effect of acetone fraction (3.4mg/kg – 6.79mg/kg) on cat blood pressure

(f)

(g)

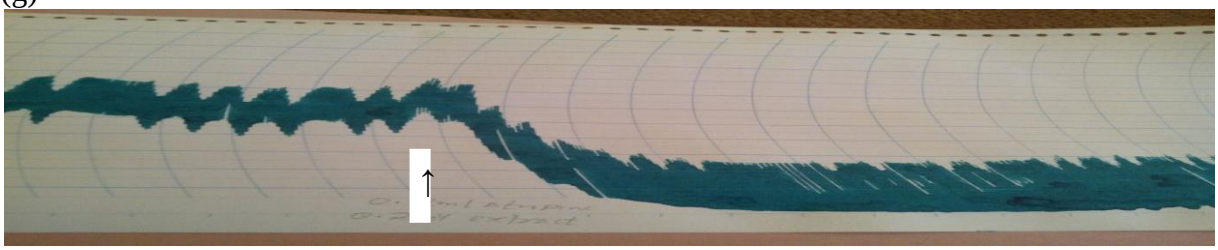


Figure 4.43f and g: Effect of acetone fraction (1.5mg/kg – 13.75mg/kg) in the presence of Atropine(0.75µg/kg – 1.5µg/kg) on cat blood pressure

(h)

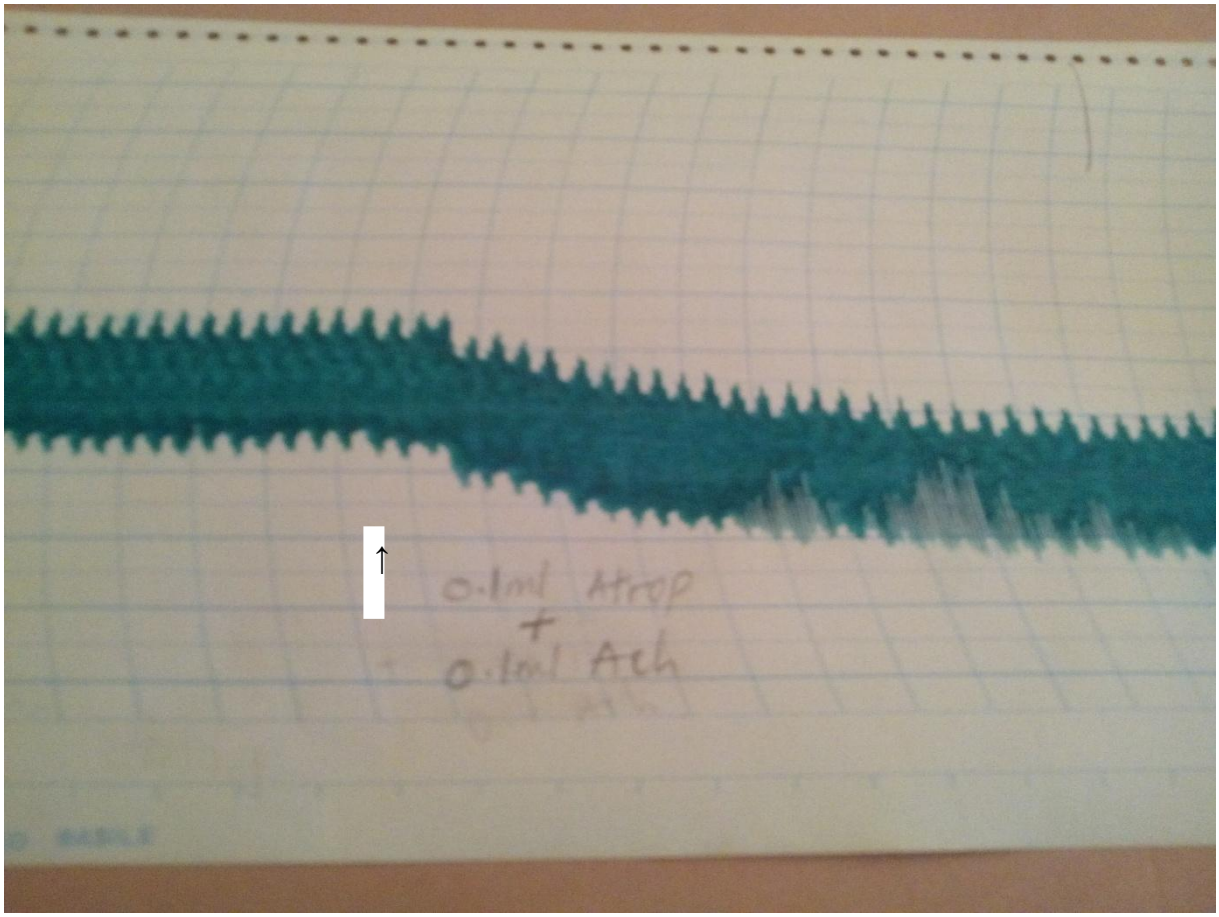


Figure 4.44 h : Effect of Atropine (0.75µg/kg) and Acetylcoline(0.75µg/kg) on cat blood pressure

(i)

