PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF OF OCHNA RHIZOMATOSA (VAN TIEGH.) KEAY [OCHNACEAE].

BY

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FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY,
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DECEMBER, 2016

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY ZARIA, NIGERIA

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DECLARATION

I declare, that the work in this thesis entitled PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF OF OCHNA RHIZOMATOSA (VAN TIEGH.) KEAY [OCHNACEAE]**,** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry, under the supervision of Prof M. Ilyas, Prof M.I. Sule and Prof A.K. Haruna. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Chinwe Euphemia Egwu

| --- | --- | --- |
| Name of Student | Signature | Date |

CERTIFICATION

This thesis entitled the PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF OF OCHNA RHIZOMATOSA (VAN TIEGH.) KEAY [OCHNACEAE]**,** by Chinwe Euphemia EGWU meets the regulations governing the award of Doctor of Philosophy in the Department , Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences of Ahmadu Bello University, Zaria and it is approved for its contribution to knowledge and literary presentation.

Prof. M. Ilyas **(**Signature) _____

Chairman, Supervisory Committee

Date _____

Prof. M.I. Sule (Signature) _____

Member, Supervisory Committee

Date _____

Prof. A.K. Haruna (Signature) _____

Member, Supervisory Committee Date _____

Dr A. M. Musa (Signature) _____

Head of Department

Date _____

Prof. K. Bala (Signature) _____

Dean, School of Post Graduate Studies

Date _____

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My profound gratitude goes to my major supervisor, Prof. M. Ilyas for his patience, guidance and useful criticism during the course of this work which he designed. I also wish to express my appreciation to my other supervisors, Prof. M.I. Sule and Prof. A.K. Haruna, for their contributions to the success of this work, cannot be over emphasized. I thank you all. I will not forget to say a big thank you sir to Dr. Y.M.Sani for your assistance and directives during the research; he has given me a deep exposure into research.

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Finally, I am forever thankful and grateful to almighty God, for His strength and mercy. Thank you God!

DEDICATION

This research work is dedicated to my husband Ogbuefi Jockin Egwu and my beloved children, Johnbosco E. Egwu , Micheal K. I. Egwu, Rosemary E. Egwu, Victor O. Egwu, Cecilia A.C. Egwu, Mercy U. Egwu, Amanda N. Egwu.

ABSTRACT

The extract of the leaf of *Ochna rhizomatosa* (van Tiegh.) Keay [Ochnaceae], a plant used in the treatment of malaria in Northern Cameroun, was subjected to preliminary phytochemical screening. The result revealed the presence of carbohydrates, steroids, cardiac glycosides, terpenoids, flavonoids, tannins, fatty acids, proteins and saponins. Alkaloids were however absent. Column chromatography of the methanol fraction of water insoluble portion of the extract afforded compound MNC and BND. One of the eluents from the column with two spots was further subjected to gel filtration using sephadex LH_{20} which resulted in the isolation of BNF and more of compound MNC. The structure of these compounds were elucidated using chemical tests, spectroscopic techniques (NMR) and by comparison with reference data. MNC was identified to be a triflavonoid compound. BND was identified to be a Catechin, with IUPAC name 2-(3, 4-dihydroxy-phenyl)- chroman-3,5,7-triol and BNF was identified to be a biflavone (Ochnaflavone derivative). The acute toxicity test gave an LD_{50} value of 86 mg/kg. Result of blood schizonticidal activity of acetone extract in suppressive test gave percent parasitaemia inhibition activity of 40.0%. While the curative test gave a dose dependent percent parasitaemia inhibition activity, highest at the highest dose administered (46.44%). The acetone extract as well as one of the isolated compounds (MNC) were also screened for antimicrobial activity using disc diffusion methods. The organisms tested were clinical isolates of *S. aureus, S. pyogenes, C. ulcerans, E. coli, S. dysenteriae, S, typhi, P. mirabilis, P. aeruginosa, K. pneumonae, C. albicans, C. ibrusei,* and *C. tropicalis*. The extract and the pure compound inhibited the growth of all the microbes tested except *S. pyogenes, C. ulcerans, P. mirabilis, P. aeruginosa* and *C. ibrusei.* Both

the crude extract and compound MNC had good activity with mean zone of inhibition > 18mm (> 27mm for compound MNC and > 23mm for the crude acetone extract). The minimum inhibitory concentration (MIC) of the crude extract and compound MNC were accessed using the broth dilution methods. Both had low MIC values though the pure compound (MNC) had lower value (12.5µg/ ml for compound MNC and an average of 15mg /ml for the crude). The low MIC value suggests that the compound and the crude extract possess good antimicrobial activity, considering that compounds with MIC values less than 100µg / ml are regarded as having strong antimicrobial activity. The minimum bacterial concentration/minimum fungicidal concentration (MBC/ MFC) for the crude and the pure compound were 60 mg/ml and 100µg/ml respectively. The use of *O. rhizomatosa* as an antimalarial and for wound healing thus has scientific basis.

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List of Abbreviations

α	Alpha
β	Beta
aq	Aqueous
^{13}C	Carbon-13
$CHCl_3$	Chloroform
Conc.	Concentration
TLC	Thin Layer Chromatography
^{0}C	Degree Centigrade(Celsius)
NMR	Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
COSY	Correlation Spectroscopy
NOESY	Nuclear Overhauser Effect spectroscopy
HMBC	Homonucear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
$FeCl_3$	Ferric chloride
g	Gram
^{1}H	Hydrogen proton
MHz	Megahertz
CD_3OD	Deuterated methanol
CH_3OH	Methanol
ml	Milliliter
mm	Millimetre

%	Percentage
cm^{-1}	Per centimeter
KCl	Potassium chloride
NaOH	Sodium hydroxide
H_2SO_4	Tetraoxosulphate (vi) acid
LD_{50}	Lethal dose
IC_{50}	Inhibitory concentration
EC_{50}	Effective concentration

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction

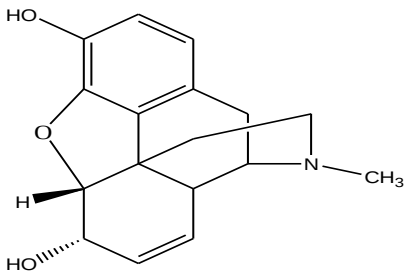
The exploration of the plant kingdom for chemical compounds of medicinal value has been going on for many centuries (Dastur, 1964). Medicinal plants have been the source of useful drugs. Classical examples include quinine, morphine, cocaine, reserpine, vinblastine and atropine to mention but a few; serve to remind us of the debt that modern medicine owes to plant. During the past century, the extra ordinary results of research have unquestionably led to success at an exponential rate that the practitioner of modern medicine now enjoys. However the exclusive use of this research oriented approach with little regard for knowledge acquired through the empirical method has served to delay the application of many potential benefits (Dastur, 1964).

Since time immemorial, humans have relied on plants for the provision of medication, food, clothing, shelter and transportation (Cragg and Newman, 2009). For thousands of years plants have been the foundation of traditional medicine systems where the knowledge on the plants has been passed on from generation to generation (Koehn and Carter, 2005). In African, Indian and Chinese communities, plants have formed the main ingredient of traditional medicines (Gurib-Fakim, 2006; Magassouba *et al.*, 2007). Over 80% of the population residing in developing countries were estimated by WHO in 2008 to be depending directly on plants for their primary medical requirements (WHO, 2008); this is attributed to the fact that plant-derived medicines can be easily accessed and are also cheap (Amin and Mousa, 2007;

Ramawat and Goyal, 2008; WHO, 2008). Even the people of the developed world are also dependent directly or indirectly on plants for their health care. In the United

States, 25% of the prescriptions given from community pharmacies consisted of plant extracts or active ingredients of plant origin (Cragg and Newman, 2009). Plant-derived medicines are often taken in the form of tinctures, teas, poultices and powders, depending on the knowledge of the use and application method of a particular plant for a given ailment (Balunas and Kinghorn, 2005; Fennell *et al.*, 2004). World Health Organization has estimated that perhaps about 80% of more than 4 biillion people on earth rely chiefly on traditional medicine for their primary health care needs (Akerele, 1990). It can safely be presumed, that a major part of traditional therapy involve the use of plant extract or their active principles (Akerele, 1990). Such treatments include the administration of infusion boiled parts, as some of the natural drugs are not usually in the form of tablet or pills (Oliver, 1960). A great number of efficacious plant extracts appears to be available for the alleviation of many symptoms involving the gastro intestinal tract. Certain plants are equally important from the commercial point of view. Plants have been used in city as plasters, as an ointment base as well as emollient, as a coloring agent for food, as an antioxidant, as an essential oil and as flavoring agents (Chambers, 2006). Thus nature is still man kinds' greatest Chemist (Chambers, 2006). Plants produce compounds of varying diversity as a means of defence against bacteria, fungi, pests and predators, hence the plants are efficient natural chemical factories, producing compounds of various structures that result in different physiological effects in the body once ingested (Edeoga *et al.*, 2005). In 1985, Farnsworth *et al*. estimated that at least 119 substances isolated from plants were used as important drugs in one or more countries and that 74% of these compounds were discovered as a result of phytochemical studies on plants used for medicinal purposes (Farnsworth *et al.*, 1985). The development of some synthetic drugs also uses plant-derived structures as leads

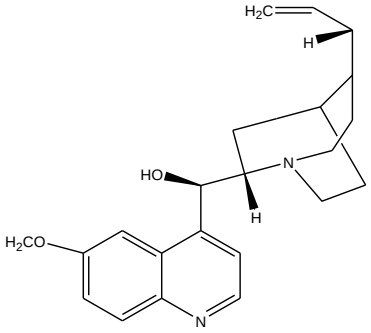
(Ganesan, 2008; Vuorela *et al.*, 2004). An analysis of the origin of new drugs that came onto the market during the period 1981-2006 indicated that only 37% of 974 new chemical entities were devoid of natural origin (i.e., purely synthetic) and that the remaining drugs are either a natural product or have a natural origin by having pharmacophores derived from a natural product (Newman and Cragg, 2007). The isolation and characterisation of natural products began over 200 years ago, when morphine (I) was first isolated from *Papaver somniferum* by a 21-year-old pharmacist's trainee called Friedrich Serturner. This was the first plant-derived pure compound with biological activity to be isolated and it formulated a foundation for alkaloid chemistry which consequently resulted in the development of more effective analgesic agents (Ramoutsaki *et al.*, 2002). After this discovery, an era began where purification and administration of plant-derived drugs were made possible.



(I)

In 1820, the French researchers, Caventou and Pelletier isolated quinine (II), an antimalarial drug, from the bark of *Cinchona l.* trees (Guerra, 1977). The bark of these trees has been used by indigenous people of the Amazon region for the treatment of fevers and in the early 1600s the bark was first introduced into Europe for the treatment of malaria. The bark was not only used for malaria treatment but also for treatment of lupus and arthritis. Nocturnal leg cramps was known to be treated by

quinine, however, recently the FDA has regarded this kind of treatment as uncertain (FDA, 2010). The discovery of II laid the foundation for the synthesis of chloroquine (III) and mefloquine (IV), which were commonly used antimalarial drugs for several years. The more effective antimalarial drug artemisinin (V) was first isolated from *Artemisia annua* (Quinhaosu), a plant that has long been used as a remedy for fever in Chinese traditional medicine. Artemisinin (V) then served as a lead structure for the development of the semisynthetic drugs artemether (VI) and arteether (VII) (Cragg and Newman, 2009; Seaquamat, 2005).



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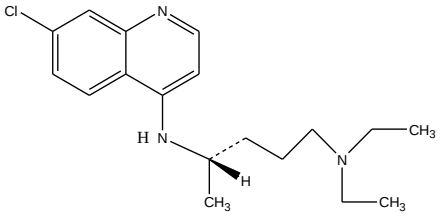

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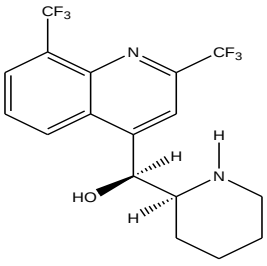

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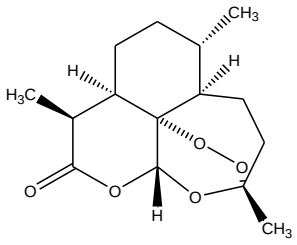

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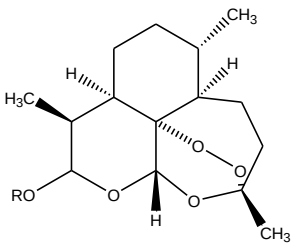
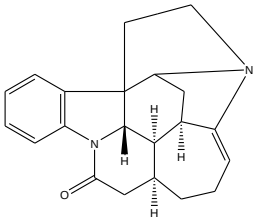


(VI) R=CH_3

(VII) R= CH_2CH_3

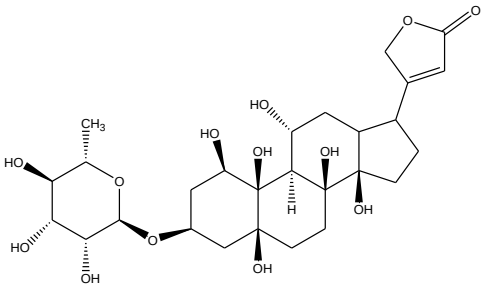
Strychnine (VIII), a colourless crystalline alkaloid was isolated from the seed extract of *Strychnos l.* species (Loganiaceae) native to India and Southeast Asia by the French botanist Leschenault de la Tour (1773-1826). The powdered seeds and decoction have been used by Hindu physicians in the treatment of certain types of cancer, digestive disorders, and diseases of the nervous system, heart diseases, for respiratory and circulatory problems and in the treatment of cutaneous diseases (ulcers infested with maggots). The plant has long been used as an arrow poison in Java, Indonesia. The structure of VIII was first elucidated by Sir Robert Robinson (Robinson, 1945) and its total synthesis was first accomplished in 1954 by Robert W. Woodward (Woodward and Brehm, 1948; Woodward *et al*., 1947; Woodward *et al.*, 1954). Small doses of VIII help in improving appetite and provide a generally strong and hopeful feeling. When dosage is increased, VIII is known to quicken and deepen the respiration and to increase pulse and blood pressure. At poisonous dosages (30 to 120 mg/Kg), VIII affects the nervous system, particularly the spinal cord and medulla (Daly, 2005; Gupta, 2009).



(VIII)

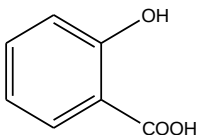
The genus *Strophanthus* DC (Apocynaceae) is found in tropical Africa and Asia, where for hundreds of years the natives have used the seeds of these plants to make poisonous arrow heads for hunting. The isolation of G-strophanthin (IX) (also known as milk for the ageing heart) and its formulation into a cardiotonic agent established a remedy for cardiac diseases. G-strophanthin is a steroidal glycoside isolated from the

dried ripe seeds of *Strophanthus* species (Apocynaceae). The compound is known to strengthen contraction of cardiac muscles and has predominant systolic action, therefore an excellent remedy for patients with pronounced cardiac dilation (Aperia, 2007; Gao *et al.*, 2002; Kracke, 2004).

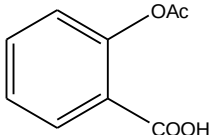


(IX)

Salicylic acid (X) was first isolated from the trees of the genus *Salix* commonly called willow trees, but later the compound was also isolated from the flowers of *Filipendula ulmaria* (L.) (meadowsweet). The meadowsweet plant has been used as a remedy for flu, rheumatism, arthritis and fevers in some communities of western Asia and Europe. The plant is also known to have anti-ulcerogenic, antitumour, anti-microbial, anti-carcinogenic, antioxidant and anti-coagulant activities. Salicylic acid, the active principle of the meadowsweet flowers has a disagreeable irritating taste when taken orally and its side effects, which limits its usage. The acetate derivative of X, acetylsalicylic acid (XI), was first synthesized in 1850 and this has become the prototypical aspirin of today (Vane and Botting, 2003; Yildirim and Turker, 2009).



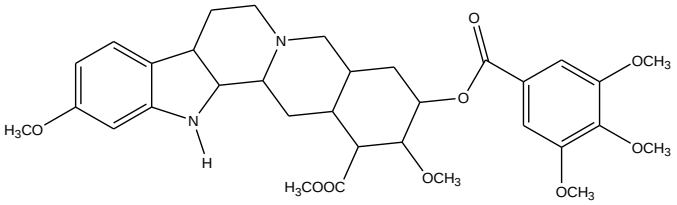
(X)



(XI)

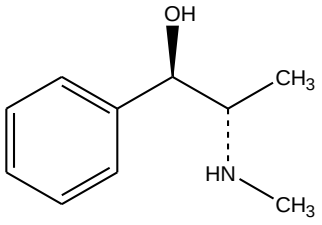
The antihypertensive agent, reserpine (XII), an indole alkaloid, was developed from the traditionally-used medicinal plant *Rauwolfia serpentine* Benth. The plant is locally used in Ayurvedic medicine for the treatment of snakebites. Reserpine (XII) has been

used for a long time as an anti-psychotic and anti-hypertensive drug. However, due to its side effects and the evolution of better drugs for the mentioned purposes, XII is rarely used currently (Carlsson, 2001; Halberstein, 2005).



(XII)

In 1887, an alkaloid ephedrine (XIII) was first isolated from *Ephedra sinica* a plant with a history of being used for the treatment of the common cold, hay fever and asthma in Chinese traditional medicine. Ephedrine (XIII), an appetite suppressant in combination with herbs containing caffeine, is known to enhance weight loss and/or to improve athletic performance. Structurally, XIII is similar to its semi-synthetic derivatives amphetamine and methamphetamine. The structure of XIII as a bronchodilator, has laid the foundation where anti-asthma compounds (beta agonists) were synthesized, *viz.* salbutamol (XIV) and salmeterol (XV) (Abourashed *et al*., 2003; Shekelle *et al*., 2003; Hackman *et al*., 2006).



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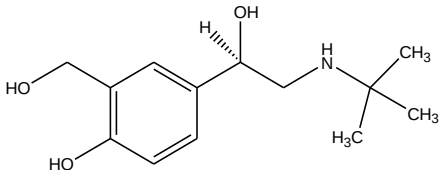

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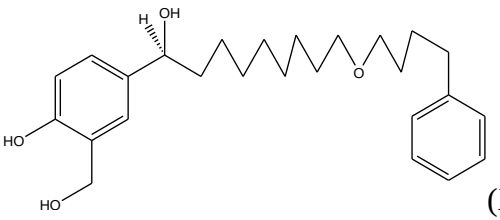
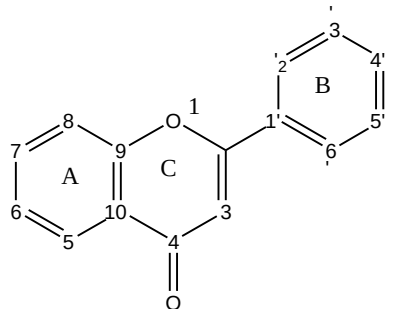


(XV)

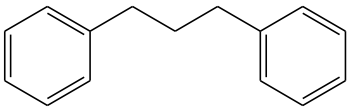
Although the structure of some plant constituents are now known, many compounds that remain undiscovered in plant are beyond the imagination of scientist The chemical constituents in medicinal plant usually explain the rational for the use of the plants in traditional medicine. The trend now is that phytochemistry exploits medicinal plant and isolation of bio-active compounds from which different analogue are synthesized with the aim of obtaining agents with better action or even different biological properties (Fansworth, 1984). We can therefore see that plants active constituent serve as template, for future drug development. The search for medicinal agents from plants is a popular procedure especially plants in fork lore medicine. Reputable laboratories world over engaged in these, which often lead to remarkable discoveries of novel agents (Fansworth, 1984). Primary species have not been left out in these numerous uses to which medicinal plants have been put to use in traditional medicine (Fansworth, 1984). Medicinal plants are believed to be important sources of new chemical substances with potential therapeutic effect (Fansworth, 1996). The research into plants with alleged various folkloric uses in the cure of various ailments should therefore be viewed as logical in the search for the treatment of various ailments (Elizabetsky *et al.,* 1995). The world health organization recognized many herbal remedies and hence encouraged the developing countries to incorporate the use of folkloric plants due to allopathic practice (WHO, 1996; WHO, 2005). In Nigeria today, ethno medical treatment using herbal remedies has succeeded in the treatment of diseases like diabetics, mental disorder, breast cancer, sickle cell anemia and other forms of viral infections (Sofowora, 2008).

1.2 Flavonoids.

The term flavonoids have been applied to embrace all compounds whose structure is based on flavone (XVI). This represents important groups of naturally occurring compounds in which 2 benzene rings are linked by a propane bridge (XVII)



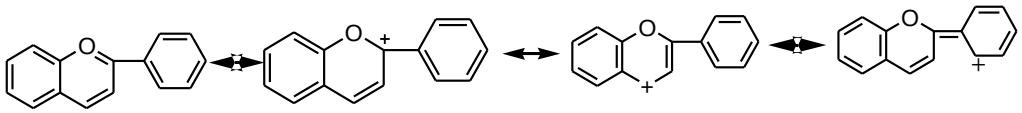
XVI



XVII

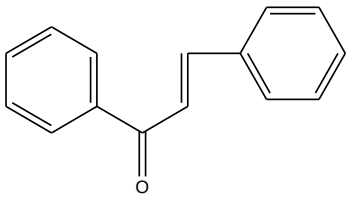
Hence they are any of the series of widely distributed plant constituent related in one way or the other to aromatic heterocyclic skeleton of flavylium cation.

The flavylium cation can be represented as a number of resonating structures (Geissmann, 1962) e.g.

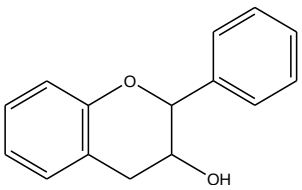


XVIII

The flavonoids include the following: -Chalcone (XIX), catechin (XX), Flavanone (XXI) Flavonol (XXII), Flavanol (XXIII), dihydrochalcone (XXIV), Isoflavone (XXV), Isoflavonone (XXVI), Aurone (XXVII),



XIX



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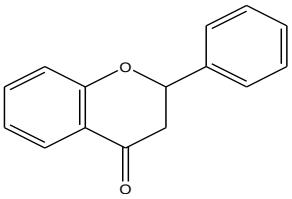
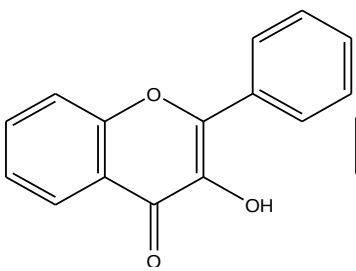
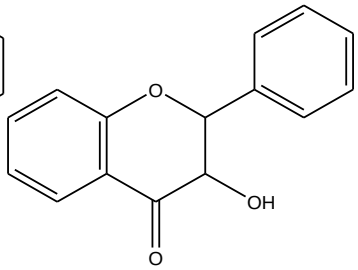


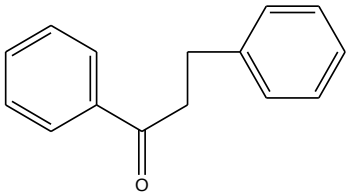
XXI



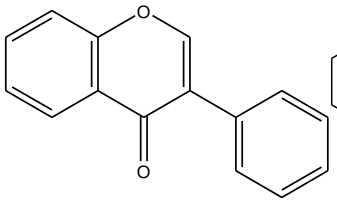
XXII



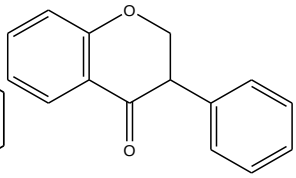
XXIII



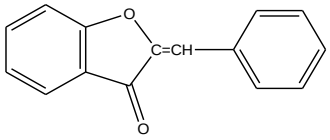
XXIV



XXV



XXVI



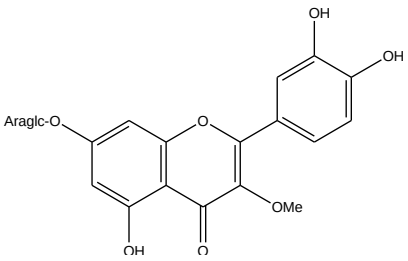
XXVII

The carbon skeleton of flavonoids compounds occurs with variation in the oxidation level of C_3 portion of the molecule. The range of oxidation level extends from the highly reduced catechin (XX) to the highly oxidized flavonol (XXII).

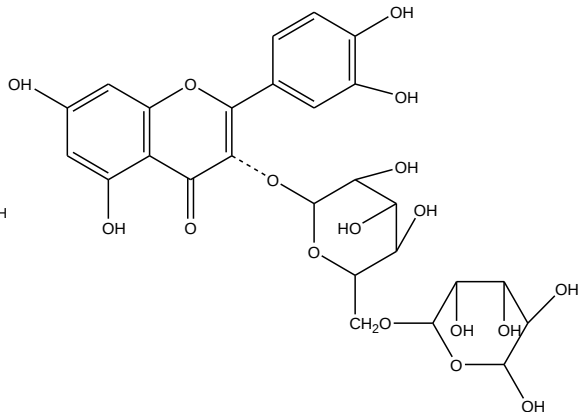
1.2.1 Flavonoids with free sugar linkage or complex sugar linkage

They Include the following:

Quercetin -3- methylether – 7 – O – α –L – Arabinofuranosyl (XXVIII) (Young *et al*., 1990) and Rutin (XXIX) .



(XXVIII)



(XXIX)

` Other types of naturally occurring flavonoids include:

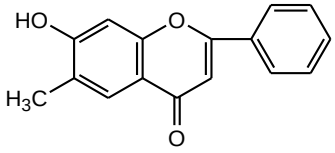
(a) C – Methylflavones

(b) Flavones with additional ring systems

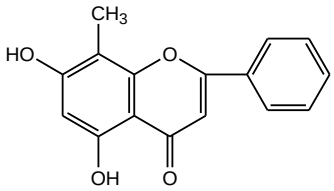
(c) Flavones with isoprenoid substituent

(a) C- methyl flavonoids.

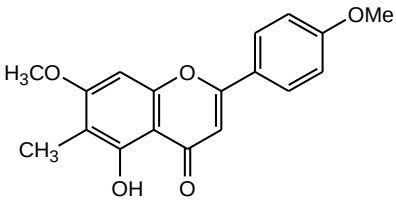
Four C-methyl flavonoids have been found in nature these include strobochrysin (6-methylchrysin) (XXX) from the heartwood of *Pines trobes* and other pine species; 6 – and 8 – methyl – 5, 7-dihydroflavones (XXXI) (Crystostrobin and stroboponin) occur in the fern *Matteuccia orientalis*; 5–hydroxy –7,4'–dimethoxy–6-methyl flavone (XXXII) (Eucalyptin); 5,4'-dihydroxy-7-methoxy–6,8-dimethylflavone (XXXIII) (Sideroxylin) from *Eucalyptus sidroxylon* (Hillis and Isoi, 1965).



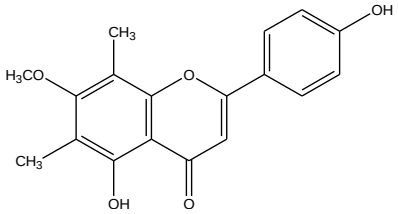
(XXX)



(XXXI)



XXXII



XXXIII

(b) Flavones with additional ring system.

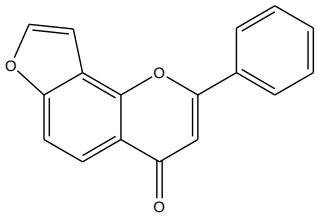
(i) Benzofuran derivatives.

(ii) Arthraxin.

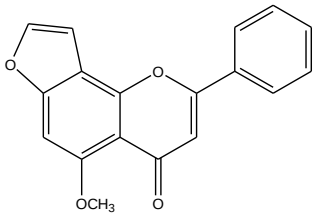
(iii) Alkaloids.

(i) The Benzofuran derivatives.

The parent furanoflavone (XXXIV) Ianceolatin occur in the root bark of *Tephrosia Ianceolata* (Rangaswani and Sastry, 1955) other include Karanjin (3 – methoxylanceolatin) isolated from *Pongamia* oil, pinnatin (XL) and gamatin (3', 4' – methylenedioxypinnatin).



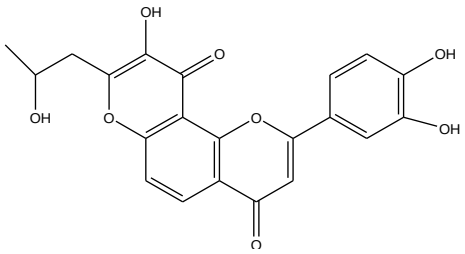
XXXIV



XXXV

(ii) Arthraxin

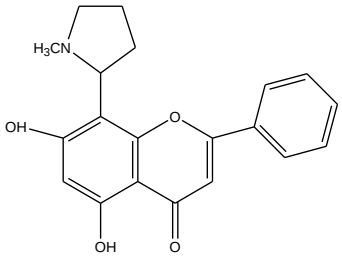
Arthraxin (LII) occurs together with luteolin and it's 7-glycoside in the leaves and stems of the herb *Hispidusmakino anthraxon* (Loranthaceae). It is clear that arthraxin is derived from luteolin by the attachment of three acetate units (CH_3 CO CH_2 CO CH_2 CO-) and subsequent reduction, hydroxylation and cyclization (Kaneta and Sugiyama, 1972)



XXXVI

(iii)Alkaloids

The first flavonidal alkaloids were isolated from *Ficus pantoniana*. The major base ficine (XXXVII) is accompanied by isoficine, the 6 – isomer (John *et al*., 1965).



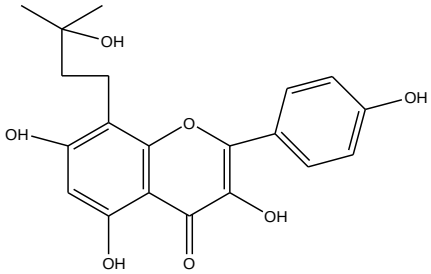
XXXVII

(c) Flavones with isoprenoid substituents.

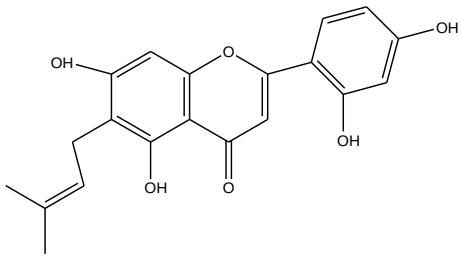
Among the 400 or more phenolic compounds with its isoprenoid substituent so far isolated from plants, only one is a flavone distinction from flavonol, if the flavone of *Artocarepus* and *Morus* are excluded.

The examples of this group include Amurensin and Icariin which are derivatives of (8-prenyluteolin) (XLII) (Geissmann,1962), Sericetin (XLXX) (Burrows and Ollis, 1960) pongachromene (XLIV) (Mukerjee *et al.*, 1969).

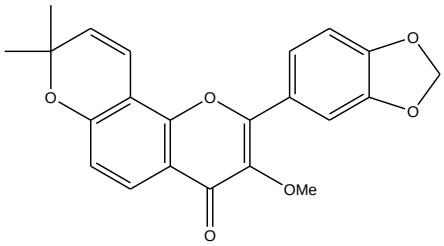
Flavonoids of the types Artocarpus and Morus are largely restricted to two families: Leguminoseae and Rutaceae. The typical example of Artocarpus flavone is Artecarpesin (XLIV) isolated from the heartwood of *Artocarpus heterophyllus* Lamk (*A. intergrifolia* L.) (Ventekaraman, 1972).The typical example of Morus flavone is mulberrin (XLV) (Deshpande *et al.*, 1968) isolated from the bark of *Morus indica.*



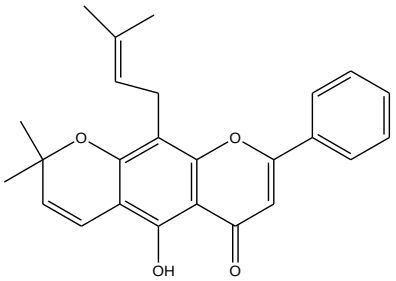
XXXIX



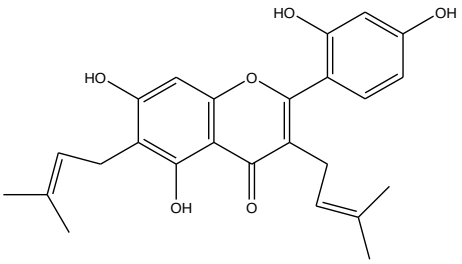
XXXVIII



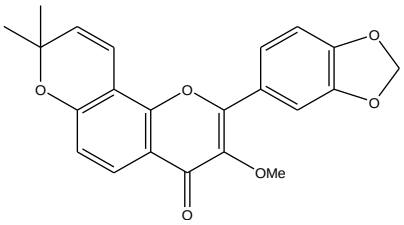
XL



XLI



XLII



XLIII

1.2.2 Identification of the different types of flavonoids by colour reactions:

Some colour reactions of flavonoids, carried out by adding the reagents (except concentrated sulphuric acid in which the solid substance is dissolved) to an ethanolic solution, are listed in Table 2.1 below. The colours mentioned are only broad indications, because within a given class the colour produced depends on the hydroxylation pattern and other substitution. There are some flavonoids, which do not respond to a given test. If the reactions are to be used for a more specific purpose such as providing identity with a known flavonoid, they must be carried out under quantitative conditions simultaneously with a sample of the authentic substance.

Table 1.1: Colour reactions of flavonoids

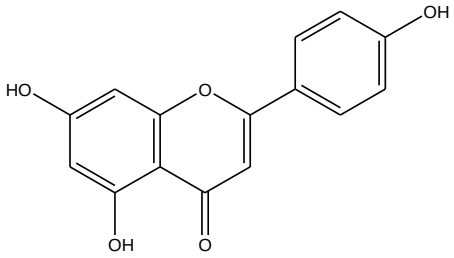
Flavonoid type	Colour reaction			
	Na OH $_{aq}$	Conc.H_2SO_4	Mg-HCl (Shinoda test)	Na–Hg then acid
Chalcone	Orange to Red	Orange Red or magenta	None	Very pale yellow
Dihydrochalcone	Colourless to pale yellow	Colourless to pale yellow	None	None
Aurones	Red to purple	Red to magenta	None	Very pale yellow
Flavanones	Yellow to orange in the cold deep red or purple on heating	Orange to crimson	Red, magenta, violet, blue	Red
Flavones	Yellow	Intense yellow to orange solution often with characteristic fluorescence	Yellow to red	Red
Flavonol	Yellow to orange (brown by air oxidation)	Intense yellow to orange solution often with characteristic fluorescence	Red to magenta	Yellow to Pale red
Flavononols	Very pale yellow, quickly changing to brown	Reddish yellow	Red to magenta	Brownish yellow
Leucoanthocyanins	Yellow	Crimson	Pink with HCl deep blue with Mg	Very pale pink
Anthocyanidin and anthocyanins	Blue to violet	Yellowish - orange	Red fades to pale pink	Yellowish orange
Catechins	Yellow changing to red and brown	Red	None	None
Isoflavone	Yellow	Yellow	Yellow	Pale red or pink
Isoflavonone	Yellow	Yellow	None	Red

(Geissmann, 1962)

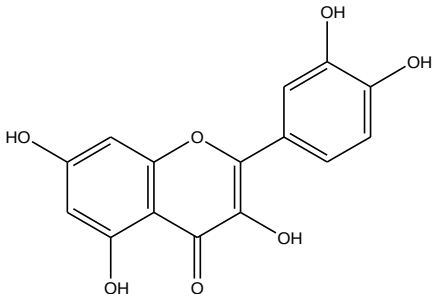
1.2.3 Occurance of flavonoids

Flavonoids are found in all groups and part of plants except few microscopic plants such as algae, bacteria and fungi (e.g. chorflavonin). Their occurrence in insects and in wings of a number of butterflies is due to their ingestion as food and subsequent failure of the insects to metabolize them.

Apigenin (XLIV) occur in the flower of *Barleria cristata* while quercetin (XLV) is very wide spread in part of plants hence no single flavonoids is the only constituent in a particular plant.



XLIV



XLV

1.3 Malaria

Malaria is an important cause of death and illness in children and adults in tropical countries. Mortality, currently estimated at over a million people per year, have risen in recent year, probably due to increasing resistance to antimalarial medicines (WHO, 2006). Malaria, a disease caused by *Plasmodium* infection, is still the most important human parasitic disease in the world: 40% of the world population is at risk with an annual fatality of 1.5 - 2.7 million; highest amongst under-five year olds (Murnigsih *et al.*, 2005). Research into the identification and production of more effective, cheaper and potentially less toxic remedies for the treatment of malaria would therefore continue to be relevant (Dibia *et al.*, 2002). This has led researchers to look for other alternatives, one of which is investigation of medicinal plants. Artemisinin-

based combination therapy (ACT) has become the standard treatment of uncomplicated malaria (WHO, 2011). Sourcing of artemisinin from *Artemisia annua* has further encouraged malaria phytotherapist to revisit the medicinal plants frequently used in the traditional management of the disease (Agbedahunsi, 1998). Neither the Cinchona plants nor *Artemisia annua* from which the most potent drugs (quinine and artemisinin) were isolated, are indigenous to sub-Saharan Africa. Tropical rainforest plants are known to have higher concentrations of natural chemical defenses and a greater diversity than plants from any other biome, thus they are potential sources of new medicines (Balick *et al*., 1996). It seems logical then to encourage studies on plants from these regions, especially since the major proportion of malaria-attributable deaths occurs in sub-Saharan African regions. Nigerian medicinal plants used for malaria treatment include *Alstonia boonei*. The species is highly priced, especially in situations where affordable antimalarial drugs are found ineffective, due to drug-resistant malaria parasites. The plant stem bark or leaves are administered as decoction or "teas" and sometimes as an ingredient in malaria "steam therapy". Recently, the stem bark extract was formulated into tablets, and made available as an antimalarial remedy (Majekodunmi *et al*., 2008). Plants of the Meliaceae family are also commonly used for malaria treatment in Nigeria, like the species *Azadirachta indica, Khaya senegalensis* and *Khaya grandifoliola*. *Azadirachta indica* is called "neem tree" and is also used in other African countries as a decoction against fever and/or malaria. It is used in traditional medical practice in form of an aqueous decoction of the leaves, stem bark and root (Obih and Makinde, 1985). Due to the importance of this plant as an antimalarial remedy in Nigeria, efforts were made to produce the tablet suspensions of the bark and leaf which exhibited high prophylactic, moderate suppressive and a very minimal curative

schizonticidal effect in mouse model of malaria (Isah *et al*., 2003). *Khaya senegalensis* and *Khaya grandifoliola* are often planted by roadsides for shade. The decoctions of their stem barks are extensively used as antimalarial remedies but they seem to have adverse effects (Adebayo *et al.,* 2003 and Bumah *et al.,* 2005). *Khaya* plants are so widely used for medicinal purposes that it is uncommon to find an intact tree near homes. *Morinda lucida* (Rubiaceae), is also largely used in malaria treatment in Nigeria. The aerial parts, stem bark or root bark of *Morinda lucida* are widely used in West Africa to treat malaria and other tropical diseases. A seasonal variation in its antimalarial activity has been reported (Makinde *et al.*, 1994). *Nauclea latifolia* (Rubiaceae) is a small spreading tree, essentially a savannah plant, used as aqueous decoction of the root bark against malaria. *Quassia amara* and *Quassia undulata* (Simaroubaceae), largely used in the southwestern part of the country (Phillipson and Wright, 1991). *Quassia amara*, called bitterwood tree, has the highest antimalarial reputation for curative and preventive purposes in the Simaroubaceae family. *Enantia chlorantha* (Annonaceae) stem bark is used against fever/malaria by traditional medicine practitioners in the forest regions. *Carica papaya* (Caricaceae), commonly referred to as pawpaw, is widely grown in the tropics for its edible fruit and also used as a weak decoction of its leaves against malaria. *Fagara zanthoxyloides* (Rutaceae) has its root widely used as chewing stick in Nigeria and West Africa in general (Odebiyi and Sofowora, 1979). The aqueous extract of the root is used for malaria treatment by the indigenous people. *Spathodea campanulata* (Bignoniaceae), popularly known as African tulip tree is native to tropical Africa, though it has now been adapted to other tropical regions around the world, mostly because of its ornamental value. It is used in southwestern Nigeria for malaria treatment by drinking the decoction of its stem bark. Most of these antimalarial plants are used in form of

monotherapy, and only a few plants are taken together in combined therapies. An example is the multi-herbal extract referred to as 'Agbo-Iba' made up of *Cajanus cajan* (pigeon pea) leaf, *Euphorbia lateriflora* leaf, *Mangifera indica* leaf and bark, *Cassa alata* leaf, *Cymbopogon giganteus* leaf, *Nauclea latifolia* leaf, and *Uvaria chamae* bark (Nwabuisi, 2002). Another multi-herbal combination is the mixture of *Carica papaya* leaves, *Cymbopogon citratus* leaves, *Anacardium occidentale* leaves and *Azadirachta indica* leaves used in 'steam therapy', in which the patients are covered with a thick blanket and made to inhale the vapour from the cooking pot. This research also tried to validate the traditional use of the plant *Ochna rhizomatosa* as an anti – malarial.

1.4 Bacterial Infections.

Infectious diseases are one of the leading causes of morbidity and mortality worldwide, especially in developing countries (Yala *et al.,* 2001). They account for approximately 50% of death cases (WHO, 2005). This might be attributed to poverty and increasing incidence of multiple drug resistance. Bacterial and viral resistances to almost all anti-bacterial and anti-viral agents have been reported (Gbodossou, 2005). The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. This might be attributed to an indiscriminate use of antimicrobial drugs commonly employed for the treatment of infectious diseases (Gbodossou, 2005). Bacteria, in general, possess the genetic ability to acquire and transmit resistance to therapeutic agents and following the massive use of antibiotics in .human therapy, bacteria have developed several resistance mechanisms including the efflux of antibiotics (Yala *et al.,*

2001). This increase in resistance has also been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters, organ

transplantation and ongoing epidemics of HIV infection (Graybill, 1988; Ng, 1994; Dean and Burchard, 1996; Gonzalez *et al*, 1996). Several mechanisms have been proposed to tackle this problem of resistance of bacteria, such as target site modification, expression of the efflux pumps, and metabolic inactivation, which contribute to the drug resistance in MDR bacteria (Hooper, 2001). Plant based natural products traditionally known to combat microbial infections are expected to play a big role in this regard (Cowan, 1999). Plant extracts and essential oils have been widely explored for therapeutic activities against most microbial infections (Nawab *et al*., 2011). *Acalypha wilkesiana* a shrub is available in tropical forest of western Nigeria and often used traditionally for the healing of wounds and dermatological as well as gastrointestinal disorders (Akinde and Odeyemi, 1987). *Phyllathus discoideus* is a small tree the bark extract is used locally to cure stomachache and lumbago. *Trema guineensis* extracts are used for the treatment of fever, bronchitis, pneumonia and gastrointestinal disorders (Ajaoyeoba, 2002, Akinyemi *et al*., 2005). *Azadirachta indica* (Dogonyaro), *V. amygdalina* (Bitter leaf), *Allium sativum* (Garlic), *O. gratissimum* (Scent leaf), and *Zingiber officinale* (Ginger) plants have been reportedly used in the treatment of ailments such as stomach disorder, cough and other ailments traditionally (Odugbemi, 2006).

Flavonoids isolated from natural dietary sources have also been investigated in combination with antibiotics as a strategy against ESBL (Extended-Spectrum Beta-lactamase) producing clinical isolates of *Klebsiella pneumoniae* (Lin *et al.,* 2005). Oregano essential oil, given in combination with fluoroquinolones, was found to enhance the activity of the drugs against ESBL-producing Escherichia coli (Si *et al.,* 2008).

In addition to the problem of drug resistance of microbes to drugs in current used, in developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and undesirable side effects, which limit their application (Maureer-Grimes., *et al* 1996). Therefore, there is need to search new infection-fighting strategies to control microbial infections (Sieradzki *et al*, 1999) and there is also an urgent need to develop new anti-microbial agents that are highly effective with less toxicity (Maureer – Grimes *et al*., 1996).

1.5 Statement of Research problems.

Despite the tremendous progress in drug development, diseases caused by bacteria, virus and parasites are still a major threat to the public health sector (WHO, 2008).