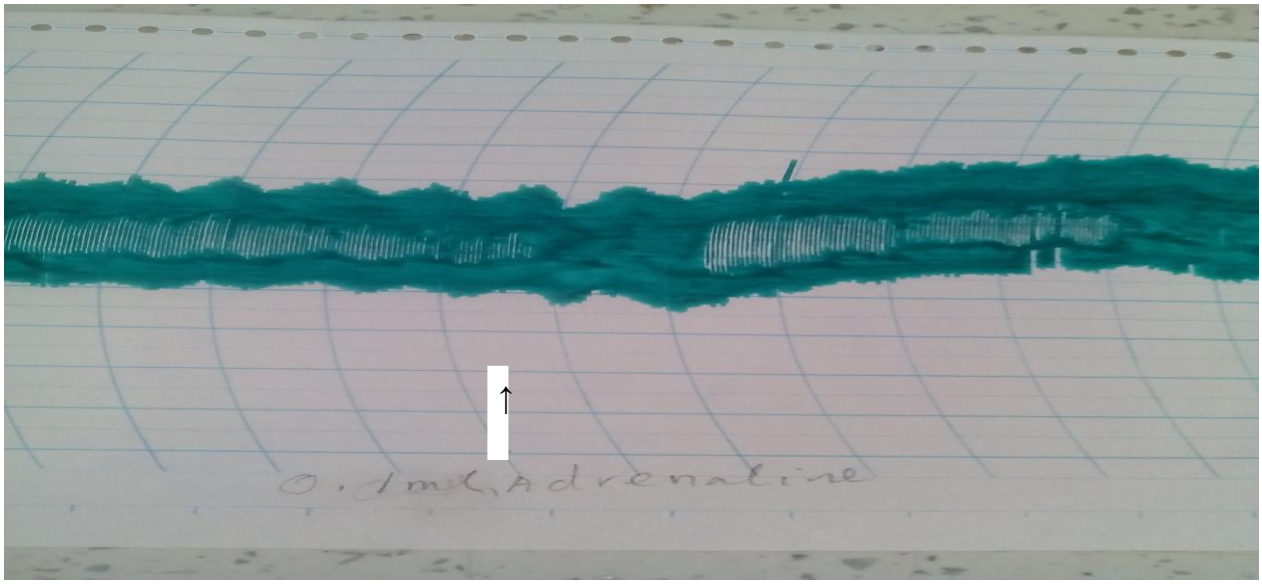
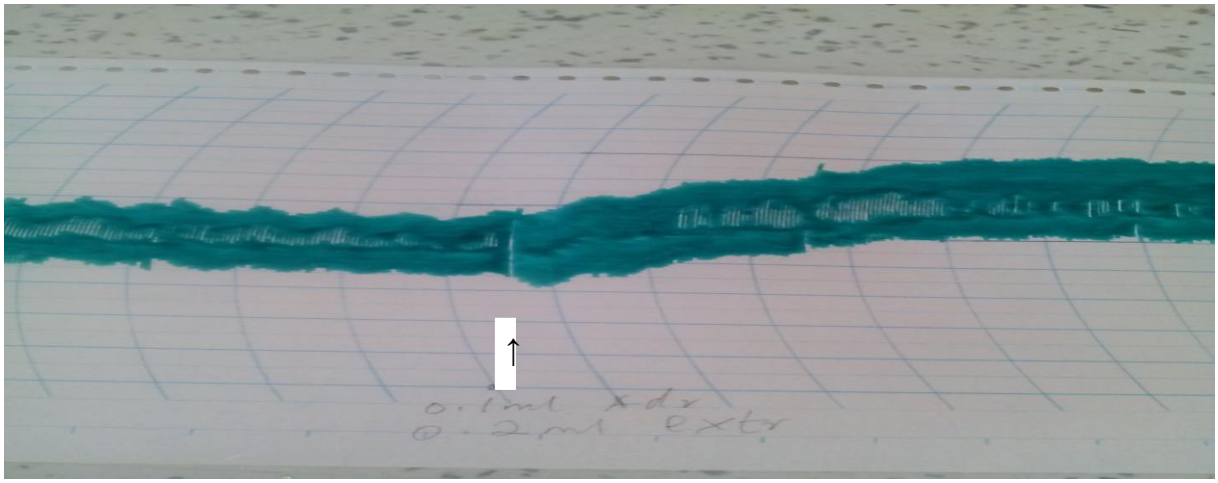


Figure 4.45i: Effect of adrenaline (1µg/kg) on cat blood pressure

(j)



(k)