Half of the world's population is at risk of malaria and an estimated 247 million cases led to nearly 881,000 deaths in 2006. The world malaria report 2008 described the global distribution of cases and deaths. New WHO recommended control strategies have been adopted and implemented in endemic countries, sources of funding for malaria control, and resent evidence that prevention and treatment can alleviate the burden of the disease (WHO, 2008). Malaria transmission occurs in six WHO regions. Globally, an estimated 3.3billion people are at the risk of being infected with malaria and 1.2 billion are at high risk (> 1 in 1000 chances of getting in a year). According to the latest estimates 198 million cases of malaria occurred globally in 2013 (uncertainties range 124-283 million) and the disease led to 584000 deaths (uncertainty range 367000 - 755000). The burden is heaviest in the Africa region, where an estimated 90% of all malaria death occurs and the children aged under 5years account for 78% of all death (WHO, 2014). Malaria is the leading cause of morbidity and mortality in sub-Saharan Africa, especially in young children and pregnant women (UNICEF, 2004). Compounding the problem are other factors that

include environmental changes, the collapse of health systems in areas of civil strife and war, resistance of malaria parasites to affordable anti-malarial drugs and limitations in national health services (Nsimba, 2006). Recently, a number of studies and campaigns have been done for the adoption of artemisinin combination therapies (ACTs) (Sutherland *et al.,* 2005; Attaran, 2004; Duffy and Mutabingwa, 2005), which have now been adopted in a number of countries including Tanzania. An estimated 65% of Nigeria's population lives in poverty and poverty is a major factor in malaria prevention and treatment (WHO, 2O11).

However, the slowly eliminated combine drugs in ACTs may sooner or later, be susceptible to development of resistance in high endemic settings (Kremsner and Krishna, 2004; Talisuna *et al*., 2004).

Antimicrobial agents are among the most commonly used and misused of all drugs. The inevitable consequences of the wide spread use of anti-microbial agents has been the emergence of antibiotic resistant pathogens, causing an ever increasing need for new drugs (Chambers, 2006). Herbal remedies used in traditional folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help to overcome the growing problem of drug resistance and also the toxicity of currently available commercial antibiotics (Al- wadh *et al*., 2001).

Hence there is need to develop new antimicrobial and antimalarial agents that are highly effective, affordable with less toxicity.

This has led to the need to search for biologically active compounds from plants that have been reported for use in conditions related to bacterial infections as well as antimalarial agents one of such plants is *Ochna rhizomatosa.*

1.6 Justifications

The prevalence of malaria and the emergence of drug resistant plasmodium parasites necessitate a continuous search and development of new antimalarial drugs.

The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing.

The antimalarial and antimicrobial drugs are associated with serious side effect, so the use of naturally occurring compounds may assist in reducing some of the undesirable effects.

The synthetic antimalarial and antimicrobial drugs are very expensive and unaffordable to majority of the populace especially in Africa.

Therefore the abundance of this plant within the African region will go a long way in providing cheaper reliable and affordable substitutes.

There is thus the need for further research into antimalarial and antimicrobial medicinal plants.

1.6 Aim of the study:

The aim of this research work is to scientifically screen the extract of the leaf of *Ochna rhizomatosa* for both antimalarial and antimicrobial properties and to isolate some of the bioactive principles responsible for the observed biological properties.

1.7 General Objectives of the study

The primary objectives of this study were to identify, isolate and structurally elucidate bioactive compounds from the leaf of *Ochna rhizomatosa* using standard phytochemical procedures. Secondly, to investigate the antimalarial properties and to determine the antimicrobial properties using clinical isolates so as to validate the ethno – medicinal uses of the plant.

1.8 Specific Objectives of the study

- T o carry out the phytochemical screening on the extract of the leaves of *Ochna rhizomatosa* (Ochnaceae).
- Isolate bioactive compounds and determine the chemical structures of the isolated compounds using spectroscopic methods.
- Determine the antimalarial activities of the crude acetone extract.
- Determine the antimicrobial activity of the crude acetone extract and an isolated compound using clinical isolates.

1 .9 Statement of research hypothesis

Ochna rhizomatosa contains bioactive compounds with antimalarial and antimicrobial activities.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of the Plant family:

Ochna rhizomatosa belongs to the family Ochnaceae. This family comprises of about 53 genera and 600 species. These species are hermaphroditic (Rendle, 1952).

Ochna genus is of the family Ochnaceae. The genus *Ochna* includes 86 species of evergreen trees, shrubs, and shrublets and is distributed widely in tropical Asia, Africa, and America, of which eleven species occur in India (Kirkitar and Basu, 1980).

2.2 Morphology of *Ochna*

Ochna rhizomatosa is a low shrub to just over 6 meters high. The shrub is decorative, with glausous, serrulate, prominently – veined leaves. It has bright yellow flowers, borne usually while the plant is leafless (Burkill, 1985).

The genus (*Ochna*) comprises 86 species of evergreen tree, shrubs and shrublets. Species of this genus are usually called Ochnas or Micky – mouse plants, a name coming from their drupaceous fruit shape. Some species especially *serrulata* are widely cultivated for decorative purposes. Several member of this genus are cultivated as decorative plants due to their colorful flowers and unusual fruits, e.g., *Ochna kirkii, O.mossambicensis, O. schweinfurthiana, O. serrulata,* and *O. thomasiana*.

Plate 2.1: Plant and plant's parts picture.

Ochna rhizomatosa (van Tiegh.) Keay [family Ochnaceae].

2. 3 Taxonomy of *Ochna rhizomatosa*

Kingdom : Plantae

Phylum : Magnoliophyta

Division : Angiosperm

Class : Magnoliopsida

Order : Theales

Family : Ochnaceae

Sub-family : Ochnoideae

Genus : Ochna`

Specie : *rhizomatosa*

Author : (van Tiegh.) Keay.

Common names

Ghana (Sisala) : Sankpenedidi

Nigeria (Gwari) **:** Belawa

(Hausa) **:** Alikama

2.4 Distribution of Plant.

Ochna rhizomatosa is of Savanna forest; from Mali to Northern Nigeria. Other members of the genus (86 species), are native to tropical woodlands of Africa, America and Asia, while some species are distributed in tropical and subtropical zones throughout the world (Rendle *et al.*, 1952, Kirtikar and Basu, 1980).

2.5 Ethno medicinal uses of *ochna rhizomatosa* and some other species in the genus *Ochna*

Literature review on the plant *Ochna rhizomatosa* revealed that no previous pharmacological investigations have been reported, however the stem bark was reported to be used for the treatment of malaria, jaundice, wound and intestinal helminthiasis (Betti, 2011).

Several members of this genus have long been used in folk medicine for the treatment of various ailments such as asthma, dysentery, epilepsy, gastric disorder, menstrual complaints, lumbago, ulcers, as an abortificient and as antidote against snake bites (Kirikikar and Basu, 1980). A number of Ochna species have a long history of use as herbal remedies in Asia and Africa. For example, *Ochna squarraosa* L. (known as 'erra juvvi'), a small shrub, has been used in indigenous systems of medicine for treating various ailments, for example, the bark as a digestive tonic and the root for its curative effect against asthma (Kirtikar and Basu, 1980). The stem bark of *O. lanceolata* syn. *O. heyneana*, a semi –evergreen tree found widely in Central and Peninsular India, is used by the Palliyar tribes as an abortifacient and for treatment of gastic complaints and menstrual disorders (Muthukumarasamy *et al.,* 2003). Similarly, *O. pumila* (called 'champa baha') root are used as an antidote to snake bites. In addition its use by Mundas for treatment of epilepsy has been reported, and the leaves have been used as a 'poutrice' for treatment of lumbago and ulcers (Muthukumarasamy *et al*., 2003). In Thai folk medicine, the bark of *O. intregerrima*, has been used as a digestive tonic and the root as an anthelmintic in Indonesia. An infusion of its roots and leaves is reputed for its antidysenteric and antipyretic properties (Perry, 1980). *O.afzelii* and *O. calodendron* have been used in traditional Cameroum medicine as a remedy for jaundice, toothache, female infertility, liver

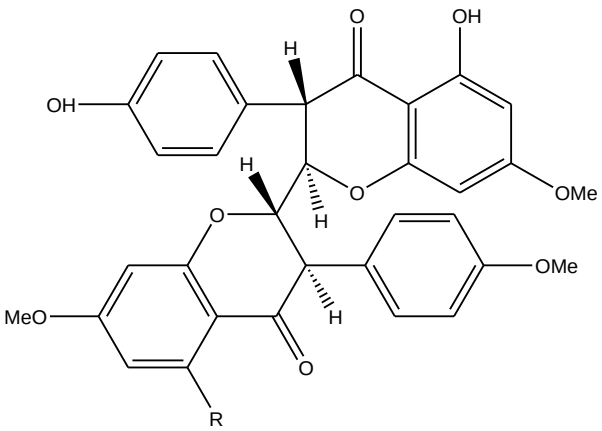
infections and dysentery (Bouquet, 1969), while in Tanzania, Washambaas used *O. schweinfurthiana* powdered bark as an antimalarial and antihelmintic. The decoction of the roots or leaves is used in wound dressing. In addition, it is used in Northern Nigeria for the treatment of measles, typhoid fever and fungal skin infections (Bouquet, 1969).

Crude extracts and isolated compounds from *Ochna* plants have been found to exhibit analgesic, anti-Hiv-1, anti- inflammatory, anti-malarial, anti-microbial and anti-toxic activities, lending support to the rationale behind several traditional uses of many members of the genus (Reddy *et al*., 2012).

2.6 Biological Activities of the Species in the Genus *Ochna*

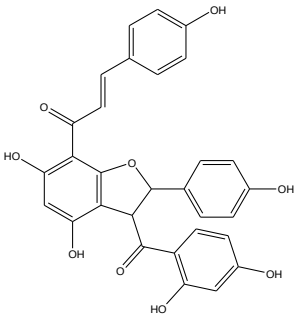
2.6.1 Antimicrobial activity.

Evaluation of the antibacterial activity of biflavonoid [Hexaspermone (XLVI), Dehydroxyhexaspermone C (XLVII), Calodenin B (XLVIII) Dihydrocalodenin B (XLIX) and Ochnone (L)] compounds, which were isolated from the ethanol extract of *O. macrocalyx* revealed antibacterial activities were made (Gibbons and Udo, 2000).

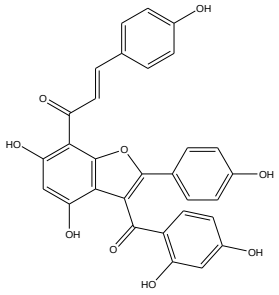


(XLVI) R = H

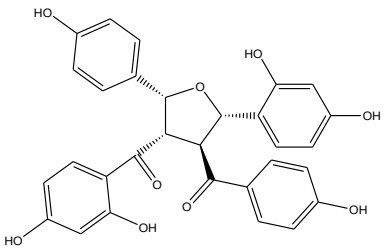
(XLVII) R = OH



(XLVIII)



(XLIX)



(L)

Among them, dihydrocalodenin B (XLIX) showed good activity against the three strains of multidrug-resistant (MDR) *Staphylococcus aureus (*RN4220, XU212, and SA-1199-B), with a minimum inhibitory concentration (MIC) value of 8µg/ml (15µm), while calodenin B was found to be active against the XU212 strain only. Compound XLV1, XLV11 and L were inactive at a concentration of 64µg/ml.These results indicated that the strong antibacterial activity of XLIX against MDR *S.aureus* may render it a good candidate for further investigation. The crude bark has traditionally been used in treatment of diarrhea; when tested against *E. coli*, which is known to cause gastrointestinal problems, especially diarrhea. The crude bark extract showed no activity at 512 µg / ml (Tang *et al.,* 2003).

More recently, the antimicrobial activities of crude acetone and methanol extracts of *O. schweinfurthiana* leaves were examined by Abdullahi *et al.,* 2010 using disc diffusion and nutrient broth – dilution techniques. Both extracts (600 µg/disc) showed

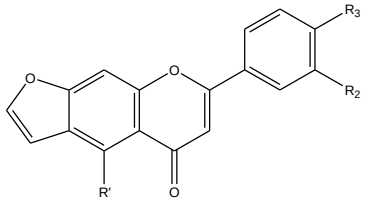
a broad spectrium of activities of susceptibility tests, with a mean zone of inhibition ranging from 15 to 21mm against *Klebsiella pneumonia* (ATCC 10031), *Pseudomonas aeruginosa* (NCTC6750), *Salmonella typhi* (ATCC 19430), and *Staphylococcus aureus* (ATCC 021001); however , no activity was observed against methicillin – resistant *S. aureus, Neisseria gonorrhea, Corynebacterium ulcerans, Bacillus subtilis* (NCTC 8236), *Escherichia Coli* (NCTC 10418), and *Candida albicans* (ATCC 10231). Sparfloxacin (100 µg/disc), a standard antibiotic, inhibited the growth of all of the organisms tested, with the exception of C. *albicans*. Except for *Streptococcus pyogenes*, the acetone extract showed higher activity against the tested organisms, as indicated by MIC and minimum bactericidal concentration (MBC) test results, with a mean zone of inhibition of 10- 20 and 20- 40ug/ml, respectively, which provides validation for the ethnomedicinal use of *O. schweinfurthiana* leaves in wound dressing and treatment of all forms of bacterial infections (Abdullahi *et al*., 2010).

2.6.2 Cytotoxic Activity

Compounds XLVI, XLVII, XLVIII, XLIX and L which were all isolated from the ethanol extract of *O. macrocalyx* were assayed for cytotoxic activity against MCF-7 breast cancer cells on 96 – well plates by the MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl-2H- tetrazolium bromide) reduction method, using doxorubicin as a reference compound (Tang *et al.*, 2003). Moderate to good cytotoxicities were observed for compounds XLVIII, XLIX and L, with IC_{50} values of 56 ± 7, 35 ± 7, and 7 ± 0.5µm. The crude extract exhibited cytotoxicity with an IC_{50} value of 52 ± 10µg/ml.

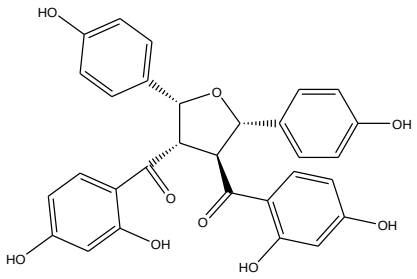
2.6.3 Analgesic and anti-Inflammatory activity.

The crude ethyl acetate fraction and the isolated constituents 3',4'- dihydroxyfurano [3'',2'':7,8] flavone (LI), 4'-Hydroxy-3'-methoxyfuranol[3'',2'':6,7] flavone , (LII) Lophirone L (LIII), isolated from the root bark of *O. squarrosa*, were examined for analgesic using tail-flick method in Swiss mice.



(LI) $R_1 = R_2, = R_3 = OH$

(LII) $R_1 = R_3 = H$, $R_2 = MeO$

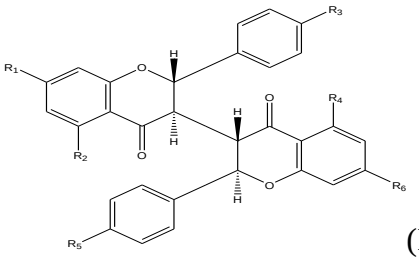


(LIII)

Anti- inflammatory carrageneenan-induced paw edema method in albino rats was also examined (Anuradha *et al*., 2006). According to the results, all the test compounds exhibited significant analgelsic activity at 25mg/kg, compared with control (1% Tween 80). Moreover, the crude extract and compound LII showed more promising protection at early reaction time and potency than at standard reaction time. The crude extract showed promising anti-inflammatory activity at 25mg/kg comparable to standard (diclofenac sodium; 2mg/kg), while compound LI, LII, and LIII were active at higher dose than standard. The potent analgesic and anti-inflammatory activities of the crude extract may be due to the synergistic effect of the mixture of compound occurring in natural proportion (Anuradha *et al*., 2006).

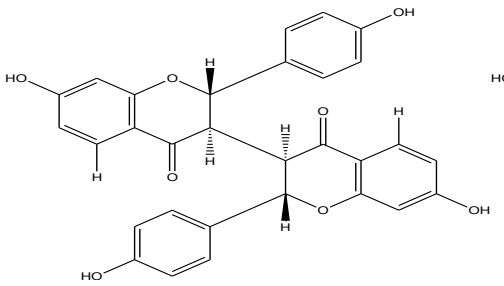
2.6.4 Antimalarial activity.

The 80% ethanol extract of the outer bark of *O. integerrima*, which preliminarily showed significant in vitro antimalarial activity against the MDR strain (KI) of *Plasmodium falciparum* and sensitive strain FCR-3 (IC50 of 6.5 and 4.5mg/kg, respectively) through bioassay – guided purification, furnished biflavones LIV (Bifavone I) and LV (Biflavone II) as new antimalarial principles (Ichina *et al.*, 2006).

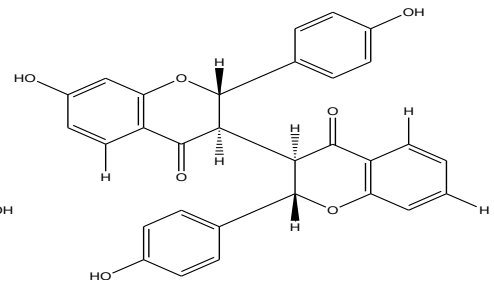


(LIV)

	R^1	R^2	R^3	R^4	R^5	R^6
(a)	Me	OH	Me	OH	Me	Me
(b)	H	OH	H	OH	H	H
(c)	H	H	H	H	H	H
(d)	Me	Me	OH	OH	Me	H



(LVa)



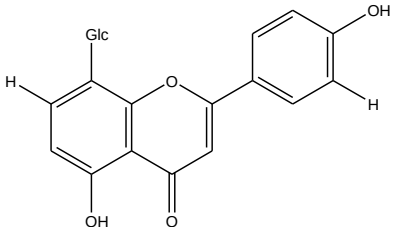
(LVb)

Test result indicated that the activity of the major active compound (LIV) ,(IC 50 80ng/ml) against the KI strain was three times higher than against FCR-3 strain, with a selectivity of 375, while its stereoisomer (LV) was 65 and 17 times weaker for both

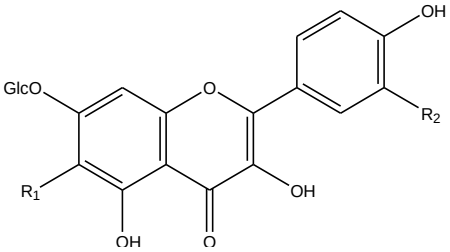
strains. It appears that LIV could be a promising compound to be used in investigations of antimalarial activity in vivo in animal models (Ichina *et al.,* 2006).

2.6.5 Anti-HIV-1 activity.

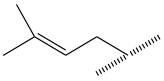
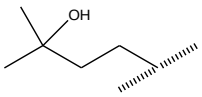
In 2007, Reutrakul *et al*, examined the anti-HIV -1 activity of crude Methanol extract , ethyl acetate fraction, and the pure isolated compounds LVI , LVII, LIX, LX, LXI, LXII, LXIII and LXIV, of the leaves and twigs of *O. interrima* (Reutrakul *et al*., 2007),

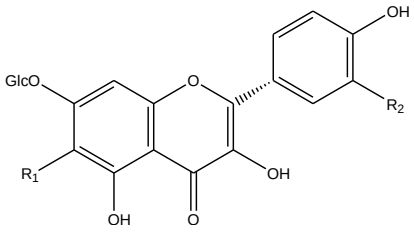


(LVI)

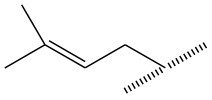
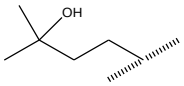
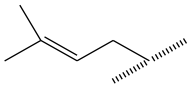


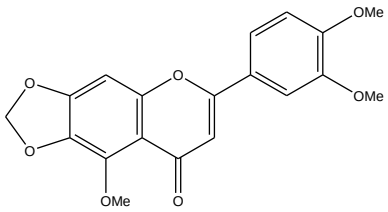
(LVII)

	R_1	R_2
(a) =		OH
(b) =		OH

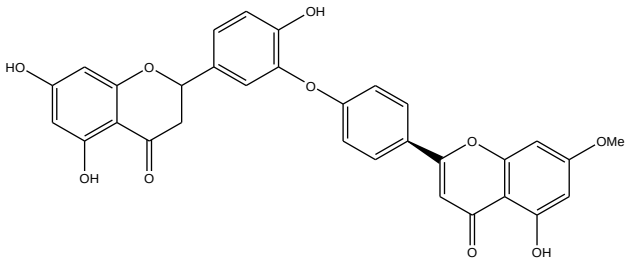


(LVIII)

	R1	R2
(a)	= 	H
(b)	= 	OH
(c)	= 	OH

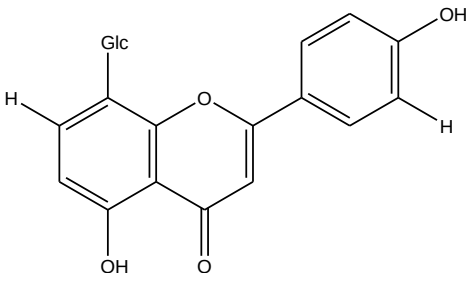


(LIX)



(LX)

Using syncytium (MC99 virus and the 1A2 cell line) (Tan *et al*., 1991) and reverse transcriptase (RT) assay methods (Nara *et al*., 1987). Flavonoids, LVII - LXI , with isoprenyl and sugar groups on ring 'A' , showed significant anti- HIV -1 activities in the syncytium assay, with EC_{50} values ranging from 14.0 to 102.4 µg/ml. biflavonoids 7'' – O - methylochnaflavone (XCIVf) and 2'',3''-dihydroochnaflavone -7''–O- methyl ether (XCVc) were found to be very active in the syncytium assay (EC_{50} 2.0 and 0.9µg/ml resp.) and demonstrated potent inhibition of HIV-1 RT (IC_{50} 2.0 and 2.4 µg/ml, resp.), comparable with a non – nucleoside RT inhibitor, nevirapine.



(LXI)

Vitexin (LXI), with only a sugar moiety on ring 'A', exhibited weak activity in the HIV -1 RT assay. The lack of anti –HIV-1 activity of (LXII) in both assays might be due to the absence of an isoprenyl moiety in its structure. Therefore, it appears that both isoprenyl and sugar moieties on ring 'A' were crucial for anti HIV – 1 activity (Reutrakul *et al*., 2007).

2.7 Phytochemistry of *Ochna Rhizomatosa* and Other *Ochna* Species

There is no phytochemical report on the specie *Ochna rhizomatosa,* but reported chemical constituents from other species in the genus *Ochna,* so far in total is 111 constituents, comprising of flavonoids including bi, tri and penta flavonoids, anthranoids, triterpenes, steroids, fatty acids and a few others compounds have been identified in the genus. The family (Ochnaceae) is characterized by the presence of flavonoids, biflavonoids and terpenoids as main secondary metabolites (Estevam *et al*., 2005).