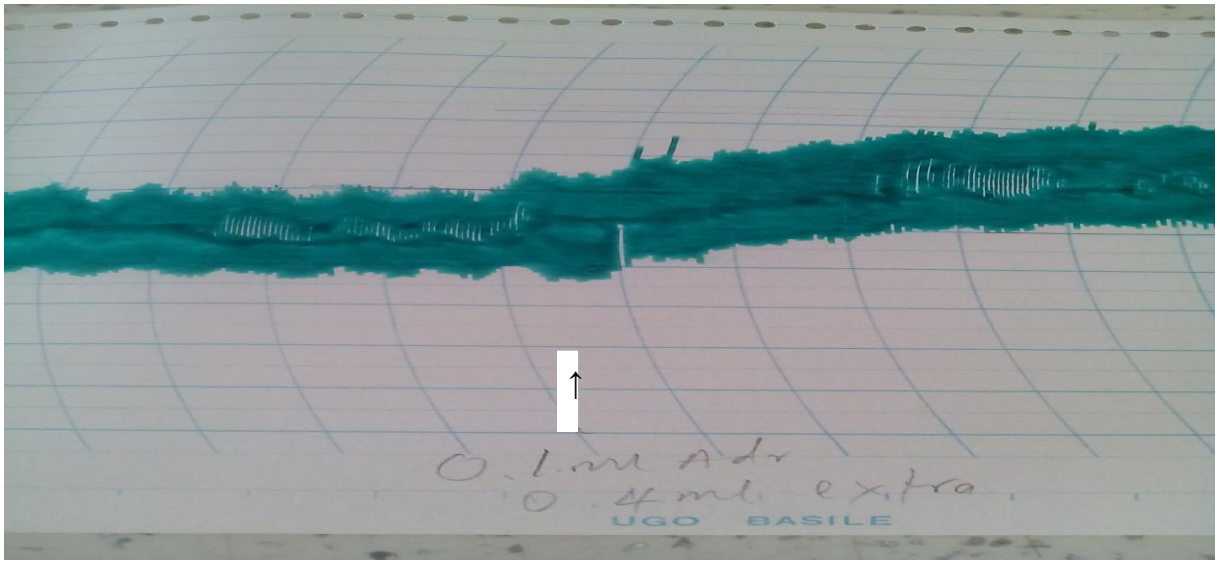
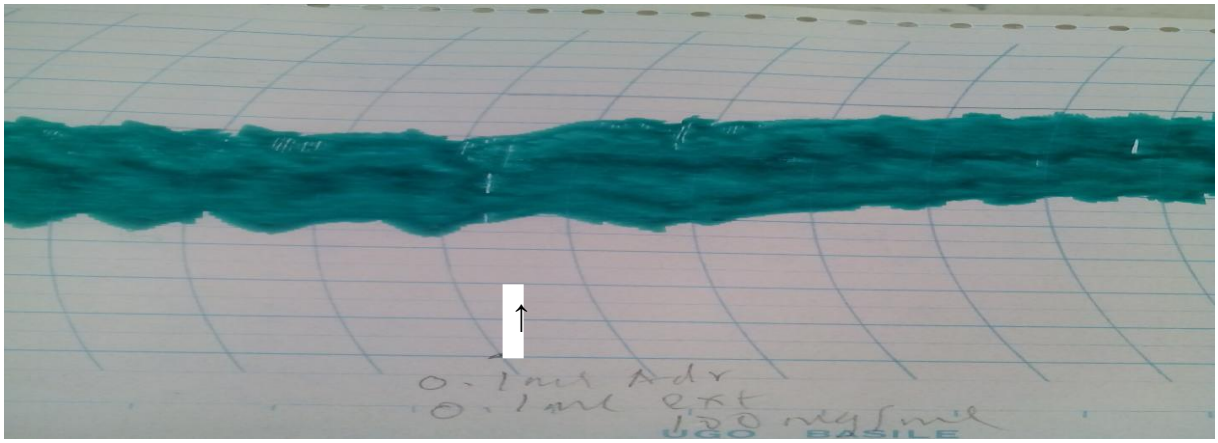


Figure 4.46 j and k : Effect of adrenaline (1µg/kg) and acetone fraction (1.5µg/kg – 6.79µg/kg) on cat blood pressure

(l)



(m)

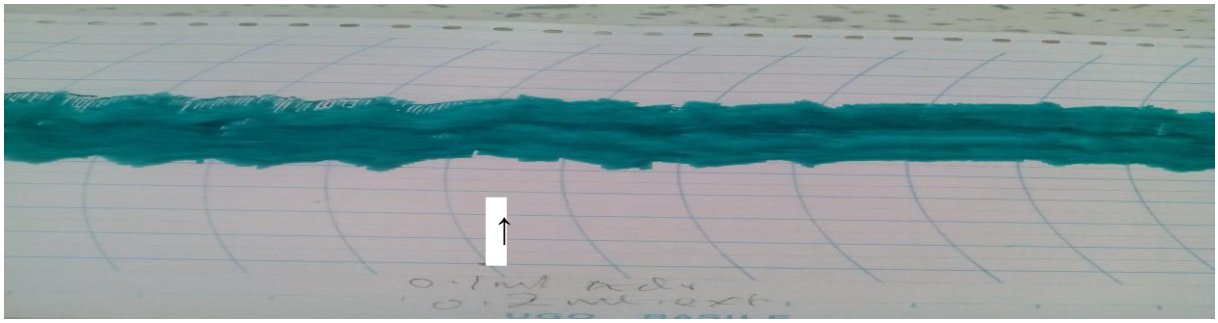


Figure 4.47 l and m : Effect of adrenaline (1µg/kg) and acetone fraction (1.4mg/kg – 13.75mg/kg) on cat blood pressure

4.7 Antimicrobial Studies

4.7.1 Antimicrobial activity of the extract and fractions

Results of the antimicrobial screening of the different concentrations of the methanolic crude extract and various fractions on the test microorganisms are shown in Table 4.9. The highest zone of growth inhibition was observed at 150mg/ml of the acetone fraction against *S. aureus* (22mm) followed by *P. aeruginosa* (20mm) for acetone fraction and methanol extract. Only the 150 and 100mg/ml concentrations of the hexane fraction had effects against all the isolates. The ethyl acetate fraction had also effects at 150 and 100mg/ml on the microorganisms, with 75mg/ml on *S. aureus.* While at 50mg/ml the extract and the fractions had no effects against all the microorganisms except *S. aureus* (12mm).

The lowest zone of inhibition was observed with 75mg/ml concentration of the crude methanol extract against *S. aureus* and *E. coli* which gave a zone of inhibition measuring 10mm; acetone gave the lowest zone with 75mg/ml on *E. coli* only at 10mm; ethylacetate gave the lowest zone with 100mg/ml concentration against *S.* typhi *and P. aeruginosa* (10mm), but *S. aureus* was observed at 75mg/ml. Hexane fraction gave the lowest zone of growth inhibition with 100mg/ml concentration against *E. coli* and *S. typhi* (10mm).

The standard antibiotic drug Cirprofloxacin at concentration of 10µg inhibited the growth of all the microorganisms with zone of inhibition between 34 – 40 mm.

Table 4.9 : Susceptibility profile of the Microorganisms against the Various fractions of the Stem Bark Methanolic extract of *Stereospermum kunthianum.*

Test Organisms	Zones of inhibition (mm) / concentrations of the extracts (mg/ml)																
	ME				AE				EAE				HE				cipr
	150	100	75	50	150	100	75	50	150	100	75	50	150	100	75	50	
S.aureus	16	12	10	00	22	18	14	12	16	12	10	00	14	12	00	00	40
E. coli	14	12	10	00	15	12	10	00	12	10	00	00	12	10	00	00	34
S. typhi	14	12	00	00	15	13	00	00	12	10	00	00	12	10	00	00	35
P.aeruginosa	20	14	12	00	20	16	12	00	13	12	00	00	16	12	00	00	40

Key: ME = methanol extract, AE = acetone extract, EAE = ethyl acetate extract,

HE = hexane extract.

The MIC of the extract/fractions on the test isolates are shown in Table 4.10. The lowest Minimum inhibitory Concentration (MIC) was observed against *P. aeruginosa* with a concentration of 9.375mg/ml for crude methanol and acetone fraction. The highest MIC was against all the isolates except *P. aeruginosa* (37.5mg/ml) in hexane fraction with a concentration of 75mg/ml. The extract had MIC of 18.75mg/ml against *E. coli*, *S. aureus* and *S.* typhi.

Table 4.10**:** Minimum Inhibitory Concentration (mg/ml) of the Extract and the fractions on the Test Organisms

Test Organisms	ME	AE	EAE	HE
S. aureus	18.75	9.375	37.5	75
E. coli	18.75	18.75	37.5	75
S. typhi	18.75	18.75	75	75
P. aeruginosa	9.375	9.375	37.5	37.5

Key: ME = methanol extract, AE = acetone extract, EAE = ethyl acetate extract, HE = hexane extract

Table 4.11**:** Minimum Bacteriocidal Concentration (mg/ml) of the Extract and the fractions on the Test Organisms

Test Organisms	ME	AE	EAE	HE
S. aureus	37.5	18.75	75	0
E. coli	37.5	37.5	75	0
S. typhi	37.5	37.5	0	0
P. aeruginosa	18.75	18.75	75	0

Key: ME = methanol extract, AE = acetone extract, EAE = ethyl acetate extract, HE = hexane extract

4.7.2 Antimicrobial study of the compound HNW3

The antimicrobial activities of the HNW3 are summarized in table (4.12). HNW3 has demonstrated a significant antimicrobial activity against *Staphylococcus aureus, Klebsiella pneumoniea, Escherichia coli*, *Salmonella typhi* for the bacteria and *Candida krusei* for the fungus. The compound showed zone of inhibition ranging from 21 – 29mm against all the tested microorganisms. The standard antibiotic drug for the bacteria erythromycin at concentration of 5µg/ml exhibited zone of inhibition on all the test microorganisms within the range of 32-35mm. But fluconazole at concentration of 5µg/ml showed no activity against the microorganisms except the *Candida krusei* with the zone of inhibition of 34mm.

Table 4.12**:** Susceptibility results of the zone of inhibition of microorganisms against the HNW3

Test Organisms	Zone of inhibition (mm)		
	HNW3	Erythromycin	Fluconazole
Staphylococcus aureus	24	35	0
Klebsiella pneumoniae	27	40	0
Escherichia coli.	22	35	0
Salmonella typhi	24	32	0
Candida krusei	21	0	34

Table 4.13**:** MIC/MBC (µg/ml) of the HNW3 on the Test Organisms

Test organism	MIC	MBC
Staphylococcus aureus	12.5	25
Klebsiella pneumoniae	6.25	25
Escherichia coli	12.5	50
Salmonella typhi	12.5	25
Candida krusei	12.5	50

CHAPTER FIVE

5.0 Discussion

The result of preliminary phytochemical screening carried out on the methanolic extract revealed the presence of flavonoids, tannins, steroids/triterpenes and saponins. These phytochemical constituyents have been reported to be associated with pharmacological activities of plants (Cowan, 1999).

The compound HW1 was isolated from hexane fraction of the crude methanol extract of the stem bark of *S. kunthianum,* it was found soluble in chloform; has a melting point range of 137^0C – 139^0C and positive to the steroidal test of Salkoswki and Liebaermann-Burchard test**.**

^{1}HNMR

The ^{1}H – NMR Spectral of compound HW1 showed the presence of six methyl proton signals that appeared as two methyl singlets at δ_H0.80 and δ_H 1.01ppm, three doublets that appeared at δ_H0.81, 0.94 and 0.93ppm; and a methyl triplet at δ_H1.36ppm. The Spectra also showed one olefinic proton at δ_H5.36ppm. The absence of proton corresponding to double bond between C – 22 and C – 23 in HW1 Spectra suggested the presence of trisubstituted double bond at C – 5 and C – 6 in its structure. The ^{1}H – NMR spectra of compound HW1 showed a proton corresponding to the proton connected to the C -3 hydroxyl group which appeared as a triplet of double of doublet at δ_H3.53ppm. and H – 6 olefinic proton showed a multiplet at δ5.14ppm.

^{13}CNMR

The ^{13}C – NMR together with DEPT showed twenty nine carbon signals including six methyls, eleven methylenes, nine methine and three quartenary carbons. From the Salkowski and Liebermann- Burchard tests for the steroids given by the HW1, it was assumed to be a sterol. The melting point was within the agreed melting point for β – sitosterol in the literature (Holland e tal., 1976). Thus, from the ^{1}H – NMR, ^{13}C – NMR, HH – COSY, the structure of compound HW1 was

assigned as β – sitosterol that was consistent to the reported literature values Conolly and Hill, (1994); Habib *et al* .(2007); Jamal *et al*.(2009); Pateh *et al*., (2009).