Of these, biflavonoids are the predominant constituents within the genus *Ochna* (Agra *et al.,* 2007). There are few reports on chemical investigations of some species of *Ochnaceae* and many compounds were isolated, most of which were phenolic derivatives such as biflavonoids and chalcones (Murakami *et al.,* 1992, Ngombing *et al*., 2006), these compounds have been reported to have anti – tumor (Murakami *et al*., 1992), anti – inflammatory (Vianna *et al*., 2003), anti – malarial activities (Li *et al*., 1995).

2.7.1 Ochna biflavonoids.

Biflavonoids have mosty been isolated from gymnosperms. Some plants belonging to Guttifereae, Euphorbiaceae, Caprifoliaceae, Archegoniateae, Selaginellaceae, Anacardiaceae and Ochnaceae, have been found to contain biflavonoids (Harbone *et al*., 1975).

Structurally these are small polyphenolic molecules comprising two identical or non-identical flavonoids units joined in a symmetrical or unsymmetrical manner through an alkyl (C-C) or an alkoxy-based (C-O-C) linker of varying length. These possibilities allow significant structural variation in biflavonoids, which is further amplified by the positions of the functional groups, e.g., OH, MeO, C = O groups or C = C bonds and stereogenic centers on the flavonoids scaffold.

Ochnaflavone (LXXa) the taxonomic marker of the genus, and its mono-methyl ether (LXXb), were reported from the leaves of *O. squarrosa* by Kanawa and co- workers in 1973. Compounds (LXXa and LXXb), reported for the first time, represent a new series of biflavone ethers linked through two phenyl rings. Later, nine more Ochnaflavone derivatives were obtained from several *Ochna* species and are classified as follows:

a) Flavone-flavone, (LXXXIVa)

b) Flavanone –flavone LXXXVa, b and c)

c) Flavone – flavonone (LXXXVIa and LXXXVIb) and

d) Flavanone – flavonone (LXXXVIa and LXXXVIb) (Jayaprakasam *et al.*, 2000, Reddy *et al.*, 2008, Rao *et al.*, 1997, Likhitwitayawuid *et al.*, 2001, Jayakrishna and Reddy., 2003, Kamil *et al.*, 1982, Gunasekar *et al.*, 1998). Among them, 7- O-methylochnaflavone (LXXXIVf) and 2’’,3’’ –dihydroochnaflavone -7-O- methyl ether (LXXXVb), isolated from *O. integerrima*, have shown anti HIV-1 activity ,

while tetrahydroochnaflavone (LXXXVIIa) , a new biflavone reported by Gunasekar *et al*., 1998, from the leaves of *O. beddomei*, has been found to exert significant cytotoxicity against human nasopharrynx carcinoma (KB) cells (Gunasekar *et al.*, 1998). Tetrahydroamentoflavone and its 7'' –O – methyl ether, (LXIIId), obtained from the leaves of *O. pumila*, were the first C-C linked biflavones reported from the *Ochna* genus (Gunasekar *et al.*, 1998).

Among recently isolated biflavones with C(3) – C (3'') linkage were chamaejasmine (LIVb) from the root bark of *O. calodendron* (Messanga *et al.*, 2001), 7,4',7'',4''-tetramethylsochamaejasmine (LIVa) from *O. lanceolata* stem bark (Reddy *et al.*, 2008)., ent-ruixiaglangdusu B (LIVd), an antipode of ruixianglangdusu B from *O.lanceolata* leaves (Khalivulla *et al.*, 2008) and biflavanones I and II (LIVc and LVa) from the outer bark of *O. integerrima*. Compound LIVc and LVa known only as biotransformation products from chalcones with peroxidases of culture plant cell have been isolated for the first time from natural plant extracts. Both LIVc and LVa displayed significant antimalarial activities *in vitro* against *Plasmodium falciparum* strains, KI and FCR-3, respectively. Biisoflavanones, hexaspermone C and its furobenzopyran derivative, cordigol, obtained from the bark of a *O. macrocalyx*, posses antibacterial and cytotoxic activities (Tang *et al.*, 2003). 5''- hydroxylophirone B 7''-O-β-D-glucoside isolated from *O. squarrosa* (Anuradha *et al.*, 2006), belongs to the lophirone series, of which is the only biflavonoid O-glucoside known so far from the genus Ochna (Kaewamtawong *et al.*, 2002). In 2003, Pegnyemb *et al*., investigated the stem bark of *O. afzelii* and reported afzelones (XLVIII) (Pegnyemb *et al.*, 2003), XLVIII was isolated from *O. squarrosa* (Anuradha *et al.*, 2006), *O.calodendron* (Pegnyemb *et al.*, 2003), *O. afzeli* and *O. integerrima* (Messanga *et al.*, 1998). Among these, calodenin C, obtained from the stem of *O. calodendron*, is

the first reported proanthocyanidin dimer from the genus *Ochna* (Messanga *et al.*, 1998). Calodenins A (XLIX) possessing structures with either a benzofuran or a dihydrobenzofuran ring system, might have been derived biosynthetically from condensation of an isoliquiritigenin unit involving the α – C- atom.

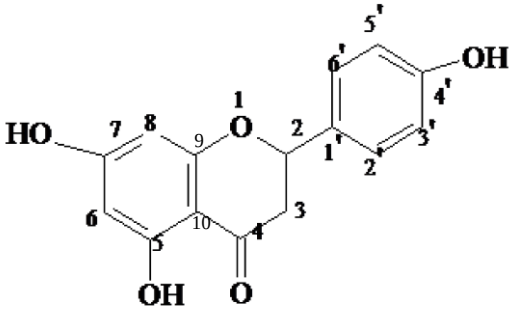
2.8 Biflavonoids

Biflavonoid is defined as dimers of apigenin and as such they were well distinguished from other flavonoid dimers like the proanthocyanidins (Harbone, 1975). However, a number of biflavonoids has been discovered afterwards which differ from the classical biflavones i.e. amento-, hinoki-, cupressu- and agathis-flavone not only in the hydroxylation pattern of the aromatic rings, but also in the oxidative level of the central heterocycle (Harbone, 1975). The biflavonoids are derived from two flavones or two flavonone of flavonone-flavone units and have been mostly isolated from gymnosperms. Among the angiosperms, some plants belonging to Guttiferae, Euphorbiaceae, Caprifoliaceae, Archegoniateae, Selaginellaceae, Anacardiaceae, and Ochnaceae, have been found to contain biflavonoids.

2.8.1 Nomenclature of biflavonoids.

In this nomenclature the generic term biflavonoids has been adopted in preference to biflavone or biflavonyl since in general the saturated system is regarded as the parent for the nomenclature. The ending oid, may then be modified to cover specific types of flavonoid dimers such as biflavonone, biflavan, e.t.c and for mixed systems flavanone–flavone. This system follows general IUPAC policy; the unmodified nomenclature is utilized as a generic term in the naming of dimeric, trimeric, tetrameric e.t.c derivative by insertion of the appropriate Greek prefix, bi-, ter-, quarter e.t.c giving biflavonoids, terflavonoid, quarter flavonoid e.t.c.

To identify specific ring position in flavonoids and their polymeric derivatives, the present long accepted system (exemplified) in (LXII) for naringenin is retained extending it in the case of polymericflavonoid by assigning to each monomer unit a Roman numeral I, II, III, e.t.c. Running in the sequence from one end of the molecules to another, the points of linkage between neighbouring flavonoid units are identified by a combination of a Roman numeral (to identify the flavonoid unit) and an Arabic numeral (to identify the position of interflavonoid linkage), the two numbers being coupled with a hyphen and enclosed within square brackets.



LXII

2.8.2 Classification of Biflavonoids.

All the Biflavonoids known to date can be classify into two main groups based on their linkages.

(a) C – C linked biflavonoids.

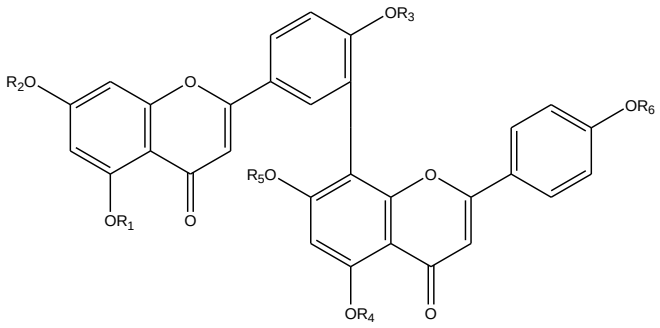
(b) C – O – C linked biflavonoids.

(a) C – C linked biflavonoids:

These have been further subdivided into the following series, based on the nature and position of the linkages of the constituent monomeric units. They include

(A) Amento flavones (I – 3', II – 8 – biflavone)

These are derived from two apigenin units with (I-3, II- 8) linkage, are represented by several members with amentoflavone (LXIIIa) as the parent compound.



(LXIII)

	R_1	R_2	R_3	R_4	R_5	R_6
(a) I-4, II-4, I-5, II-5, I-7, II-7 - hexahydroxy I-3,II-8-biflavone(Amentoflavone) (Pelter *et al.*, 1970)	H	H	H	H	H	H
(b) I-7-O- methyl(Sequiaflavone) (Miura and Kawano, 1968b)	H	Me	H	H	H	H
(c) I-4'-O-methyl (Bilobetin) (Baker *et al*., 1963)	H	H	Me	H	H	H
(d) II-7-O-methyl (Sotetsuflavone) (Chang *et al*., 1969)	H	H	H	H	Me	H

(e) II-4'-O-methyl (PodocarpusflavoneA)

(Chexal *et al*., 1970) H H H H H Me

(f) I-4', I-7-Di-O-methyl (Ginkgetin) (Pelter *et al*.,

1970) H Me Me H H H

(g) I-4',II-4'-Di-O-methyl (Isoginkgetin)(Baker

et al., 1963) H H Me H H Me

(h) I-7,II-4'-Di-O-methyl(PodocarpusflavoneB)

(Miura *et al.*, 1969. H Me H H H Me

(i) I-4', II-7-Di-O-methyl (Beckmann *et al*., 1971) H H Me H Me H

(j) I-7,II-7-Di-O-methyl (Rahman *et al.*, 1972) H Me H H Me H

(k) I-7,II-7,II-4'–Tri-O-methyl(Heveaflavone)

(Chandramouli *et al*., 1971) H Me H H Me Me

(l) I-4', II-4', I-7,-Tri-O-methyl (Sciadopitysin)

(Baker *et al*., 1963) H Me Me H H Me

(m) I-4',II-4'II-7-Tri-O-methyl (kayaflavone) (Baker

et al., 1963) H H Me H Me Me

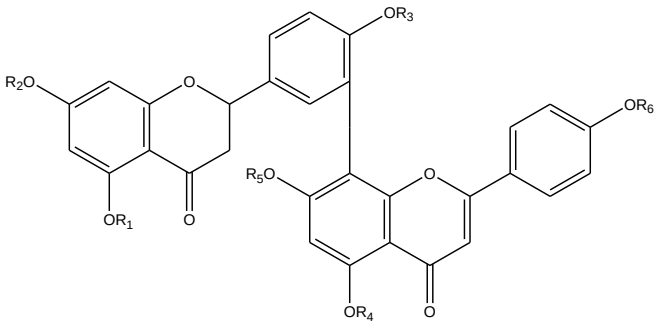
(n) I-4',I-7,II-7-Tri-O-methyl (Rahman *et al*., 1972) H Me Me H Me H

(o) I-4',II-4',I-7,II-7-tetra-O-methyl (Pelter *et al*.,

1969a) H Me Me H Me Me

(p) I-4', II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl

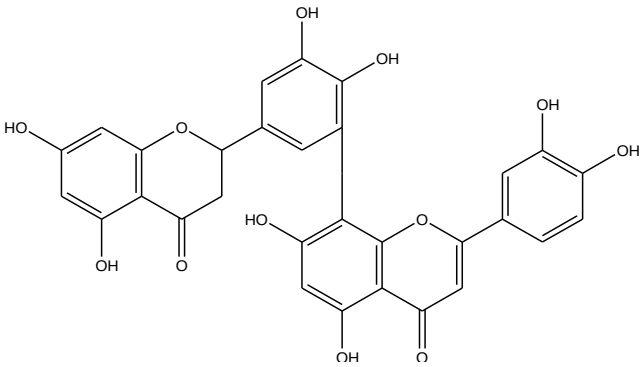
(Dioonflavone) (Dossaji *et al*., 1973) Me Me Me Me Me Me



(LXIV)

	R_1	R_2	R_3	R_4	R_5	R_6
(q) 2, 3-dihydroamentoflavone (Geiger and Degroot - pfeider, 1971)	H	H	H	H	H	H
(r) II-4', II-7-Di-O- methyl (Geiger and Degroot – pfeider, 1971)	H	H	H	H	Me	Me
(s) I-7, II-7-di-O-methyl (Sheng-Hong *et al*., 2003)	H	Me	H	H	Me	H
(t) I-4', II-4',I-7-Tri-O-methyl (2-3-dihydrosciatopitysin) (Beckmann *et al*., 1971)	H	Me	H	H	Me	Me
(u) I-4', II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl (Beckmann *et al*., 1971)	Me	Me	Me	Me	Me	Me

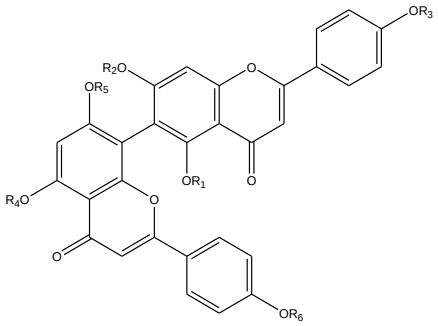
(v) Biluteolin (LXV) (Nilson, 1973).



(LXV)

B. Agathisflavone series

These are derived from two apigenin units with [1-6,II-8] linkage and are represented by seven members with agathisflavone (LXVIa) as parent compound. They include: -



(LXVI)

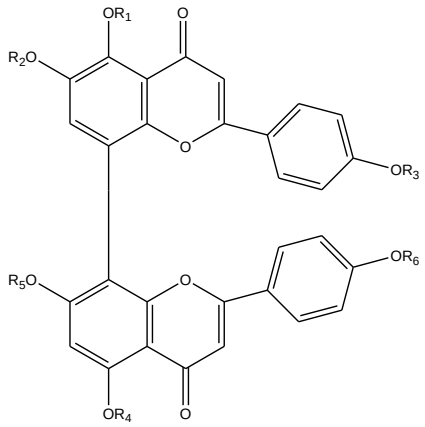
	R_1	R_2	R_3	R_4	R_5	R_6
(a) I-4', II-4', I-5, II-5, I-7, II-7 - hexahydroxy I-6 ,II-8-biflavone(agathisflavone) (Khan *et al*.,1972)	H	H	H	H	H	H
(b) I-7-O- methyl (Pelter *et al*., 1969c)	H	Me	H	H	H	H
(c) I-7,II-7-di-O-methyl (Khan *et al*., 1972)	H	Me	H	H	Me	H
(d) I-7,II-4'-Di-O-methyl (Pelter *et al*.,1969c)	H	Me	H	H	H	Me
(e) I-7, II-7,II-4'-Tri-O-methyl (Rahman *et al*.,1972)	Me	Me	H	H	H	Me
(f) I-4',II-4',I-7,II-7-Tetra-O-methyl (Handa *et al*.,1971a)	H	Me	Me	H	Me	Me
(g) I-4',II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl (Pelter *et al*., 1969c)	Me	Me	Me	Me	Me	Me

C. Cupressuflavone series.

These are derived from two apigenin units with [1-8,II-8] linkage and are represented by seven members where cupressuflavone (LXVIIa) is the parent compound while the others are its partial methylether.

This cupressuflavone has been isolated from *Araucaria cunnighamii* (Ilyas *et al.,* 1968) and *Araucaria cookiisome* examples of this group are Mesuferone-A and

Mesuferone –B derived from two naringenin units with [I-8, II-8] linkage and are optically active atropisomers (R_1= R_2 = R_3 = R_4 =R_5 = R_6 = H)

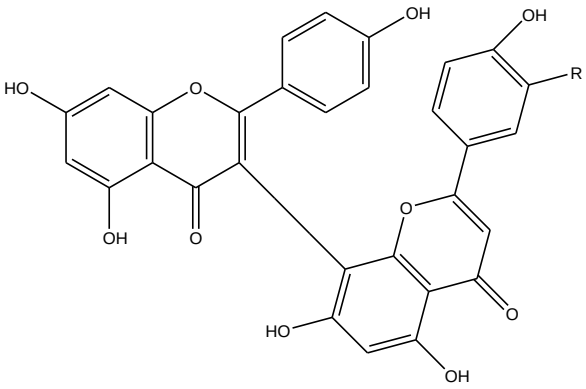


LXVII

	R_1	R_2	R_3	R_4	R_5	R_6
(a) I-4[1], 11-4[1], I-5, II-5, I-7, II-7 - hexahydroxyl I-8,II-8-biflavone (Ilyas *et al.*,1968)	H	H	H	H	H	H
(b) I-7-O-methyl (Khan *et al.*, 1972)	H	Me	H	H	H	H
(c) I-7,II-7-Di-O-methyl (Ilyas *et al.*,1968)	H	Me	H	H	Me	H
(d) I-4',I-7,II-7,II-4'-Tri-O-methyl (Khan *et al.*, 1972)	H	Me	Me	H	Me	H
(e) I-4',II-4',I-7,II-7-Tetra-O-methyl (Ilyas *et al.*, 1968)	H	Me	Me	H	Me	Me
(f) I-4',II-4',I-5,I-7,II-7-Penta-O-methyl(Ilyas *et al.*, 1968)	H	Me	Me	Me	Me	Me
(g) I-4',II-4',I-5,II-5,I-7,II-7-hexa-O-methyl (Ahmad and Razaq, 1971)	Me	Me	Me	Me	Me	Me

D. Garcinia biflavone (WGH) series. [I-3, II-8].

These are derived from two apingenin units with [I-3.II-8] linkage. In this group biflavonoidal glycoside have been found and are all optically active. They include:



LXVIII

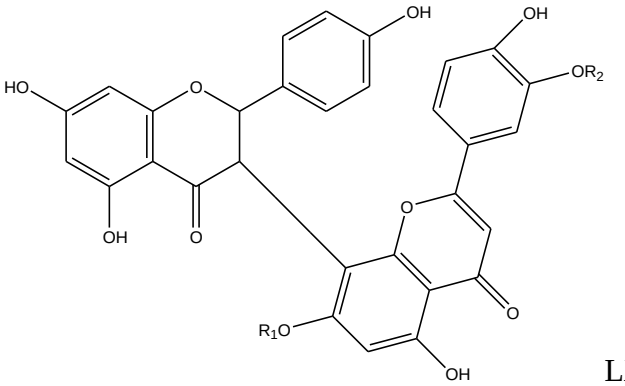
R

(a)I-4',II-4',I-5,II-5,I-7,II-7-Hexahydroxy(I-3,II-8)biflavone

(WGH III) (Jackson *et al*., 1971) H

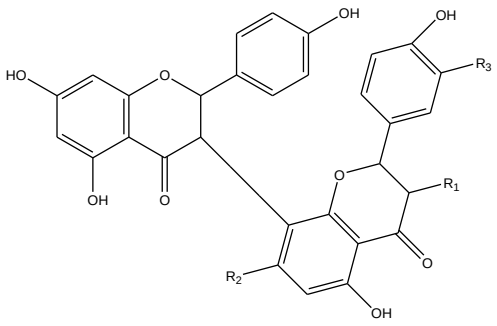
(b)I-3',II-3',I-5,II-5,I-7,II-7-Hexahydroxy[I-3,II-8]biflavone

' WGHII) (Pelter *et al*., 1971a) OH



LXIX

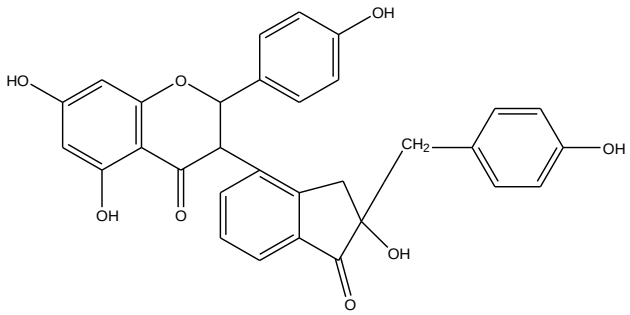
	R_1	R_2
(a) Volkensiflavone (Pelter *et al*., 1969a)	H	H
(d) Spicatiside (Konoshima and Ikeshiro, 1970)	β - D – glc	H
(e) Fukugetin (Pelter *et al*., 1969a)	H	OH
(f) I-3' – 0 – methyl fukugetin (Konoshima *et al*.,	H	OMe
(g) Fukugiside (Konoshima and Ikeshiro, 1970)	β - D - glc	OH



LXX

	R_1	R_2	R_3
(h) GB1 (Jackson *et al.,* 1971)	OH	H	H
(i) GB1a (Jackson *et al*., 1971)	H	H	H
(j) GB2 (Jackson *et al*., 1971)	OH	H	OH
(k) GB2a (Jackson *et al.,* 1971)	H	H	OH
(l) Xanthochymisside (Konoshima *et al*., 1970)	OH	β-D-glc	H

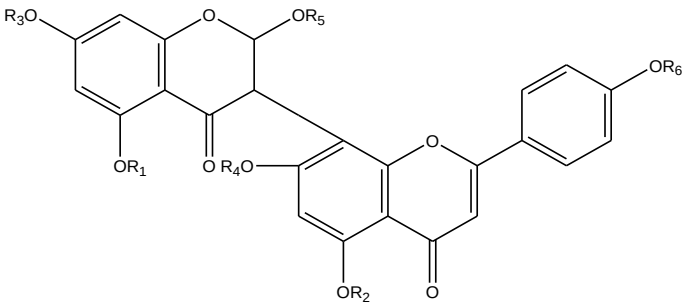
(m) Zeyherin (LXXI) (Dur-volsteed and Roux, 1971)



(LXXI)

E. BGH series

These are derived from naringenin and an apigenin or luteolin units with [I-3, II-8] (flavonone-flavone) linkage

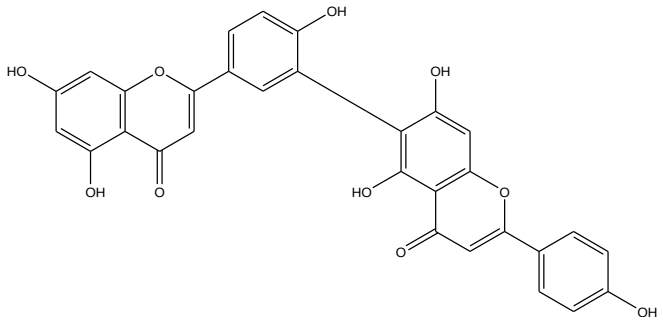


LXXII

	R_1	R_2	R_3	R_4	R_5	R_6
(a) I-3,II-8 (Morelloflavone/Fukugetin) (Pelter *et al.*, 1969a)	OH	H	H	H	H	H
(b) II-3'-O- methyl (Konoshima *et al*., 1969)	OMe	H	H	H	H	H
(c) II-3',I-4',II-4',I-5,I-7,II-7-Hexa-O-methyl (Konoshima *et al*., 1969)	OMe	Me	H	Me	Me	Me
(d) II-3',I-4', II-4',II-5,I-7,II-7-Hexa-O-methyl (Konoshima *et al*., 1969)	OMe	H	Me	Me	Me	Me
(e) II-3',I-4',II-4',I-7,II-7- Penta -O-methyl (Konoshima *et al*., 1969)	OMe	H	H	Me	Me	Me
(f) II-3',I-4',I-7,II-7- Tetra-O-methyl(Konoshima *et al*., 1969)	OMe	H	H	Me	H	Me
(g) BGH –III(Talbotaflavone/Volkensiflavone) (Pelter *et al*., 1969a)	H	H	H	H	H	H

F. Robustaflavone series (I-3', II-6) LXXIII

This class has been recognized and is represented only by robustaflavone (LXXIII) as the parent compound and its mono and dimethyl ethers, characterized only as their complete methyl ethers. These are derived from two apigenin units with [I-3', II –6] linkage (Chexal *et al*., 1970).