Stigmast-5-en-3β-ol or β – sitosterol a phytosterol, is a very common chemical constituent of medicinal plants, which possesses valuable biological activity, such as gastroprotective activity in several experimental ulcer model in rats.

β – sitosterol alone and in combination with similar phytostesrol, reduces blood levels of cholesterol, and is sometimes used in treating hypercholesterolemia. β – sitosterol inhibits cholesterol absorption in the intestine (Matsuaka *et al*., 2008). When the sterol is absorbed in the intestine, it is transported via lipoproteins and subsequently incorporated into the cellular membrane (Awad *et al*., 2008).

Phytosterol and phytostanols both inhibit the uptake of dietary and biliary cholesterol, and reduce the level of LDL and total serum cholesterol. Due to the similarity between structure β – sitosterol and that of the cholesterol, β – sitosterol takes the place of dietary and biliary cholesterol in *micelles* produced in the intestine lumen *(*Moreau *et al., 2002).* This at the end reduces cholesterol absoption in the blood. β – sitosterol has been reported to be used in herbal therapy especially for benign prostatic hyperplasia (Berges *et al*., 1995).

β – sitosterol having its side effects should be avoided during pregnancy and breast-feeding, since not enough is known about its effects on unborn and newborn children. It is also not reconmmended for individuals with sitosterolemia, a rare inherited fat storage disease, this is because, people with this condition have too much β – sitosterol and related fats in their system, therefore, taking it will only worsen this condition (Assmann *et al*., 2006).

The UV spectrum of HNW2 showed two major absorption bands at 340.00nm and 256.00nm, which indicated the presence of flavonol structure (Fathiazad *et al*., 2005).

The IR spectrum revealed a strong absorptions at 3419cm^{-1} (OH) and 1641cm^{-1} (C=O) (Ahmad *et al*,. 1998).

The ^{1}HNMR spectrum of HNW2 as shown in figure (4.11) revealed a meta-coupled system at δ6.22 (1H,d, J= 2.0Hz) and δ6.41(1H, d, J= 2.4Hz), these are assigned to H-6 and H-8 respectively. A tri-substituted coupling system was sighted a typical of ring B at δ7.64 (1H, d, J=2.0Hz), δ6.89 (1H, d, J= 8.4Hz) and δ7.43 (1H,dd, J= 2.0, 8.4Hz), these are the signals assigned to H-2', H-5' and H-6' respectively. The coupling system indicates a 3, 5, 7, 3' 4' penta-oxygenated flavonol with a quercetin nucleus (Mabry, *et al*., 1970; Markham *et al*., 1978 and Abdullahi *et al*., 2010). The ^{1}HNMR spectrum also revealed a cluster of signals at δ5.12 – 3.25 and 1.12, typical of sugars (Abdullahi *et al*., 2010); it also supported the presence of rhamnose and glucose moieties with rhamnose and glucose anomeric downfield protons signals at δ5.12(1H, d,J= 7.6Hz) and δ4.52(1H d, J= 1.2Hz). The coupling systems of these anomeric protons indicated α and β- configuration respectively (Wu *et al*., 2007).

The ^{13}C – NMR spectrum analysis revealed the presence of 27 carbon atoms; 17 in the aromatic region and the others formed a cluster around δ77 – 67 (two the sugar regions), this also confirmed the presence of two sugar units. There is an upfield signal at δ_C17.96 corresponding to δ_H 1.12(d, 3H, J= 6.4) this was assigned to C-6''' of the rhamnose (Abdullahi *et al*., 2010). The DEPT experiment (figure 4.15) confirmed further the multiplicity of the carbon as having 10 quarternary, 15 methine and 1 methylene and methyl each. The presence of methylene and methyl groups was an indication of two sugar units as glucose and rhamnose. The signals at δ_H 4.52 and 5.12 are characteristics for anomeric protons attributable to sugar moieties i.e. Glucose and Rhamnose (Shengmin, *et al*., 2003).

From the spectrum of ^{1}H - ^{1}H COSY (figure 4. 16) correlations were established such as 6.42 (H-8) and 6.23(H-6); 6.87(H-5□) and 7.66(H-6□); 4.52(H-1□ □ □) and 3.63(H-2□ □ □); 5.12 (H-1□ □) and 3.47(2□ □). From these correlations, it can be deduced that HNW2 has four fragments as rings A and B, together with the presence of rhamnose and glucose.

Using HSQC (figure 4.20) spectrum allowed the establishment of various protons to their carbons. Few of these attachments are δ_H 6.41 # δ_C 94.96 (H-8 # C-8); δ_H 7.64 # δ_C 117.78 (H-2□ # C-2□); δ_H 5.12 # δ_C 104.77 (H-1□ □ # C-1□ □) and δ_H 4.52 # δ_C 102.52 (H-1□ □ □ # C-1□ □ □) for anomeric protons and carbons for glucose and rhamnose.

The results of HMBC (figure 4.24) showed a long range correlation between δ_H 5.12 (H-1□ □) and δ_C 102.52 (C-3). This confirming the linkage of aglycone to the glycosyl chain at C-3. While the correlation between δ_H 4.52 δ_C 68.65 (C-6□ □) confirmed the linkage of rhamnose and glucose sugar moities.

The 1D and 2D spectra of HNW2, the IR and UV data with comparison with data obtained from literature (Chien-Chang et al., 1998 and Marzieh et al., 2008) allowed the suggestion of the structure of the compound to be 5, 7, 3□ 4□- tetrahydroxy-3-O-α-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl flavones or Quercetin 3-O-β-D- glucopyranosyl(1→6)-α rhamnoside (Rutin), with a molecular formula as $C_{27}H_{30}O_{16}$.