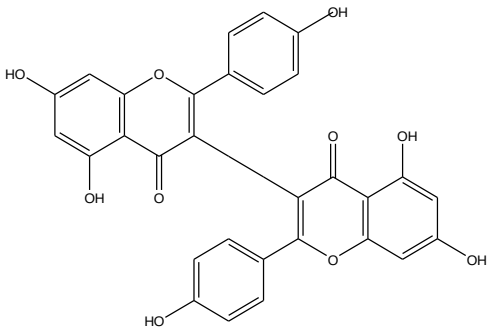
LXXIII

G. Miscellaneous biflavonoids

They include: - Biflavonoids that either occur naturally or are only synthesized.

(i) I-4’,II-4’,I-5,II-5,I-7,II-7-Hexahydroxy (I-3,II-3) biflavone

The series comprising of only one member has recently been synthesized by oxidative coupling of apigenin (LXXIV) (Molyneux *et al.,* 1970).

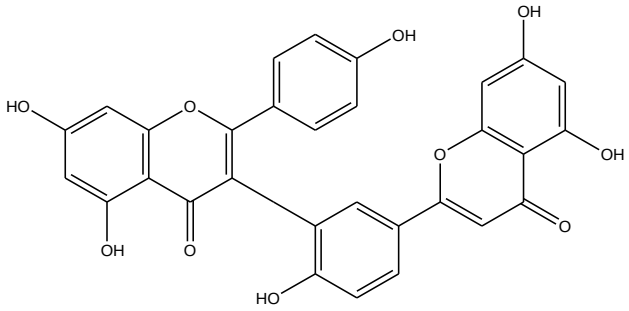


LXXIV

(ii) Taiwania flavone (I-3, II-3’) (Molyneux *et al*., 1970)

I-4’,II-4’,I-5,II-5,I-7,II-7-Hexahydroxy (I-3,II-3’) biflavone (LXXV)

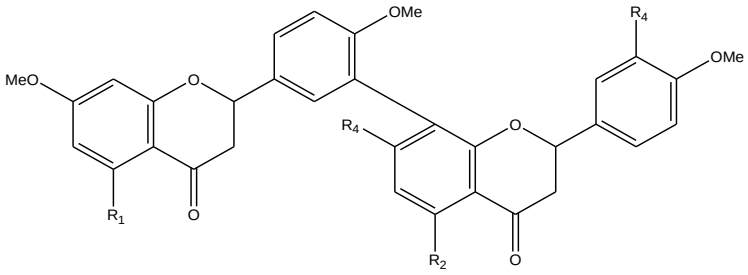
This series of naturally occurring biflavone was isolated from *Taiwania criptomeriadine* or the parent and its, mono and dimethyl apigenin unit with (I-3, II-3’) linkage (Molyneux *et al*., 1970).



LXXV

(iii) Sequioaflavone (I-3',II-8) flavonone –flavonone (Biflavone)

This class represented by only three members and characterized as their partial/complete methyl ether (LXXVI) (Rao *et al*., 1973).

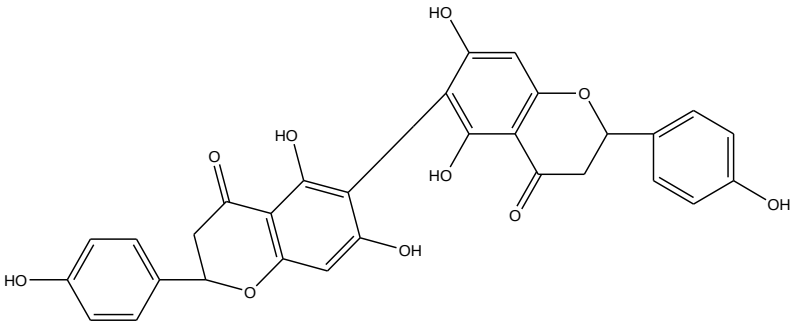


LXXVI

	R_1	R_2	R_3	R_4
(a) I-4',I-4',I-7-tri-O-methyl (Rao *et al*., 1973)	OH	OH	OH	H
(b) II-3',I-4',II-4',I-7-Tetra-O methyl (Rao *et al*., 1973)	OH	OH	OMe	H
(c) I-4',II-4',I-7,II-7-Tetra-O-methyl (Rao *et al*., 1973)	H	H	H	Me

(iv) Succedania flavone (LXXVII)

This is derived from two naringenin units with (I-6, II-6) linkage.



(LXXVII)

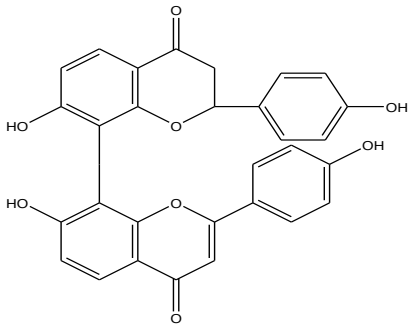
They include.

(a) 5,5'-deoxy cupressuflavone (LXXVIII) (Chandramouli *et al*., 1972)

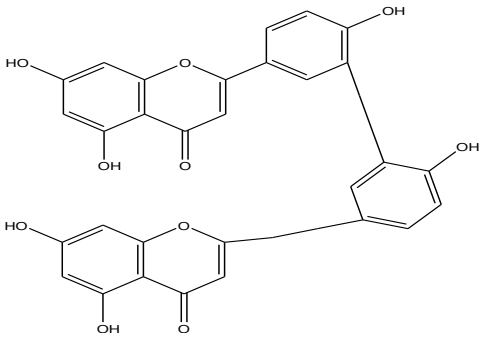
(b) 3',3''' –biapigenin (LXXIX) (Nakazawa, 1962)

(c) 3, 3''' – biapigenin (LXXX) (Molyneux *et al*., 1970)

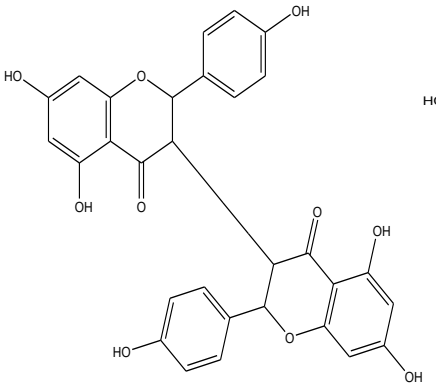
(d) 3, 3'' – biapigenin (LXXXI) (Molyneux *et al*., 1970)



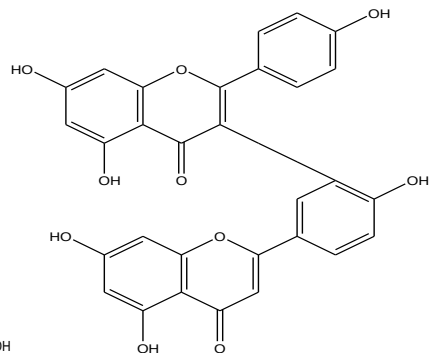
LXXVIII



LXXIX



LXXX



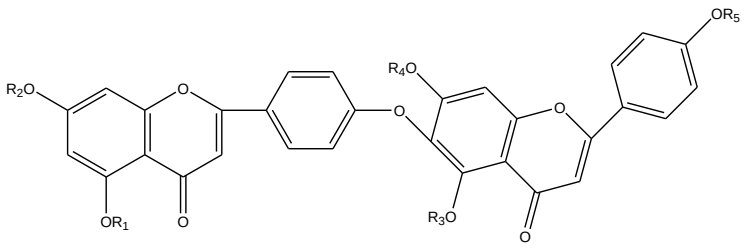
LXXXI

(b) C - O – C linked biflavonoids.

Here the interflavonyl links is not a C – C bond but a diaryl ether linkage.This group of biflavonoid includes: -

A. Hinokiflavone series

These are derived from two apigenin units with [I-4'-O-II-6] linkage; an example of Hinokiflavone has been isolated from *Metasequinia glyptotrobodies* and *Cycas* species. Hinokiflavone (LXXXIIa) is the parent compound of this series.

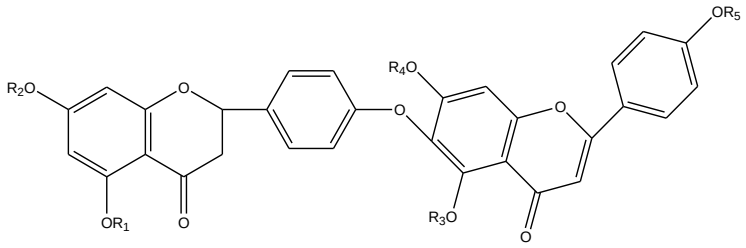


LXXXII

	R_1	R_2	R_3	R_4	R_5
(a) II-4', I-5, II-5, I-7, II-7 pentahydroxyI-4'-O-II-6-biflavone (Hinokiflavone) (Pelter *et al*., 1970).	H	H	H	H	H
(b) I-7– O- methyl (Neocryptomerin) (Miura and Kawano, 1968c)	H	Me	H	H	H
(c) II-7-O-methyl (Isocryptomerin) (Miura *et al.,* 1968)	H	H	H	Me	H
(d) II-4'-O-methyl (Cryptomerin-A)(Miura *et al*., 1968)	H	H	H	H	Me
(e) I-7,II-7-Di-O methyl(Chamaecyparin) (Miura and Kawano, 1968c)	H	Me	H	Me	H
(f) II-4',-II-7-Di-O-methyl (CryptomerinB) (Miura *et al.,* 1970).	H	H	H	Me	Me
(g) II-4',I-7,II-7-Tri-O-methyl (Miura and Kawano,1968a)	H	Me	H	Me	Me
(h) II-4',I-7-di-O-methyl (Miura and Kawano, 1968a)	H	Me	H	H	Me

(i) II-4',I-5,II-5,I-7,II-7-Tetra –O-Methyl (Nakazawa,

1968) Me Me Me Me Me



LXXXIII

R_1 R_2 R_3 R_4 R_5

(j) II-4',I-5,II-5,I-7,II-7-pentahydroxy 2,3-dihydroHinokiflavone

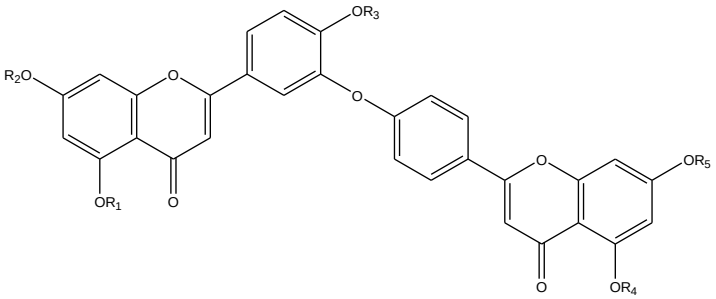
(Beckmann *et al*., 1971) H H H H H

(k) II-4',I-5,II-5,I-7,II-7-Penta – O- Methyl (Beckmann

et al., 1971) Me Me Me Me Me

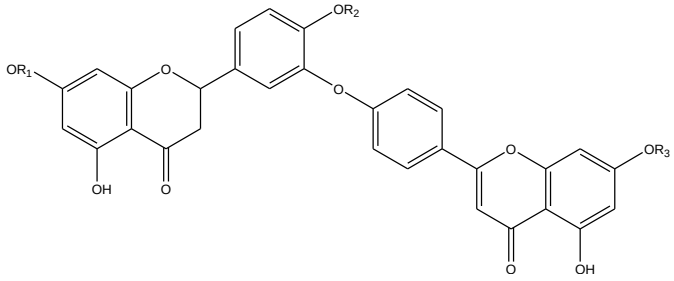
B. Ochnaflavone series

In this group ochnaflavone (LXXXIVa) is the parent compound with three others as its partial methyl ethers, they are derived from two apigenin units with (I-3'-O-II-4') linkage. Members of this group have been isolated from *Ochna squarrosal* (Ochnaceae).



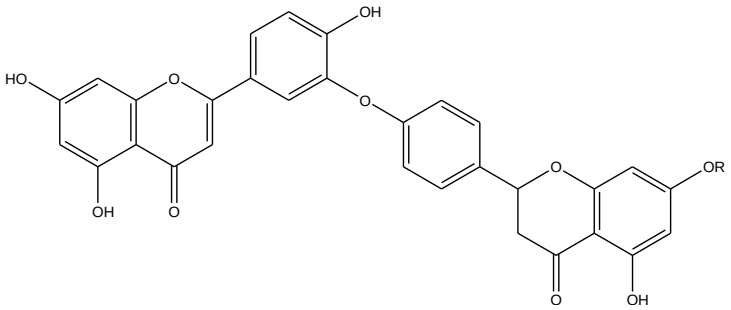
LXXXIV

	R_1	R_2	R_3	R_4	R_5
(a) I-4', I-5, II-5, I-7, II-7 - hexahydroxy I-3'-O-II-4 '- biflavone (Ochnaflavone) (Okigawa *et al*.,1973)	H	H	H	H	H
(b) I-4'–O- methyl (Okigawa *et al*.,1973)	H	H	Me	H	H
(c) I-4',I-7-Di-O-methyl (Okigawa *et al*., 1973)	H	Me	Me	H	H
or	H	H	Me	Me	H
(d) I-4',I-7,II-7-Tri-O- methyl (Okigawa *et al*., 1973)	H	Me	Me	H	Me
(e) I-4',I-5,II-5,I-7,II-7-penta-O-methyl (Okigawa *et al*., 1973)	Me	Me	Me	Me	Me
(f) 7'' - Methylochnaflavone (Reutrakul *et al.,* 2007)	H	H	H	H	Me



LXXXV

	R^1	R^2	R^3
(a) 2, 3-Dihydroochnaflavone (Jayaprakasam *et al.*, 2000)	H	H	H
(b) 2, 3-Dihydroochnaflavone -7"-O-methyl ether (Rao *et al.,* 1997)	Me	H	H
(c) 2, 3-Dihydroochnaflavone-7-O-methylether (Reddy *et al.*, 2008)	H	H	Me
(d) 2,3-Dihydroochnaflavone-7,4',7''-tri-o-methyl ether (Likhitwitayawuid *et al.,* 2001)	Me	Me	Me

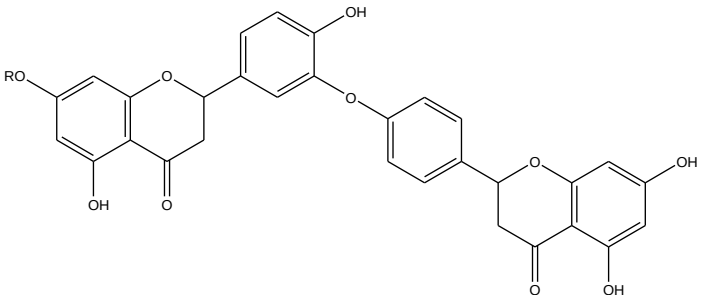


(LXXXVI)

R

(a) 2’’,3’’-Dihydroochnaflavone (Jayakrishna and Reddy, 2003) H

(b)2’’,3’’-Dihydroochnaflavone 7-O-methyl ether (Kamil *et al.*, 1983) Me



(LXXXVII)

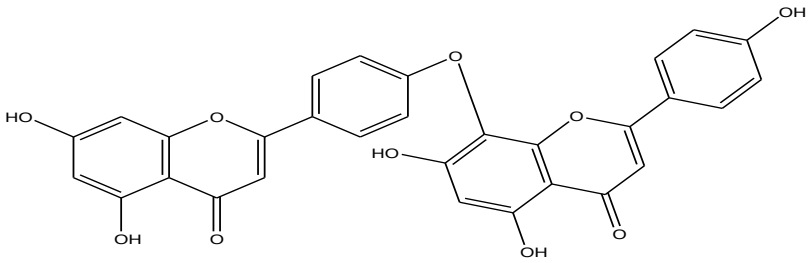
R

(a) Tetrahydroochnaflavone (Gunasekar *et al.*, 1998) H

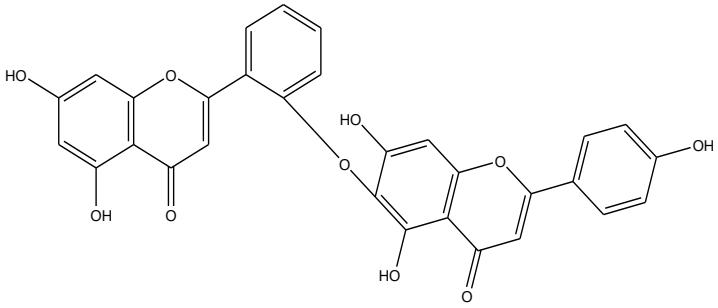
(b) 7-O-Methyltetrahydroochnaflavone (Gunasekar *et al.*,1998) Me

Other C-O-C linked miscellaneous include

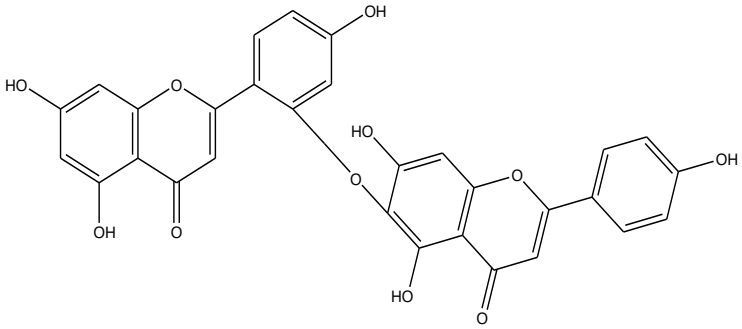
4”’, 5, 5”, 7, 7”- pentahydroxy – 4 – 8 - biflavonylether or I- 4’, I-5, II-5, I-7, II-7-pentahydroxyl (LXXXVIII); 3’’’desoxydicranolomin (LXXXIX); 2,3-dihydro-3’’’-desoxydicranolomin (XCI) (Rampendahl *et al*., 1996).



(LXXXVIII)



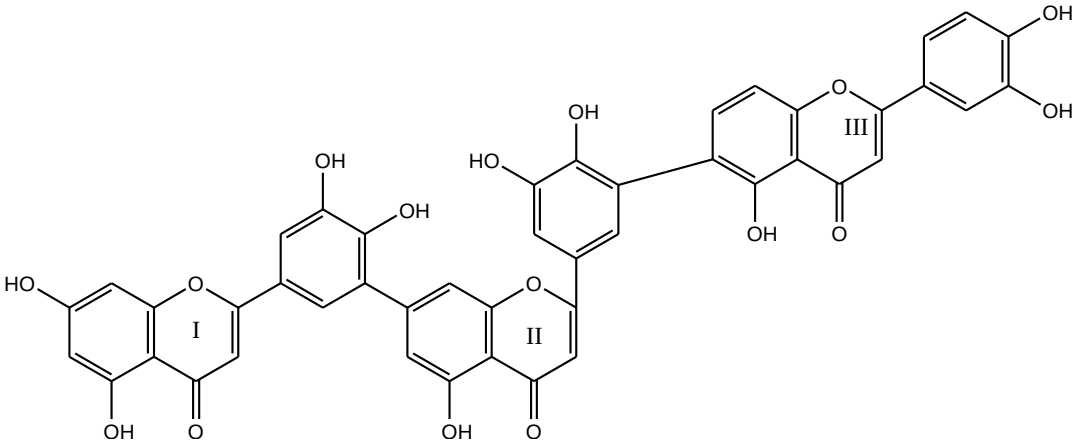
(LXXXIX)



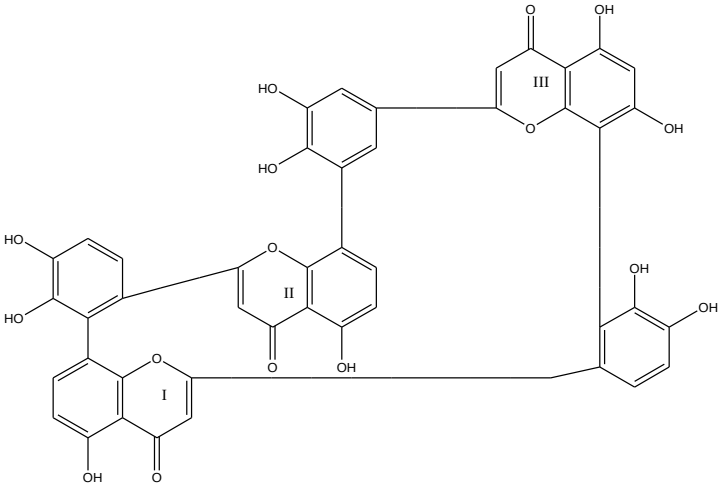
(XC)

2.9 Triflavonoids.

The following triflavonoids have been isolated, mostly from the moss family. Aulacomniumtriluteolin (XCI) was isolated from *Aulacomnium palustre* (Hahn, 1995). Cyclobartramiatriluteolin (XCII) was isolated from *Bartramia stricta* (Geiger, 1995).

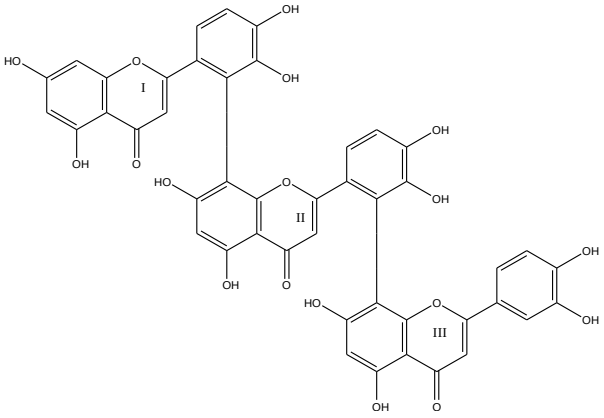


(XCI)

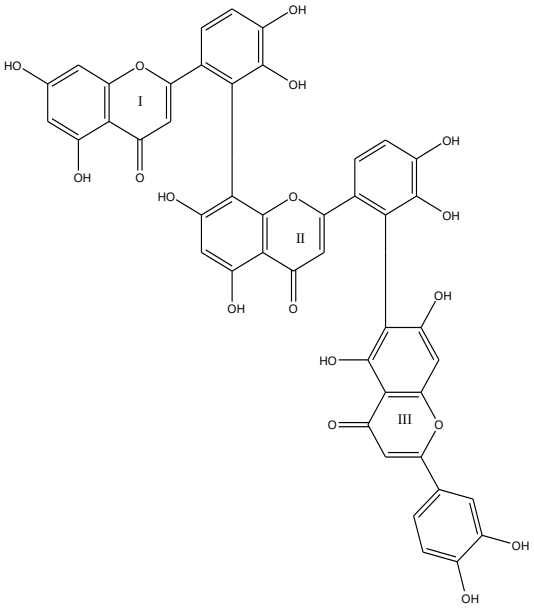


(XCII)

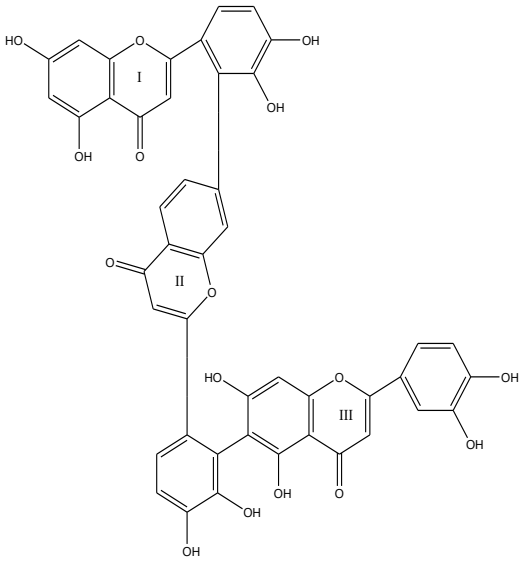
Epibartamiatriluteolin atropisomers (XCIII) and Strictatriluteolin atropisomers (XCIV) isolated from *Bartramia pomiformis* and *Bartramia stricta* all from moss family (Seeger, 1995). The triflavonoids Distichumtriluteolin (XCV) and Rhizogoniumtriluteolin (XCVI) were isolated from *Rhizogonium distichum* (Hakim, *et al.*, 2002).



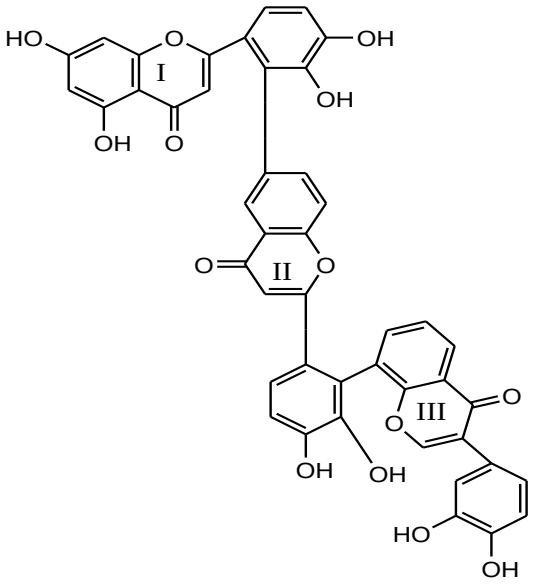
(XCIII)



(XCIV)

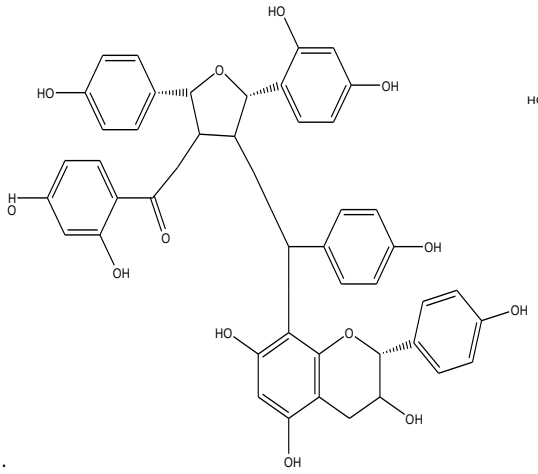


(XCV)



(XCVI)

Two new triflavonoid pigments, caloflavan A (XCVII) and caloflavan B (XCVIII) resp.), were isolated from the dichloromathane extract of the leaves of *O. calodendron.* From a biogenetic point of view, both XCVII and XCVIII may arise from condensation of isombamichalcone with afzelechin, either at C(6) or C(8) of ring A, respectively (Messanga *et al*., 2002).



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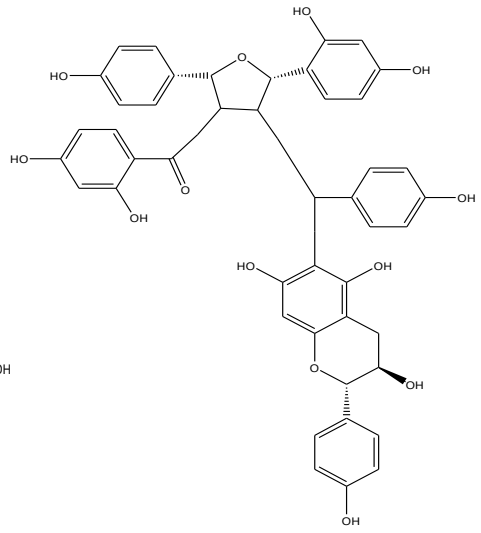
XCVII



XCVIII

2.10 Pharmacological properties of flavonoids.

Flavonoids that exhibited beneficial effects on abnormal capillary permeability and fragility were once known as vitamin P. Recent research has revealed that flavonoids act on blood cell aggregation a phenomenon that generally accompanies illness and injury. Rutin (XXXIV) and its derivatives possess coronary dilating action (Owada *et al.,* 1969). Other derivatives have been claimed to act as contraceptive drug (Galea *et al*., 1968), as antiphogistic, cholerectic, spasmolytic and antihistamine activity (Szirmai, 1965), as heart stimulant (Voigt, 1961), anti convulsant, analgesic and bronchodilator activity (Staufferr, 1968). Baicalein phosphate are useful for treatment of allergic diseases (Imai *et al*., 1970), Acacetin decrease formalin-induce inflammation and reduce capillary fragility in mice, while in rabbit it increase urinary excretion by 75% in the first 25-30min (Khadzhai *et al*., 1969) many of the glycoside have been isolated, purified and employed in the form of medicine.

2.10.1 Anti fungal and anti bacterial (anti microbial) activity of flavonoids.

Some flavonoids may help control growth in some plants. Other class of flavonoids can protect this plant from infection by viral, fungal and bacterial.

Flavonoids of many types have antiviral effects in animals. In human cell lines (Hela cells) Herpesvirushominis is inhibited by quercetin at levels of 300μg ml^{-1} (Pusztai *et al*., 1966). Most of the flavonoids investigated have an inhibitory activity toward one or more bacteria or fungi studied. Antihelmitic activities have also been reported for flavonoids (Laliberte *et al*., 1967).

2.10.2 Pharmacological properties of biflavonoids:

Cupressuflavone, agathisflavone, hinokiflavone and robustaflavone were found to inhabit cyclic nucleotide phosphodiesterases. Amentoflavone was found to be potent inhibitor of nucleotide phosphodiesterase. It was found to also show antifungal

activity. Amentoflavone was also found to inhibit the growth of Aspeigillusfumigatus, Botrytiscinerea and trichodermaglaucum (Harbone and Baxter, 1993).

Four new biflavonoids, robustaflavone - 4'- methyl ether, robustaflavone -7,4'-dimethyl ether, 2'', 3''- dihydrorobustaflavone 7, 4', - dimethyl ether, and 2'',3''-dihydrorobustaflavone 7,4', 7'' - trimethyl ether as well as two known biflavonoids, robustaflavone and amentoflavone, and three caffeoylquinic acids, were isolated from *Selaginella delicatula*. The cytotoxic activity of these compounds on various tumor cell lines was evaluated, and both compounds (robustaflavone 4'- methyl ether and 2'', 3''-dihydrorobustaflavone 7, 4', -dimethyl ether) significantly suppressed the growth of Calu-1 tumor cell lines (Lie-Chwen *et al.,* 2000). Several natural biflavonoids including ochnaflavone and ginkgetin inhibit phospholipase A2. Most importantly, certain biflavonoids exhibit anti-inflammatory activity through the regulation of proinflammatory gene expression in vitro and in vivo. Recently, several synthetic approaches yielded new biflavonoid molecules with anti-inflammatory potential. These molecules also exhibit phospholipase A2 and cyclooxygenase-2 inhibitory activity. Although the bioavailability needs be improved, certain biflavonoids may have potential as new anti-inflammatory agents. This is the first review of biflavonoid pharmacology to date (Kim *et al.,* 2008).

Ochnaflavone and ochnaflavone 7-O- methyl ether isolated from *Ochna pretoriensis* leaf extract were tested for antibacterial activity. Both biflavonoids which belong to the group ochnaflavones were previously characterized from Ochna obtusata. These compounds have anti-atherosclerotic, anti-inflammatory, and anti-tumor activity. They also inhibit lymphocytes proliferation, archidonic acid release and phospholipase activity. Moreover, ochnaflavone 7-O- methyl ether was reported to inhibit HIV-1 activity as well as HIV-1 reverse transcriptase activity. The

antibacterial activity, and potential cytotoxic, genotoxic and antigenotoxic effects of the isolated compounds were determined. The MIC values ranged from 31.3 to 250 μg/ml for ochnaflavone against *P.aeruginosa* and *E. faecalis* and ochnaflavone 7-O-methyl ether against *P. aeruginosa* with an MIC of 31.3 μg/ml. The isolated compounds were much less active than the positive control gentamycin. The compounds had low cytotoxic activity, with LC50 values of 125.9 μg/ml for ochnaflavone and 125.9 μg/ml for ochnaflavone 7-O- methyl ether against vero cells (Makhafola, 2009).

Two new biflavonoid derivatives (4, 4', 7-tri-*O*-methylisocampylospermone A and 4"'- de –*O* – methylafzelone A) were isolated. These compounds were isolated along with the known compounds ochnaflavone, 2", 3"-dihydroochnaflavone. The growth inhibitory effect of these compounds was evaluated against three cancer cell line panel of TK 10 (renal), UACC62 (melanoma) and MCF7 (breast) using a sulforhodamine B (SRB) assay. Ochnaflavone inhibited the growth of melanoma cancer cells at 12.91 μM. Lophirone A, a rearranged biflavonoid, showed TGI against these cancer cells at 58.96 μM. The new biflavonoid, 4,4',7-tri-*O*-methylisocampylospermone A demonstrated the highest antimalarial activity against chloroquine-resistant strains of *Plasmodium falciparum* (FCR-3) with IC50 of 11.46 μM, followed by ochnaflavone (17.25 μM). The compound, 2",3"-dihydroochnaflavone exhibited the lowest antimalarial activity (61.86 μM) (Ndoile, 2012).

2.10.3 Extraction of biflavonoids.

The solvent used for the extraction of biflavonoids depends largely upon the nature of the biflavonoids and the nature of plant materials, since all biflavonoids are more or less strongly absorbed in the tissue in which they occur. Saturated aliphatic

hydrocarbons are the only solvents in which biflavonoids are insoluble and so they can be used for preliminary de-fatting. Ether extraction in most cases yields all the biflavonoid present, although the unmethylated types are sometimes extracted rather slowly. In this case acetone, ethyl methyl ketone, methanol or ethanol may be used. Preliminary purification is done by distribution between two immiscible solvents eg. Water-soluble material can be removed by distribution between ethyl methyl ketone and water, whereas lipids may be removed by distribution between dimethyl formamide and light petroleum. Column and preparative thin layer chromatography may be used not only for resolving biflavone mixtures but also for the elimination of by-products. The result of all operations has to be followed by TLC (Jackson *et al.*, 1970).

2.10.4 Methods of Isolation of biflavonoid compounds

2.10.4.1 Thin layer chromatography

Thin layer chromatography almost completely replaces paper chromatography for the separation of biflavonone, with silica gel cellulose and polyamides as supports. In most TLC system separation of biflavonoids into groups having the same degree of methylation normally offer no problems.The system cellulose and n-butanol/ammonia is especially effective for the separation of isomeric methyl ethers since it responds to the difference in acidity of the remaining free hydroxyl group (Bakers *et al*., 1963).

Preparative TLC on silica gel layer is an excellent means for the isolation and purification of weighable amounts of biflavonoids; especially the more highly methylated ones. Visualization of biflavone is effected in the same way as for monoflavonoids. Biflavones with free hydroxy group in the 5-position appear in U.V. light as dark spots quenching any fluorescence of the layer, and after spraying with ferric chloride etc. with which they form chelate, they fluorescence-yellow, green or

brown. These colours are sufficiently different for it to be possible to distinguish between various biflavonoids, if they are on the same chromatogram, but if they are not, different enough to be described unequivocally. Biflavone permethylether fluorescence brightly in U.V. light without any spray but the colours are describe quite subjectively (Beckmann *et al*., 1971) Determination of the fluorescence maxima would be very useful. Biflavonoids containing at least one flavone moiety may be visualized as orange or red spots (Horowitz, 1957) by spraying with a solution of sodiumborohydride in isopropanol and subsequent exposure of the plate to hydrochloric acid fumes. This reaction may faint when analytical grade isopropanpol is used, in this case, a small drop of acetone must be added to the spray solution.

2.10.4.2 Making derivatives of biflavonoids:

Acetates, methyl ethers and deuteromethyl ethers are the derivatives usually prepared during identification and structural elucidation of biflavonoids. With biflavones these derivatives are prepared by standard methods. Deuteromethyl ethers are prepared in the same way as the methyl ethers, using the deuters analogue of the methylating agent (Beckmann *et al*., 1971).

2.10.4.3 With known compounds:

If an authentic sample is at hand, identification may be achieved by any of the usual methods like co-chromatography, mixed melting points of the substance itself and its derivatives, UV, IR, NMR and mass spectrometry.

2.10.4.4 Counter current distribution.

This is an excellent means for the separation of very similar compounds. Since most of commercial machines allow recycling, several thousand transfers are normally possible. In the biflavone field, two phases –system have been described.

The system ethyl methyl ketone/borate buffer was introduced by Baker *et al.*, (1963) while the phase pair aqueous dimethyl formamide and ether were used by Miura *et al*., (1968), Khan *et al.,* (1972) and Beckmann *et al*.,(1971).

In Beckamann's system, the biflavonoids are separated into groups containing about the same number of methoxyl groups where as Baker's system also separates isomeric pairs that differ in the acidity of the free hydroxy group e.g. ginkgetin and isoginkgetin.

2.11 Methods of Determination of Biflavonoid Structure

In structural determination of the compounds present in plants both colour reaction, chemical and physical methods can be employed.

The chemical methods include the phytochemical screening using several analytical reagents, usually employed in testing for flavonoids; also degradation and synthesis while the physical methods involve mass spectroscopy, U.V., I.R., and N.M.R. etc.

2.11.1 Ultra violet spectroscopy

U.V. spectra, which are very useful in the monoflavone series are of limited value with biflavonoids because in this case there are two independent chromophores, which responds independently to various shift reagents. Only where both moieties are alike, can conclusion be drawn. Also from the fact that R_f values and U.V. spectra are in many cases very similar, this proves the difficulty encounter in isolation and identification of biflavonoids. e.g. flavones absorbs between 304 –350 nm while biflavones absorb in the range 322-385nm, amentoflavone, a biflavone give a band at λmax 335nm in methanol.The only structural feature of biflavonoids for which chemical degradation cannot supply the answer to the question whether in a given case C- 6 or C- 8 is involve in the interflavonyl link, these can only be done by synthesis or countercurrent.

2.11.2 Infra red spectroscopy

I.R. is use mainly in the detection of the presence, type and number of functional groups, which have characteristic bands of absorption in the I.R. spectra. e.g I-5 hydroxy biflavonoid show band at 1660cm^{-1} as do the corresponding mono –5 – hydroxyflavonoids.

2.9.8.3 Nuclear magnetic resonance spectroscopy

2.9.8.3.1 Proton Magnetic Resonance

The P.M.R. technique uses for this purpose involve the benzene induced methoxy shift using a proton magnetic resonance. The application of these data to biflavonoids structure analysis has resulted in the ready distinction of the I-6, II-8 – linked agathisflavone series from I-8, II-8 – linked cupressuflavone series and the

I-3',II-8–linked amentoflavone. From this method cupressuflavone and amentoflavone being of the same molecular weight could not be distinguished.

2.11.3 HSQC ^{13}C - ^{1}H correlation.

Heteronuclear single quantum correlation (HSQC) is used for the determination of substitution patterns at the different carbon atoms, correlation of carbon shifts, multiplicity and proton shifts can be compiled from a two dimensional heteronuclear correlation spectrum. The combined spectral information allowed a view of carbon atoms showing different functionalities and substitutions.

2.11.4 COSY (^{1}H - ^{1}H 2D).

Two dimensional spectra are obtained by recording a series of conventional NMR in which two parameters are changed incrementally. The most readily established connections between the individual carbon atoms are derived from connectivities through couplings between the protons as compiled from a COSY spectrum. From

these correlations, the main parts of the proton spin systems are outlined and several structural fragments are identified.

2.11.5 HMBC long range $^{1}H - ^{13}C$ correlation.

Heteronuclear multiple bond correlation (HBMC) experiment provided information on the direct ^{13}C and ^{1}H heteronuclear connectivity. The method relies on the indirect detection of the ^{13}C by observing their effects on the more sensitive proton nuclei to which they are coupled. It not only shows the connection of unprotonated carbon atom to the proposal elements, but also indicates vicinal proton relationships not resolved in the COSY spectrum.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials / Reagents / Equipments and Analytical procedure

3.1.1 Apparatus and equipment.

Test tube
Conical flask
Mortar and pestle
Weighing balance
Round bottom flask
Boiling tube
Extraction apparatus
Distillation apparatus
Measuring cylinder
Water bath/tank
Retort stand and clamp
Capillary tube
Filter paper and funnel
Spatula
Litmus paper
Crucible
Syringe and needle (1ml, 2ml, 5ml)
Pipette Microscopic slides
Slide box
Petri dish
Watch glass
Glass rod
Glass plates
Iodine Tank
Chromatographic tank, Ultraviolet (U.V. visible) spectroscopy Unicam S.P 1700
U. V. Light (254nm)
Gallenkemp (Melting point apparatus)

Distillation Apparatus

I . R. Spectrometer

^{1}H NMR and ^{13}C NMR Spectroscometer.

Light microscope.

3.1.2 Reagents

Methyl alcohol, Potassium chlorate, Chloroform, Ferric chloride, Potassium dichlorate, Magnessuim chips ,Toluene , Ethanol , Zinc chips, Sodium chloride, calcium chloride**,** Silica gel, Magnesium chloride, Formic acid, AnilinePhthalic acid, Pyridine, Benzene, Acetone, Acetic acid, distilled water , Sodium hydrogen phosphate di-hydrate ($NaH_2PO_42H_2O$), Sodium hydrogen carbonate ($NaHCO_3$), Concentrated Hydrogen Chloride (conc. HCl), Concentrated sulphuric acid (conc. H_2SO_4) , Magnesium sulphate hexa hydrate ($MgSO_4$. $7H_2O$), NaOH, KOH, phenylamine, Lead acetate , KBr, KCl, Mayer's reagent, n-butanol, Dragendoff's reagent, Fehlings solution A & B, Wagner's reagent, Glacial acetic acid, Barfoed's reagent, Ammonia solution. Normal saline distilled and de-ionized water, Giemsa stain. Chloroquine powder was obtained from May and Baker.

3.1.3 Spray and test reagent for phytochemical screening

3.1.3.1 The composition of the test reagent and their methods.

The T.L.C plates after spraying with 10% H_2SO_4 were heated to $110^{o}C$ for about 15mins.

(a) Anisaldehyde reagent:

Anisaldehyde (2g) acetic anhydride 50ml was mixed with sulpuric acid 50%, 50ml (Lisboa, 1984).

(b) Cerrium ammonium sulphate reagent:

Cerium ammonium sulphate 2g water 90ml were mixed with conc. H_2SO_4 10ml (Jacouljevic *et al.*, 1964).

(c) Liebermann – Burchard reagent:

Acetic anhydride 20ml, conc. H_2SO_4 10ml and chloroform 50ml was mixed together (Tschesche, 1962).

(d) Ferric chloride reagent:

Ferric chloride 9g was dissolve in 100ml of water

(e) Molybdophosphoric acid reagent:

Molybdophosphoric acid 20g was dissolved in 10% 100ml ethanol (Kritchersky and kirk, 1952).

(f) Methanolic potassium hydroxide:

Potassium hydroxide 10g was dissolved in 100ml methanol (Fink and Fink, 1949).

(g) Dragendoffs reagent:

Bismuth nitrate 8g was dissolved in nitric acid 20 ml, potassium iodide 27.2g all was dissolved in 50 ml water separately. The two solutions were mixed and allowed to stand. When potassium nitrate crystallized out, the supernatant was decanted and the solution was made up with water to 100ml (Bregoff *et al.*, 1953).

(h) Mayer's reagent:

Mercuric chloride, 1.36g was dissolved in 60ml water. To this was added a solution of potassium iodide 5g in water 100ml. The final solution was diluted with water to 100ml (Bregoff *et a*l., 1953).

(i) Wagner's reagent:

Iodine 1.27g and potassium iodide 2g were dissolved in 5ml water. The solution was diluted with water to 100ml (Bregoff *et al*., 1953) .

3.2 Collection of Plant Material:

3.2.1 Collection, identification and preparation of plant materials.

The leaf part of *Ochna rhizomatosa* (van Tiegh.) Keay (Ochnaceae) was colllected from Karaukarau village, Zaria in the month of June, 2012. The plant was identified and authenticated by Mallam Umar Gallah of Herbarium Section of Biological Science Department, Ahmadu Bello University, Zaria, Nigeria, as *Ochna rhizomatosa*, after comparism reference specimen with a voucher number of 2760. The fresh plant leaves were air-dried and made into powder using pestle and mortar and subsequently referred to as powdered plant leaf.