Among the benefits of rutin, it has been used as a colouring agent, food additive in various food preparations and drinks and for various purposes in cosmetics. Other uses of Rutin include the following:

Rutin inhibits platelet aggregation as well as decreases capillary permeability, making the blood thinner and improving circulation. Rutin shows anti-inflammatory activity in some animal and *in vitro* models (Chan Hun *et al*., 2007).

Rutin inhibits aldose reductase activity. Aldose reductase is an enzyme normally present in the eye and elsewhere in the body. It helps change glucose into the sugar alcohol sorbitol. Recent studies show rutin could help prevent blood clots, so could be used to treat patients at risk of heart attacks and strokes. Some evidence also shows rutin can be used to treat hemorrhoids, varicosis, and microangiopathy. Relatively high amount of rutin increases thyroid iodide uptake in rats and decreases serum T3 and T4 level. The decreased hormone level can be explained by its inhibitory effect produced on Thyroid peroxidase enzyme (TPO). Rutin is also an antioxidant; compared to quercetin, acacetin, morin, hispidulin, hesperidin, and naringin, it was found to be the strongest. However, in other trials, the effects of rutin were lower or negligible (Chan Hun *et al*., 2007).

The IR spectrum of HNW3 revealed a strong absorptions at 3419cm^{-1} (OH) and 1641cm^{-1} (C=O) (Ahmad *et al*,. 1998).

The compound HNW3 was isolated from acetone fraction of the crude methanol extract of the stem bark of S. kunthianum, it was found be soluble in acetone and methanol and a melting point range of 216^{0}C – 219^{0}C and was positive to the ferric chloride test on the chromatogram.

^{1}HNMR

The ^{1}HNMR Spectrum of HNW3 (figure 4.33) exhibited the characteristics signals at δ_H 7.50 for the iridoid structure and showed the existence of a methoxy functional group at δ_H 3.73, and a tertiary methyl group at δ_H 1.23. The H – 1 signal at δ_H 5.81 which was shifted downfield due to glycosidation, indicated the attachment of the β – glucopyranose unit at C – 1 position of the iridoid aglycon. The chemical shift values of H – 3 (δ7.50) and H – 9 (δ_H2.50) were suggestive of C – 4, C -5 and C – 8 to be substituted, therefore, the methoxy carbonyl group was assigned to be positioned at C – 4, due to highly deshielded signal of the H – 3 proton, and quaternary carbon resonance at δc71.6 was attributed to C -5 (Yoshiyasu *e tal*., 2004). Signals observed at δ_H 4.61, δ_H 3.21, δ_H 3.33, δ_H 3.34, δ_H 3.55 and δ_H 3.67ppm are all protons attributed to glycopyranose. The H-H COSY (figure 4.36), exhibited the correlation of H-1 of the cyclopentano pyran ring system to H-1□, H-12

and H-10. The H-1□ was also found to be correlated to H-5□, H-4□ and H-1□ respectively (Nan-zhang *et al.*, 2008).

The HSQC correlation (figure 4.37), shows that δ_C 92.8ppm/δ_C 98.2ppm C/C□ are coupled to δ_H 5.89ppm/δ_H 4.52ppm of H/H□ (Dharma *et al.*, 2001).

The HMBC (figure 4.38) confirmed the point of attachment for the β- glycopyranose unit at C-1 and correlation between (H-1□/C-1) of δ_H4.52ppm/ δ_C92.8ppm to (H-1/C-1□) of δ_H5.89/δ_C98.2ppm (Masaki *et al.*, 2001).

^{13}CNMR

The ^{13}CNMR spectrum of HNW3 (figure 4.34) exhibited 17 carbon signals, six of which could be attributed to a β-glucopyranosyl moiety and 11 to the aglycone.Signals at δ_C98.2ppm, δ_C73.0ppm, δ_C76.0ppm, δ_C70.2ppm, δ_C77.0ppm and δ_C61.4ppm are all characteristic carbon signals for glucopyranose unit (Yong and Peng, 2003). A conjugated methyl ester δ_H 3.72ppm; δ_C50.2ppm, 166.6ppm, which was confirmed by HMBC correlation between the singlet olefinic proton at δ_H 7.50ppm and the ester carbonyl resonance at δ_C 166.6ppm.The HMBC correlations of the H-1 signal at δ_H 5.89ppm to the C-1 anomeric carbon at δ_C98.2ppm and the C-3 olefinic carbon at δ_C 151.2ppm indicated that a β-glycopyranose unit was attached to C-1 which was further linked to C-3 through ether bond (Yoshiyasu, *et al*., 2004). The Complete analysis of the ^{1}H and ^{13}C NMR spectral data of HNW3 allowed the assignment of the Multiplet signals observed at δ_H 2.15/2.26 and δ_H 1.56/2.05 to the methylene protons at C-6 (δ_C 37.4) and C-7 (δ_C 38.9934), respectively.The multiplicity of H-9 was also an indicative of a totally substituted C-8. Therefore, the chemical shift value of the tertiary methyl group (δ_H 1.1732, s) suggested its attachment at C-8. However, the chemical shift values of both C-8 (δ_C 77.5058ppm) and H-10 also indicated the presence of a tertiary hydroxyl function at C-8 position (Robert, 1989). By the complete analysis of NMR data of HNW3, and comparison with data given in the table above, compound HNW3 was determined to be the same as Ipolamiide (Zuhal *et al.*, 2006; Tayfun *et al ., 2002*).

Iridoids are associated with a wide range of health benefits. They provide support against a broad range of physical, chemical or biological stressors. They have a normaling effect on the human body that targets imbalances without affecting properly functioning systems. The benefits include:

(1) Neuroprotective: Several iridoids have been found to protect the nervous system against a number of environmental and chemical stressor, pesticides, or heavy metals found in amalgam tooth filling (Liang *et al*., 2009). Studies have also found iridoids to have memory enhancing effects (Choi *et al*., 2011).

(2) Anticancer and anti-timour: Asperulosidic acid, extracted from dried *Morinda*, has been reported to inhibit the formation of melanoma (skin cancer) cells (Masuda *et al*., 2012). Aucubin, a newly discovered iridoid, displays significant anti-tumour effects on cell cultures of leukemia, breast, colon, lung, skin, nose and throat cancers (Saracoglu and Harput, 2012).

(3) Anti-inflammatory: Several iridoids have been found to have strong anti inflammatory properties both when applied topically and when taken orally (Haznagy-Radnai *et al*., 2001).

(4) Antioxidant: One of the most powerful properties of iridoids is their antioxidant power, which is believed to be much greater than those of more 'traditional' plant antioxidants such as flavonoids or polyphenols from plant sources such as citrus fruits and green tea (Kumarasamy *et al*., 2003).

(5) Antibacterial: Some iridoids from plant family, possess such strong natural antibacterial activity that makes them comparable to antibiotic drugs (Kumarasamy *et al*., 2003).

(6) Cardiovascular property: Oleacei and oleurupein, two iridoids present in olive oil, have been found to have powerful cardioprotective properties. Consumption of olive oil lessen abdomoinal and hepatic fat deposition, as well as less fat deposition in heart and

liver tissue, less and less cardiac stiffness. There is also less suffering from cholesterol problems and hypertension (Poudyal *et al*., 2010). Iridoids in olive oli have been shown to protect the heart and arteries from the detrimental effects of toxicity generated by stress (Andreadou *et al*., 2009).

(7) Blood Sugar control: Iridoids also seem to protect the pancreas which is useful for diabetic patients (Lin *et al*., 2001). Iridoids isolates from different plants have been found to have blood sugar control properties comparable to diabetic drugs

(Sandaram *et al*., 2012).

Effect of acetone fraction on cat blood pressure

Acetylcholine (ACh) is one of the many neurotransmitters in the autonomic nervous system (ANS). It acts on both the peripheral nervous system (PNS) and central nervous system (CNS) and is the only neurotransmitter used in the motor division of the somatic nervous system. Acetylcholine is also the principal neurotransmitter in all autonomic ganglia.

In cardiac tissue, acetylcholine neurotransmission has an inhibitory effect, which manifested as lowering of heart rate. However, acetylcholine also behaves as an excitatory neurotransmitter at neuromuscular junctions in skeletal muscle (Campbell and Reece, 2002).

In cardiac tissue, ACh has an inhibitory effect when it is released from vagal parasympathetic neurons. It has three primary effects upon the heart: decreased heart rate (negative chronotrophic effect) and decrease in force of contraction (negative inotropic effect). The dilatation of vascular beds is due to the presence of muscarinic receptors in the endothelium, which when stimulated released some factors (endothelium derived relaxing factor) which diffused through the smooth muscle and causes relaxation. It decreases spontaneous depolarization by reducing the rate of phase 4 depolarisation and hyperpolarizing the resting cell membrane in sinoatrial (SA) node. It also

slows conduction in atrial muscles, atrioventricular (AV) node, bundle of His and Purkinje system (Brown and Laiken, 2010).

Atropine is commonly classified as an anticholinergic or anti-parasymphathetic drugs. It is termed an antimuscarinic agent since it antagonizes the muscarinic actions of acetylcholine and other choline esters. Atropine inhibits the muscarinic actions of acetylcholine on structures innervated by postganglionic cholinergic nerves, and on smooth muscles which respond to endogenous acetylcholine but are not so innervated. As with other antimuscarinic agents, the major action of atropine is a competitive or summountable antagonism which can be overcome by increasing the concentration of acetylcholine at receptor sites of the effector organ. The receptors antagonized by atropine are the peripheral structures that are stimulated by muscarine. Responses to postganglionic cholinergic nerve stimulation also may be inhibited by atropine but this occurs less readily than with responses to injected choline esters (Brown and Laiken, 2010).

The results show that acetylcholine lowered blood pressure of anaesthetized cat. Unlike acetylcholine, adrenaline elevated the blood pressure. Acetone fraction in this study decreased blood pressure like acetylcholine and also blocked the action of adrenaline as observed. The blood pressure lowering effect of the acetone fraction was not blocked with the muscarinic receptor antagonist, atropine. Atropine acts by blocking or antagonising muscarinic receptors, this suggests that the acetone fraction might be acting via different mechanism and not through muscarinic receptors interaction; therefore, blood pressure reducing effect of acetone fraction can be observed in this study and non-antagonism of effect by Atropine suggesting non-involvement of muscarinic receptors.

It has been reported that chronic oral administration of saponins from *Herniaria glabra* decreased the arterial blood pressure and effected salt and water transport in renal tubules (Rhiouani *et al*,. 1999). Also, dietary saponins can lower plasma cholesterol concentrations. They act either directly, by inhibiting absorption of cholesterol from the small intestine, or indirectly reabsorption of bile

acids (Oakenful and Sidhu, 1990). A flavonoids is another metabolites of signicant health benefit. Lisa (2010) has shown that flavonoids, as an antioxidant, has significantly decreased systolic and diastolic blood pressure and also reduced total cholesterol. Hodgson and Croft (2010), reported that both green and black teas are rich dietary sources of flavonoids therefore, it decreased the risk of cardiovascular diseases. The decrease in the blood pressure observed from this study might be attributed to the presence of bioactive compounds such as phenolic components, flavonoids and saponins present in the plant extract.