3.2.2 Extraction

The powdered plant leaf (500g) of *Ochna rhizomatosa w*as extracted with acetone to exhaustion using cold maceration to obtain acetone extract. The solvent was removed *in-vacuo* to afford dark greenish gummy mass marked as 'X' (Ohta and Yagishita, 1970).

The extract 'X' (51.15g) was treated with hot water. The water soluble portion was partitioned with ethyl acetate, n-Butanol and in each case dried to afford ethyl acetate (A) and n-Butanol (B) soluble portions. (Ya-ching *et al.*, 2004; Shengmin *et al.*, 2003). Residual aqueous layer was discarded.

The water insoluble portion was successively washed with Petroleoum Ether (60 – 80 ^{0}C), chloroform and methanol Coded 'C', 'D' and 'E' respectively.

The fractions A, B, C, D and E were tested for flavonoid (Table 4.4). The fractions were examined using TLC (Plate 4.1). Fraction E on examination revealed three promising spots and was subjected to column chromatography.

3.3 Preminary Phytochemical Screening.

The following constituents were tested for, with small portion of the different fractions of the acetone extract, coded as ethyl acetate (A), n-butanol (B), petroleum ether (C), chloroform (D), and methanol (E).

To test for flavones and phenolic group, a small quantity of the sample was put in a test tube and a few drop of ethanol was added to it to make it alcoholic.

3.3.1. Test for flavonoids

(a) Shinoda's test.

Pieces of magnesium chips were added to alcoholic solution of the extract followed by two drops of conc. hydrochloric acid. Effervescence was observed with the dark brown solution changing to a deep red solution. This confirmed the presence of flavone in the plant (Trease and Evans, 2002).

(b) Ferric chloride test.

About 5ml of distilled water was added to the extract and boiled on water bath for about 2 minute and then filtered. To 2ml of the filtrate, few drops of 10% alcoholic ferric chloride solution were added. Effervescence occurred and the dark brown solution change to green, blue to violet colouration indicate the presence of phenolic group (hydroxyl group) (Trease, and Evans, 2002).

(c) Lead acetate test.

A small quantity of the extract was dissolved in water and filtered. Few drop of 10 % lead acetate was added to 5mls of the filtrate. A buff coloured ppt. Indicate the presence of flavonoids (Brain and Turner, 1975).

(d) Sodium hydroride test.

Filtered extract (2ml) was dissolved in 10% NaOH of solution to give a yellow colour. A change in colour from yellow to colour-less on addition of dilute hydrochloric acid, indicate the presence of flavonoids (Trease and Evans, 2002)

3.3.2 Test for carbohydrate

(a) Molisch's test (general test for carbohydrate):

A few drops of molisch's reagent was added to the extract dissolved in distilled water.

This was followed by addition of 1ml of conc H_2SO_4 down the side of the test tube, so that the acid formed a layer beneath the aqueous layer. Mixture was allowed to stand for 2min and then diluted with 5ml of water. Formation of a red to dull violet colour at the interface of the two layers is taken as positive (Trease and Evans, 2002).

(b) Barfoed's test for sugar.

One millilitre of aqueous filtrate of the extract was mixed with 1ml of Barfoed's reagent in a test tube. The test tube was then heated on the water bath for few minutes. A red ppt of cuprous – oxide indicated the presence of a monosacharide sugar (Brain and Turner, 1975).

(c) Test for reducing sugar:

(Fehling's test) small amount of the powdered leaves was dissolved in distilled water and allowed to extract for some time. The mixture was then filtered and filtrate was heated with 5ml of equal volume of Fehling's solutions A and B, for few minutes (5mins). Formation of a red ppt of cuprous oxide indicates the presence of free reducing sugar (Trease and Evans, 2002).

(d) Test for combined reducing sugar.

The plant extract was hydrolyzed by boiling with 5ml dilute hydrochloric acid and the resulting solution neutralized with sodium hydroxide solution. Few drops of Fehling's A and B solution was added to it and heated on a water bath for 2 minutes. Formations of reddish brown ppt of cuprous oxide indicate the presence of combined reducing sugar (Trease and Evans, 2002).

3.3.3 Tests for cardiac glycosides

(a) Test for steroidal / terpenoidal nucleus.

(i) Lieberman – Burchard test:

Small sample of the extract was dissolved in chloroform and filtered. The filtrate was mixed with 2ml of acetic acid and cooled in ice. Few drops of conc. Sulphuric acid were then carefully added down the side of the test tube. A colour change from violet to blue or bluish green indicates the presence of steroidal nucleus (Stahl, 1969).

(ii) Salkowskii test:

Small amount of the extract was dissolved in 2ml chloroform followed by the addition of conc. H_2SO_4 to form a lower layer. A reddish brown colour at the interphase indicate the presence of a steroidal or triterpenoidal nucleus (Sofowora, 1982).

(b) Test for cardenolide.

(i) Small quantity of the extract was dissolved in pyridine and a few drops of sodium nitroprusside together with a few drops of 20% sodium hydroxide solution were added. A deep red colour, which fades to brownish yellow, indicates the presence of cardenolides (Sofowora, 1982).

(ii) Keller – kiliani test

To test for digitalis glycoside small quantity of the extract was dissolved in 2ml of 3.5% ferric chloride in glacial acetic acid. This was then transferred to the surface of the 2ml conc. H_2SO_4. A reddish – brown ring obtained at the interphase of the liquid indicate the presence of a digitoxose sugar component (deoxysugar) characteristics of cardenolide (Trease and Evans, 2002).

3.3.4 Test for saponins

To test for saponins,1ml of the extract was shaken with distilled water in a test tube, frothing which persist on warming indicates the presence of saponins (Sofowora, 1982).

3.3.5 Test for tannins

Small quantity of the extract was mixed with distilled water and heated on a water bath. The mixture was filtered while conc. H_2SO_4 and 5% ferric chloride were added to the filtrate. A blue-black, green or blue-green ppt indicates the presence of tannins (Trease and Evans, 2002).

3.3.6 Test for phlobatannins

Small quantity of the extract was boiled with water and filtered. 5ml of 1% hydrochloric acid was then added to the filtrate. A red ppt indicates the presence of phlobatanins (Trease and Evans, 2002).

3.3.7 Test for anthraquinones.

Borntrager's test for Anthraquinone derivatives; Small quantity of the extract was boiled with 2ml of 10% HCl for 3min the mixture was filtered while still hot and the filtrate allowed to cool. The cool filtrate was then shaken with equal volume of chloroform to extract the anthraquinone. The chloroform layer was then transferred into a clean test tube and treated with equal volume of 10% ammonia solution. The

mixture was then shaken and the colour of the upper layer noted. Colourless layer indicates that anthracene derivatives i.e free or combined anthraquinone is absent (Trease and Evans, 2002).

(b) Small quantity of the extract was shaken with 10ml of benzene. The content was filtered and 5mls of 10% ammonia solution added to the filtrate the presence of a pink, red or violet colour in the ammoniacal (lower) layer indicates the presence of free anthraquinone (Trease and Evans, 2002).Small quantity of the extract was boiled with 10ml aqueous sulphuric acid and filtered. The filtrate was shaken with 5ml benzene and 10% ammonia solution. A pink, red or violet colouration in the ammoniacal layer indicates the presence of combined anthraquinone (Trease and Evans, 2002).

3.3.8 Test for alkaloids

The extract (3ml) was stirred with 5 ml of 1% aqueous Hydrochloric acid and filtered. The filtrate was divided into 3 portion of 1ml each. The first portion was added few drops of Dragendoff's reagent. An orange red precipitate indicates the presence of alkaloids. The second potion was added Wagner reagent. A reddish brown colour indicates the presence of alkaloids. The third portion was added few drop of Mayer reagent. A buff precipitate indicates positive test for alkaloids (Trease and Evans, 2002).

(a) Test for morphine alkaloid:

To test for morphine alkaloids, 1ml of the extract was evaporated to dryness and the residue dissolved in 0.66ml of 1% sulphuric acid, 2ml of distilled water was added and 2 drops of 10% sodium nitrate solution, this solution was made alkaline with dilute ammonia solution. A reddish brown solution shows the presence of morphine alkaloid (Brain and Turner, 1975).

(b) Thalleiquine test for quinoline alkaloids:

0.5ml of conc. Hydrochloric acid and a few crystals of potassium chlorate were added to the extract. It was carefully evaporated and a few drops of strong ammonia was added to it. a green colour is taken as positive (Brain and Turner, 1975)

(c) Test for indole alkaloid:

Few drops of conc. H_2SO_4 and a crystal of potassium dichlorate was added to the extract. A violet colour indicates that indole alkaloid is present (Brain and Turner, 1975).

(d) Vitali – Morin test for tropane alkaloids:

The extract was evaporated to dryness the residue was dissolved in a few drop of fuming nitric acid. This was then evaporated to dryness and allowed to cool. It was dissolved in 2ml acetone and few drops of fresh 3% alcoholic potash solution was added. A violet colour indicates that tropane alkaloid is present (Brain and Turner, 1975).

3.7.9 Test for terpenoids:

Five milliliter of the extract is mixed with 2 ml of chloroform in a test tube. Three milliliter of concentrated H_2SO_4 is carefully added to the mixture to form a layer. An interface with a reddish brown coloration is formed if terpenoids constituent is present (Ayoola *et al*., 2008).

3.7.10 Tests for Proteins and Amino acids:

(a) Biuret test:

To the methanolic extracts, 4% sodium hydroxide and 1% copper sulfate solution were added and formation of violet or pink color indicated the presence of proteins (Trease and Evans, 2002).

(b) Million's test:

To the methanolic extracts, Million's reagent (mercury in nitric acid) was added. Formation of white precipitate which turned red on heating indicated the presence of proteins (Trease and Evans, 2002).

(c) Ninhydrin test:

The extracts were heated with 5% Ninhydrin (in butanol) solution in boiling water bath for 10min and development of purple or bluish color indicated the presence of amino acids (Trease and Evans, 2002).

3.7.11 Test for fatty Acids:

The extract (0.5ml) was mixed with 5 ml of ether. These extract was allow it for evaporation on filter paper and dried the filter paper. The appearance of transparence on filter paper indicates the presence of fatty acids (Ayoola *et al*., 2008).

3.4 Analytical Procedure

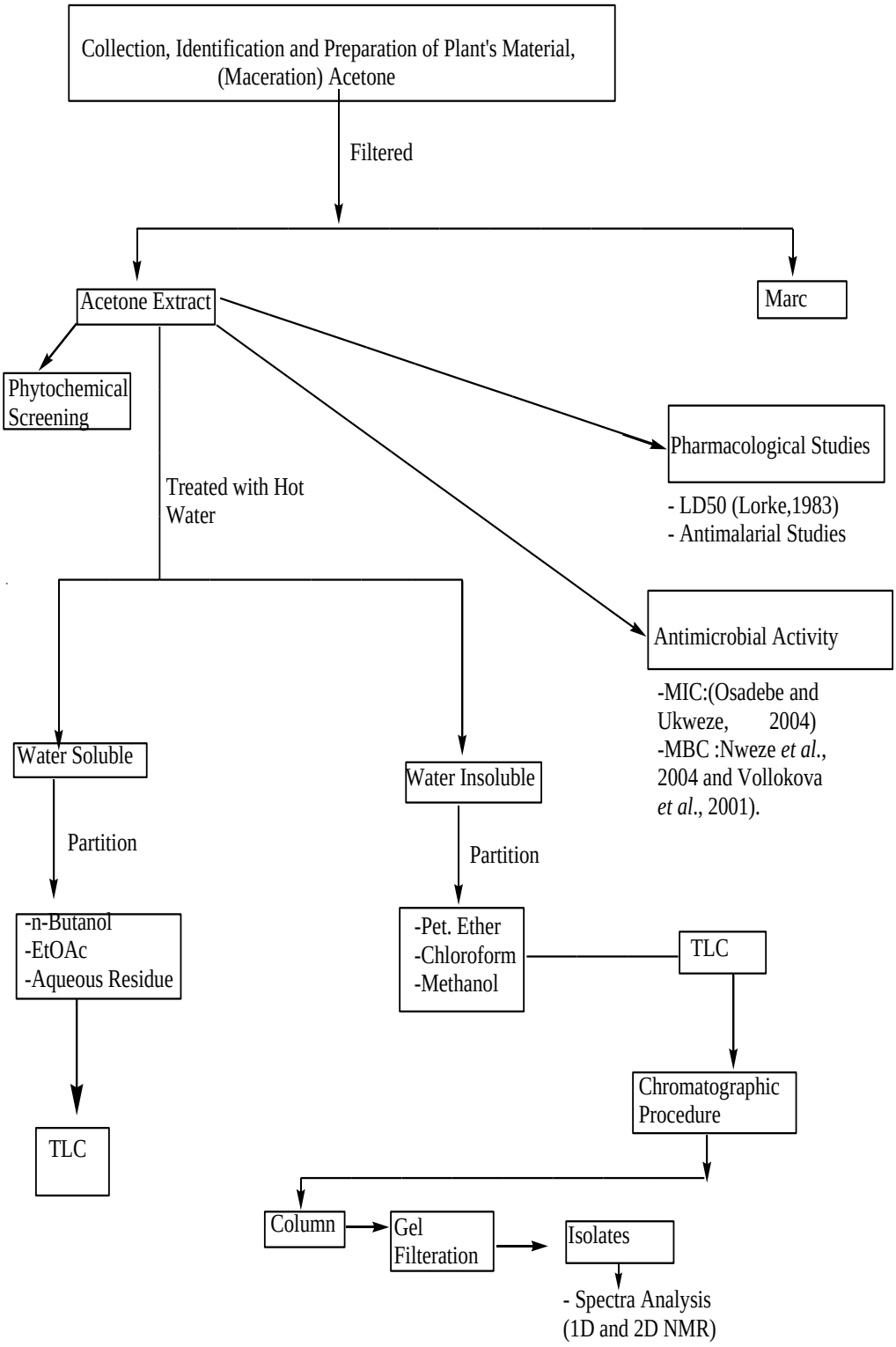


Figure 3.1: Chromatographic and Pharmacological studies of *Ochna rhizomatosa.*

3.5 Chromatographic Procedures

3.5.1 Thin layer chromatography (TLC)

Thin layer chromatography was carried out on silica gel pre-coated aluminium plates Pf_{254} with layer thickness of 0.2mm.

Technique: One way ascending.

Spotting and development: Spots were applied manually

Technique: Ascending

Stationary phase absorbent: silica gel G.

Spotting and development: The spots were applied to the origin manually using glass capillary tubes. Plates were dried using air blower and were developed at room temperature using Shandon chromatographic tank ($22x2.1x9cm^3$) for distance of about 10 – 15cm.

Mobile phase solvent system: Different solvent systems were used.

Visualization / Detection:

The developed chromatograms (plates) were dried in fume cupboard and then

(a) Viewed under ultraviolet light at 254nm wavelength, and then sprayed with a suitable chromatogenic reagents.

(i) alcoholic ferric chloride)

(ii) with 10% sulphuric acid followed by heating at 110^0C for 5-10 minutes

(b) Exposed to iodine vapour in a closed chamber.

3.5.2 Column chromatography

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

(a) Technique: Constant volume gradient elution technique

(b) Column: Sintered glass column (100 x 4 cm) with sintered disc at the bottom of various dimensions.

(c) Stationary phase (adsorbent) - silica gel, 60 – 120 mesh .

(d) Column packing*:* wet slurry method.

(e) Sample loading*:* The sample was applied using dry load method; the sample was dissolved in small amount of suitable organic solvent. It was then mixed with a small quantity of silica gel, triturated and then loaded on top of the column (Richard, 1998).

(f) Mobile phase (eluting solvent): Various solvent systems were used depending on the polarity of the material. Elution was carried out using one or mixture of the following solvents:

I – Petroleum ether

II - Chloroform

III – Ethyl acetate

IV - Methanol

3.5.3 Gel filteration chromatographic techniques.

Gel filteration was performed using Sephadex LH-20 (Sigma).

Packing method: The Sephadex LH-20 was suspended in methanol (100%) as the eluent. This was then allowed to swell for 24 hours prior to use. It was then introduced as slurry down the column and allowed to settle overnight.

Sample application: The sample was dissolved in small volume of the eluent and applied on to the top of the column, using a syringe.

Eluting solvent: Methanol.

3.5.4 Melting point (m.p) determination.

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

3.6 Chromatographic Separation of MNC, BND and BNF.

3.6.1 Thin layer chromatographic examination of fraction A, B, C, D and E.

Thin layer chromatography was carried out on silica gel pre-coated aluminium plates Pf_{254} with layer thickness of 0.2mm and silica gel 60-100 mesh were used for column chromatography. Spots on plates were visualized by spraying with alcoholic ferric chloride or 10% H_2SO_4, followed by heating the in oven. A small quantity of the fractions was dissolved in small quantity of methanol and spotted manually on the baseline of the plate, using a capillary tube. The plate was developed in a mixture of Benzene: Ethylacetate: Acetic acidic (8:4:1) solvent system. After development, on examination of the chromatogram, fraction 'B' revealed the presence of three promising spots. To isolate these compounds in this fraction, column chromatography was employed.

3.6.2 Column Chromatography of the methanol Fraction (E) of *O. rhizomatosa.*

The methanol fraction (E) was chromatographed over glass column (100 cm × 4 cm) packed with silica gel (60-120 mesh size). The column was eluted continuously using petroleum ether (100%), chloroform, chloroform: ethyl acetate (mixture), ethyl acetate (100%) and finally with methanol (100%) by gradient elution technique. The progress of elution was monitored using thin layer chromatography. A total of 99 fractions of 100 ml aliquot were collected. Fractions were pooled based on their TLC profile to afford 12 major fractions (E_1-E_{12}). Fractions E_3 (21-26) and E_5 (31-35) consisting of one spot each were coded BND and MNC respectively.

Fraction E_8 (66-68) consisting of two major spots was subjected to a repeated gel filtration's technique using Sephadex LH-20 and eluted with methanol to obtain 3 sub – fractions (F_1-F_3). Sub fraction F_2 and F_3 gave single spots when subjected to TLC using two different solvent systems (Benzene: ethyl acetate: acetic acid 8:4:1) and

chloroform: ethyl acetate: acetic acid (4:8:2) to afford compound BNF (F_3) and more of compound MNC (F_2).

The purity of isolated compounds (MNC, BND and BNF) was confirmed on TLC, using different solvent systems as Benzene: ethyl acetate: acetic acid (8:4:1) and chloroform: ethyl acetate: acetic acid (4:8:1), Toluene: pyridine: acetic acid (10:1:1) etc to obtain a single spot in each case.

3.6.3 Solubility test:

The solubility of compound MNC, BND and BNF was determined in chloroform, Ethyl acetate, Acetone and Methanol at room temperature and they were found to be soluble in acetone and methanol.

3.6.5 Nuclear Magnetic Resonance (NMR) analysis.

The nuclear magnetic resonance spectrum of the isolated compounds was determined using the Varian Unity Inova 600MHz. Approximately 10 - 15mg of each of the compounds were used. ^{1}H NMR and ^{13}C NMR were performed on Bruker spectrometer 600 MHz. Spectra were refered to the CD_3OD solvent signals □ 3.80 (^{1}H) and 49.00 (^{13}C) with TMS as an internal standard. Chemical shift values (□) were reported in parts per million (ppm) in relation to the appropriate internal solvent standard (TMS). The coupling constant (j-values) was given in Hertz. Both the one dimension and two dimension spectra were generated. The spectra results were then used to elucidate the structures of the isolated compounds. Analysis and interpretation of the spectroscopic data were made.

3.6.6 Phytochemical investigation of compound MNC, BND and BNF

Compound MNC, BND and BNF were subjected to flavonoids test as described earlier. They all showed a positive result.

3.7 Pharmacological Studies

3.7.1 Experimental Animals

The animals were obtanined from National Institute for Tryponosomiasis Research, Kaduna and bred in the animal house of Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria to attain a weight between 15 – 25g.

3.7.2 Infected animals

Swiss albino mice infected with *Plasmodium berghei* parasites were obtained from Nigerian Institute of Medical Research, Lagos, Nigeria. The animal were fed with standard mouse cubes and tap water. The parasite stock was maintained by continuous re-infection in the bred mice.

3.7.3 Standard Drug

Chloroquine used as positive control was obtained from May and Baker.

3.7.4 Instrument

Light microscope was used to count the parasite in the blood smear.

3.7.5 Acute toxicity study (LD_{50}) of the Acetone Extract.

LD_{50} determination was carried out using the method of Lorke (1983). Thirteen mice were used for this study in the first phase, three doses of the extract (10, 100 and 1000 mg/kg) were administered intraperitoneally (i.p) to three groups each containing three mice each. In the second phase, four doses of the extract (600, 370, 225 and 140 mg/kg) were administered through the same route to four groups containing one mouse each. The median lethal dose (LD_{50}) value was calculated as the thirty percent of the square root of the product of the highest non lethal dose and the lowest lethal dose of the extract.

3.7.6 Antimalarial activity study of acetone extract of leaf of *O. rhizomatosa*

3.7.6.1 Antimalarial studies experimental design:

The experimental design involved two distinct experimental protocols:

3.7.6.1.1 Evaluation of blood schizonticidal activity of the acetone extract in suppresive test.

This was done using a method similar to that described by Knight and Peters (1980). A total of 30 mice were used for this study on blood schizontiadal activities. Each mouse was subsequently given standard intra- peritoneal inoculums of $1.02x10^5$ *P. berghei* parasites (Chloroquine sensitivity) with the aid of a 1ml disposable syringe. This was done to all the mice. The animals were then divided into five groups of six mice each. The treatments were all given intraperitonially. Group 1 to 3 animals were given 21.5, 43 and 86 mg/kg per day of the extract respectively. Group 4 animals were given 5 mg/kg per day of Chloroquine and group 5 animals were given 0.2ml of normal saline. All the extracts, drug and distilled water were given for three days. Group 4 served as the positive control group while group 5 served as the negative control group. All the extracts, drugs and distilled water were given for 3 days. On the fourth day, thick blood smears were made from the blood samples obtained from the tails (caudal veins) of the animals. The smears were stained with Giemsa stain and examined under the light microscope for the levels of parasitaemia. The average percentage suppression of parasitaemia was calculated in comparism to negative control using this formular;

Chemosuppression of parasite growth = 100 – [Mean parasitemia treated / mean parasitemia control] x 100

3.7.6.1.2 Evaluation of blood Schizonticidal activity in curative tests:

This was determined using a method similar to that described by Ryley and Peters (1970). The mice were divided into 5 groups (a - e) consisting of 6 mice each. All the

animals were inoculated intraperitoneally with $1.02x10^5$ *Plasmodium berghei* parasites with the aid of a 1ml disposable syringe. Treatment was commenced on the fourth day (72hrs later) using 21.5, 43 and 86 mg/kg per day of extract for group a, b and c respectively. Group'd' animals were given 5mg/kg per day of Chloroquine intraperitoneally and group 'e' animals were given 0.2ml of normal saline. All the extracts drug and distilled water were given for three days. Group 'e' served as the negative control group. The treatment using the extract, drug and normal saline were given for three days. After 24 hours of completion of the treatments, thick blood smears were made and the level of parasitaemia determined as described above.