Antimicrobial study of the extract and fractions

In this study, the results obtained indicated that the extract of the *Stereospermum kunthianum* inhibited the growth of the test microorganisms except *S.* typhi. This, therefore, shows that the extract contains substance(s) that can inhibit the growth of some microorganisms. Other workers have also shown the extract of the plants inhibits the growth of various microorganisms at different concentrations (Esimone *et al*., 1998; Akujobi *et al*., 2004, and Nweze *et al*., 2004).

Plants with pharmacological active metabolites such as tannin; flavonoids; saponins; triterpenes; anthraquinones and alkaloids have been found *in vitro* to have antimicrobials properties (Cowan, 1999, Draughon, 2004; Aliyu *et al*., 2009). The antimicrobial activity of crude methanol extract and the fractions could be attributable to the various phytochemicals detected in the plant. Saponins may act by damaging cell membranes causing leakage of cellular materials, ultimately leading to cell death (Mshvildadze *et al*., 2000). Therefore the activity displayed by the methanol extract and the fractions can be explained by the complementary effects of the secondary metabolites present or due to synergism.

The large zones of inhibition, exhibited by the extract against *S.aureus* and *P. aeruginosa* justifies their use by traditional medical practitioners in the treatment of sores, burns and open wounds. *S. aureus* and *P. aeruginosa* have been implicated in cases of boils, sores and wounds (Braude, 1982). Also, the moderate growth inhibition against *E. coli* justifies its use in the control of diarrhea and

dysentery. *E. coli* is the common cause of traveller's diarrhea and diarrhegenic infections in human (Ogbulie *et al*., 2007).

The low MIC exhibited by the extract against *S. aureus* is of great significance in the health care delivery system, since it could be used as an alternative to orthodox antibiotics in the treatment of infections caused by these microorganisms, especially as they frequently develop resistance to known antibiotics (Singleton, 1999).

HNW3 was found to be active against *Staphylococcus aureus, Streptococcus pneumoniea, Klepsiella pneumoniae, Escheria coli* and *Salmonella typhi* for the bacteria and *Candida ikrusei* for the fungi.

The low MIC of the plant extract and that of the HNW3 showed their activities against the microorganisms which are associated with different type of infections which include urinary tract infections notably *S. aureus* and typhoid fever *S.* typhi. *S. aureus* is noted to be responsible for a wide range of diseases, such as Pneumonia, skin and soft tissue infections, and diabetic foot infections (Shorr, 2007). Similarly, *P. aeruginosa* is a common pathogen that is associated with burn wound infections, keratitis and respiratory tract infections (Marquart *et al*., 2005). The extract and HNW3 showed activity against *E. coli* which is the common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infection in young women (Usman *et al*., 2007).

The very low MIC and MBC of this compound indicates its potential as antimicrobial agent. It is impotant to note that the activity of the compound HNW3 against *Candida ikrusei* indicates that the plant can serve as a source of an antifungal agent.

Findings lends credence to the traditional use of the plant as a remedy for wounds, ulcer, abdominal upsets and pains, diarrhoeal infections, skin eruption etc. which are caused by *Staphylococcus aureus* and *Escherichia coli* (Oyewale *et al*., 1999).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical investigation of methanolic extract of the stem bark of *Stereospermum, kunthianum* revealed the presence of sterol/triterpenes, coumarin, saponins, flavonoids, steroids, tannins, carbohydrates while alkaloids are absent.

Extensive phytochemical investigations using silica gel column chromatography, gel filtration and preparative thin layer chromatography led to the isolation of Stigmast-5-en-3β-ol or β – sitosterol, ipolamiide and flavonoid diglycoside (Rutin).

Pharmacological studies on the stem bark of methanolic extract of actone fraction showed that the extract exhibited antihypertensive activity; this could find some relevance in the treatment of hypertension in clinical medicine.

In addition, the extracts both crude and the fractions showed antimicrobial activities against the microorganisms tested. The Ipolamiide isolated from the acetone fraction showed tremendous activity on tested microorganisms. It exerts the highest activity on *Streplococcus pneumoniae*. Having activity on *Candida ikrusei* confers on the compound the antifungal activity.

6.2 Conclusion

It can be concluded from the findings in this study, that the use of Stereospermum kunthianum in treatment of hypertension has a scientific basis. Clinicians and pharmacologists will find this study highly relevant with a view to actually formulating a medicament from the acetone fraction that could be effectively used in humans for the treatment of hypertension. The dose-dependant decrease in blood pressure, indicating that the acetone fraction might lower the blood pressure by reducing the total peripheral resistances and/or by decreasing the cardiac output via a reduction in the heart rate. While the antimicrobial activities of the crude methanol and the fractions could be linked to the presence of saponins, tannins, flavonoids, cardiac glycosides and terpenoids contained in the

stem bark of the plant. To the best of our research, this is the first time the isolation of Iridiod (Ipolamiide) was reported from the stem bark of *Stereospermum kunthianum* and the first time the compound was been investigated antimicrobially.

6.3 Recommendations

There is the need to carry out extensive toxicity studies on the plant this is in order to rule out the possibility of any side effect that may arise as a result of the prolong use of the plant.

Further studies should be carried out to isolate flavonoid glycosides and saponins from the same polar portion of the extract.

The need to carry out studies so as to screen the plant for antileprosy, antijaundice antiulcer, antirheumatism and antiviral activities is highly recommended.

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