3.7.8 Statistical analysis:

Results obtained were presented as mean ± standard error of mean. Statistical analysis was done using student's t-test. A 'p' < 0.05 were considered significant. The mean survival period for each group within a 28days period was determined and noted

3.8 Antimicrobial Assay

3.8.1 Microorganism

The isolates used in this experiment were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Zaria. These include *Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium ulcerans, Escherichia coli, Shigella dysentriae, Salmonella typhi, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans, Candida ibrusei,* and *Candida tropicalis.* These isolates used were checked for purity and maintained in slants of nutrient agar.

3.8.2 Antimicrobial Susceptibility testing

The disc diffusion method (Sardari *et al*., 1998) was used for the test. Filter paper disc (6 mm in diameter) impregnated with sample solutions were placed on blood agar

plates which have been inoculated with test organisms according to standard protocol described by National Committee of Clinical laboratory standard (1993). 0.6g of the crude and 0.001mg of the pure compound (MNC) was weighed and dissolved in methanol were tested at a concentration of 60mg and 100µg of the extract and pure compound /disc respectively. The plates were incubated at 37°C for 24 hrs, after which the diameters of the inhibition zones were measured and recorded in millimetres using a transparent ruler. Filter paper disc containing extraction solvents without any test extract served as control and no inhibition was observed. The reference antibacterial drug ciprofloxacin (100 mg/ disc) and reference antifungal fluconazole (5 mg/disc) were used as standard drugs (NCCLS, 1993).

3.8.3 Determination of minimum inhibitory concentration (MIC)

Determination of the MIC was carried out on the extracts that showed inhibitory effect on the test micro-organism. Broth dilution method was used (Ibekwe et al., 2001). Nutrient broth was prepared according to the manufacturers instructions. 2mls of the medium was dispensed in screw-capped test tubes and sterilized at 121°C for 15 min. Mc-Farland's turbidity standard scale (0.5) was prepared by adding 9.95 ml of 1% H_2SO_4 and 0.05 mls of 1% $BaCl_2$ to give a turbid solution. 10 mls sterile normal solution was used to make a turbid suspension of the micro-organism. Dilution of the organism suspension was done continuously using normal saline until the turbidity marched that of Mc-Farland's scale by visual comparison. At that point, the concentration of the micro-organisms was about 1.5 x 10^8 cfu/ml. Two-fold dilution with nutrient broth was done to give concentrations of 60, 30, 15, 7.5 and 3.25 mg/ml for the crude and 100µg, 50µg, 25µg, 12.5µg and 6.25µg/ml for the pure compound. 0.2 mls of the micro-organism suspension was inoculated into the different concentrations of the extract and pure compound in test tubes. The tubes

were incubated at 37°C for 24 h and at 25°C for 48 h for bacteria and fungi respectively after which the plates were observed for growth. The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each micro organism.

3.8.4 Determination of minimum bactericidal/minimum fungicidal concentration

Blood agar plates were prepared according to the manufacturers instructions. The contents of the MIC tubes and the following tubes in the serial dilution were sub-cultured into appropriately labeled blood agar plates by dipping a sterile wire loop into each test tube and streaking the surface of the labeled blood agar plates. The plates were then incubated at 37°C for 24 hr after which they were observed for growth. The MBC was the plate with the lowest concentration of the extract and pure compound in serial dilution without growth (Meenakshi *et al.*, 2001).

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical Screening Result.

The results of the phytochemical screening of ethyl acetate (A), n – butanol (B), petroleum ether(C), Chloroform (D), and methanol (E), fractions are summarized (Table 4.1).

Table 4.1: Results of Phytochemical Screening.

Phytochemical constituents	**Various Fractions**				
	A	**B**	**C**	**D**	**E**
Flavonoids	+	+	-	+	+
Cardiac Glycosides	+	_	-	+	+
Saponins	+	+	+	+	+
Tannins	+	+	+	+	+
Steroids	-	-	+	+	-
Terpenoids	+	+	-	+	+
Proteins	+	+	-	+	+
Carbohydrates	+	+	-	-	+
Fatty acids	+	+	-	+	+
Alkaloids	-	-	-	-	-

Key : + = Present, - = absent

4.2 **Yield and Colour of the Fractions**

The results of the yield and the colour of the various fractions of the extract are presented in the table below.

Table 4.2: The yield and colour of the fractions.

Solvent	Nature of fractions	Mass (g)	% of Fraction
(1) Petroleum ether (60 - 80 ^{0})	Greenish gummy mass	9.26	18.10
(2) Chloroform	Light yellow solid	0.47	0.92
(3) Ethyl acetate	Reddish-Brownish solid	1.00	1.94
(4) Methanol	Brownish solid	6.47	12.65
(5) n - Butanol	Dark red gummy mass	33.95	66.37

4.3 Results of Flavonoids Test of Fractions.

The result of the test for flavonoids on the various fractions are presented in the table below.

Table 4.3: Results of flavonoids test of fractions

Portion	Reagents			Inference
	Mg-HCl	Alc. FeCl3	Zn- HCl	
Petroleum ether	-	-	-	-
Chloroform	Orange	Dark green	Red	+
Ethyl acetate	Orange	Dark green	Red	+
Methanol	Orange	Dark green	Red	+
n- Butanol	orange	Dark green	Red	+

Key : + = Present, - = absent

4.4 Result of Column Chromatographic

The results of the column chromatography separation of the methanol fraction using gradient elution technique are as presented in the table below.

Table 4.4: Result of Column Chromatographic separation of methanol fraction (E) on silica Gel.

S/N	Fraction	Eluting solvent	Number of spots
E_1	1 – 7	$CHCl_3$: EtOAC (9.1)	-
E_2	8 - 20	$CHCl_3$: EtOAC (8:2)	-
E_3	21 – 26	$CHCl_3$: EtOAC (7:3)	1 (BND)*
E_4	27 – 26	$CHCl_3$: EtOAC (7:3)	2
E_5	31 - 30	$CHCl_3$: EtOAC (7:3)	1 (MNC)*
E_6	36 – 35	$CHCl_3$: EtOAC (6:4)	2
E_7	61 - 65	$CHCl_3$:EtOAC (5:5)	3
E_8	66 – 68	$CHCl_3$:EtOAC (4:6)	2
E_9	69 – 82	$CHCl_3$: EtOAC (3:5)	3
E_{10}	83 – 84	$CHCl_3$: EtOAC (2:8)	3
E_{11}	85 – 86	$CHCl_3$:EtOAC (1:9)	3
E_{12}	87 – 99	MeOH (100%)	3

Key:

* = Pure isolates

$CHCl_3$ = Chloroform

EtOAc = Ethyl acetate

MeOH = Methanol

Table 4.5: Results of gel filteration separation of column fraction (E_8) on sephadex LH-20

S/N	Fraction	Eluting Solvent	Number of spots

F_1	1 – 10	Methanol	100%	NIL
F_2	11 – 16			1 (MNC)
F_3	17 – 30			1 (BNF)
F_4	31 – 60			2

Plate 4.1: TLC profile of compound MNC with methanol fraction (E).

Solvent system is Benzene: EtOAc: Acetic Acid (8:4:1), sprayed with alcoholic ferric chloride solution.

4.5: Some Properties of MNC

Table 4.6: Some Properties of MNC

Property	Observation
Quantity	30mg

Appearance	yellow amorphous solid
Solvent of solubility	methanol
M.P	240 -241 ^{0}C
RF value	0.31
Solvent system	Benzene: Ethyl acetate: Acetic acid (8: 4:1)
Chemical test	Positive to shinoda's test.

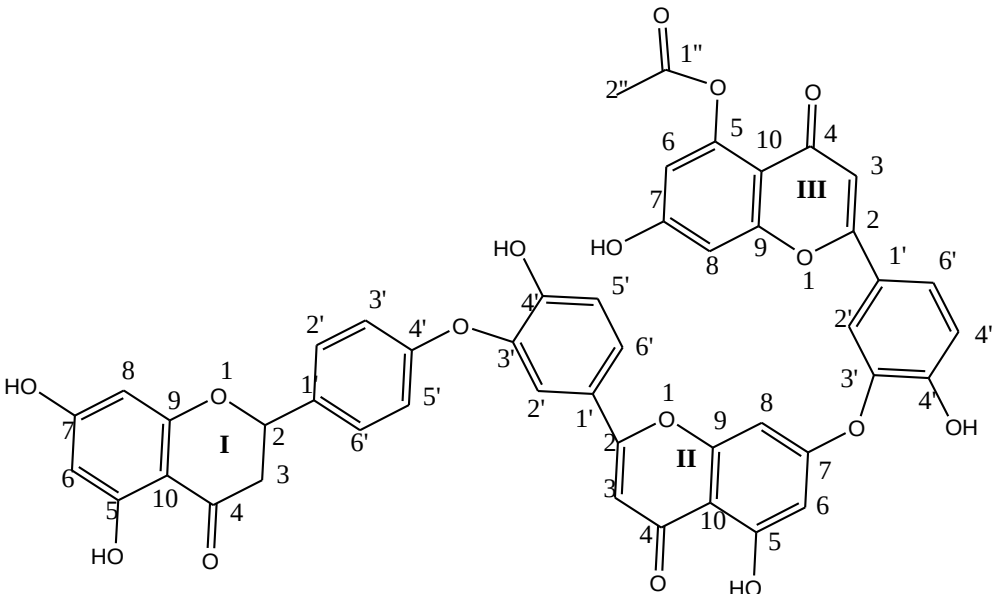
4.5.1 Spectral Analysis of Compound MNC

4.5.1.1 Proton magnetic resonance Analysis of MNC

The ^{1}H NMR spectrum of compound MNC shows the following resonances as shown below (Figure 4.1a – 4.1c and Table 4.7).

Table 4.7: ^{1}H NMR Analysis of MNC

H (ppm)	J-value(Hertz), Position
5.44	IH, dd, J=12.6, 2.6Hz, I-H-2
2.78	1H, dd, J = 2.6, 17.0Hz, I – H – 3a
3.11	IH, dd, J = 17.1, 12.6Hz, I – H – 3e
6.17	1H, S, I-H-6
6.36	1H, S, I-8
7.49	2H, d, J= 8.2Hz, I – 2', 6'
7.04	2H, d, J = 8.2Hz, I -H – 3',5'
6.53	1H, S, II-C-3
6.17	1H, S, II-H-6
6.36	1H, S, II-8
7.57	1H, S, II-H-2'
7.09	1H, d, J = 8.3Hz, II - H – 5'
7.72	1H, d, J = 8.3Hz, II - H -6'
6.53	1H, S, III-C-3
5.87	IH, S, III-6
5.90	IH, S, III-8
7.57	1H, S, III-H-2'
7.09	1H, d, J = 8.3Hz, III - H – 5'
7.72	1H, d, J = 8.3Hz, III - H -6'
1.8	3H, S, III – H – 2''



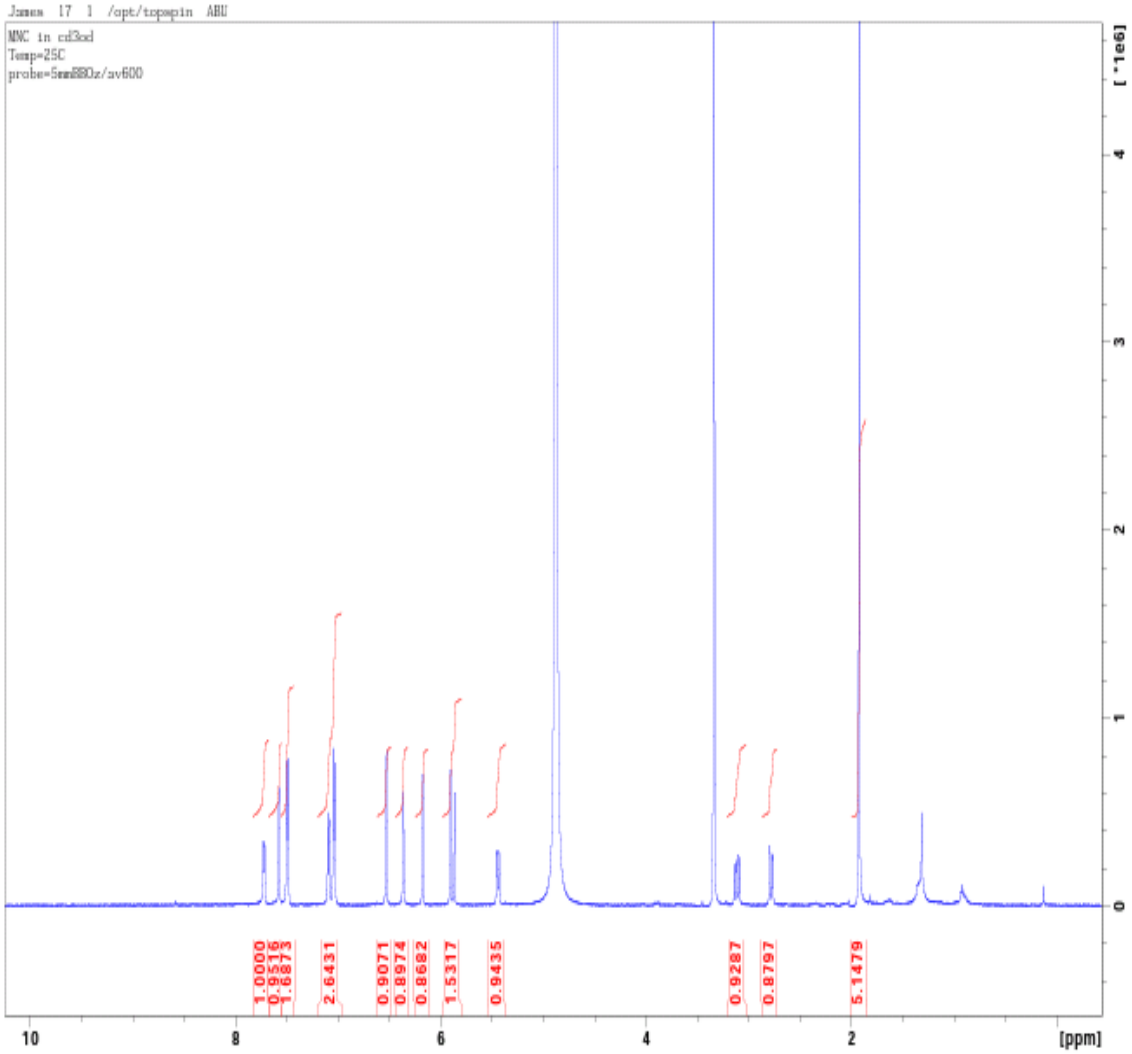
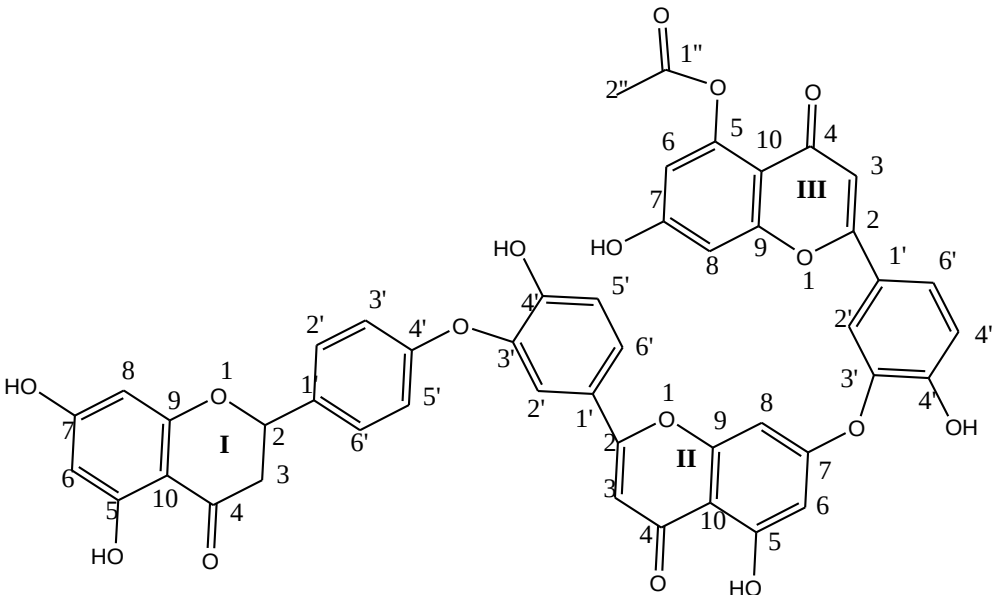


Figure 4.1a: ^{1}H NMR Spectrum of compound MNC



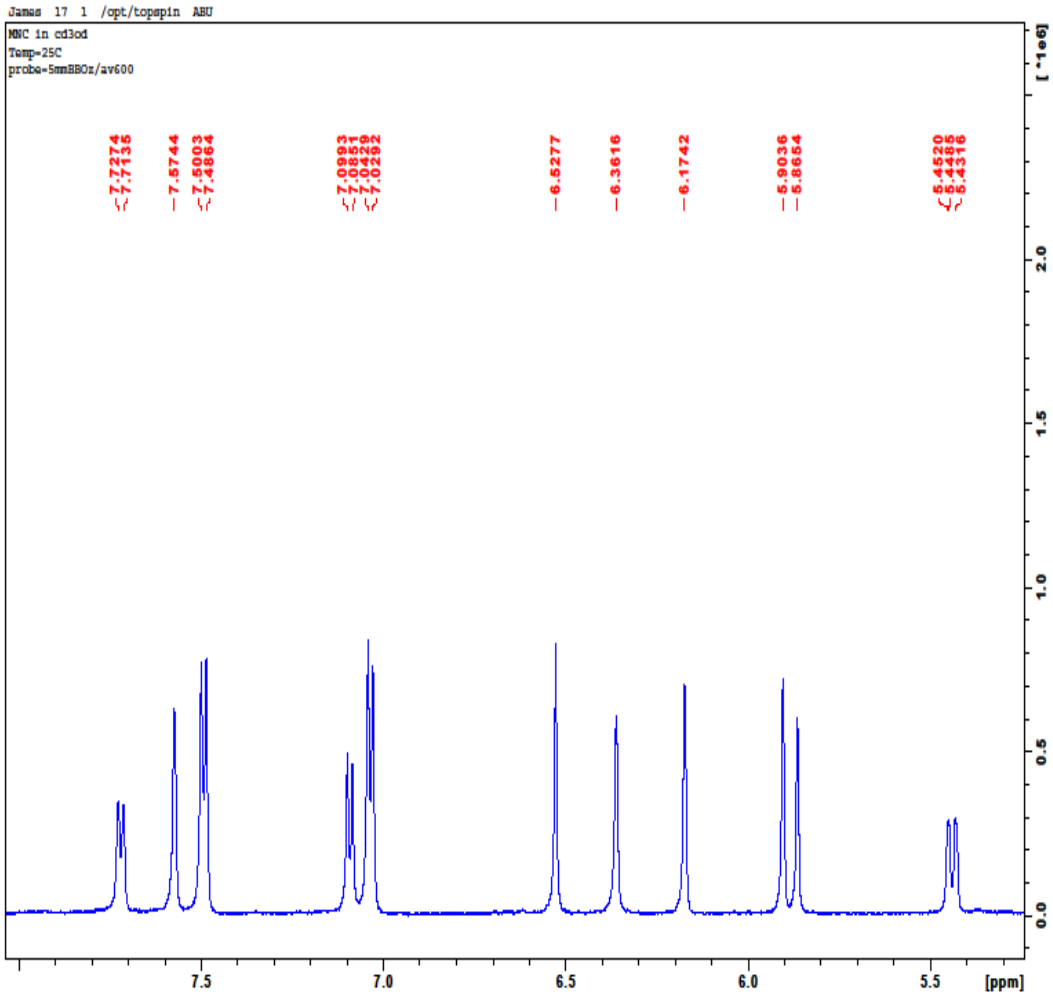
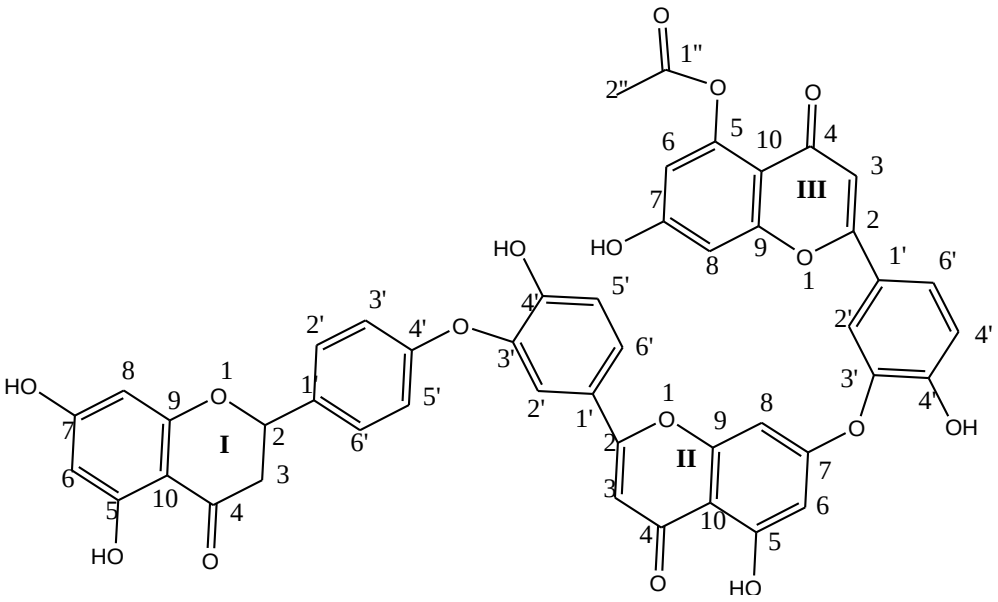


Figure 4.1b: ^{1}H NMR Spectrum of compound MNC (expanded -1).



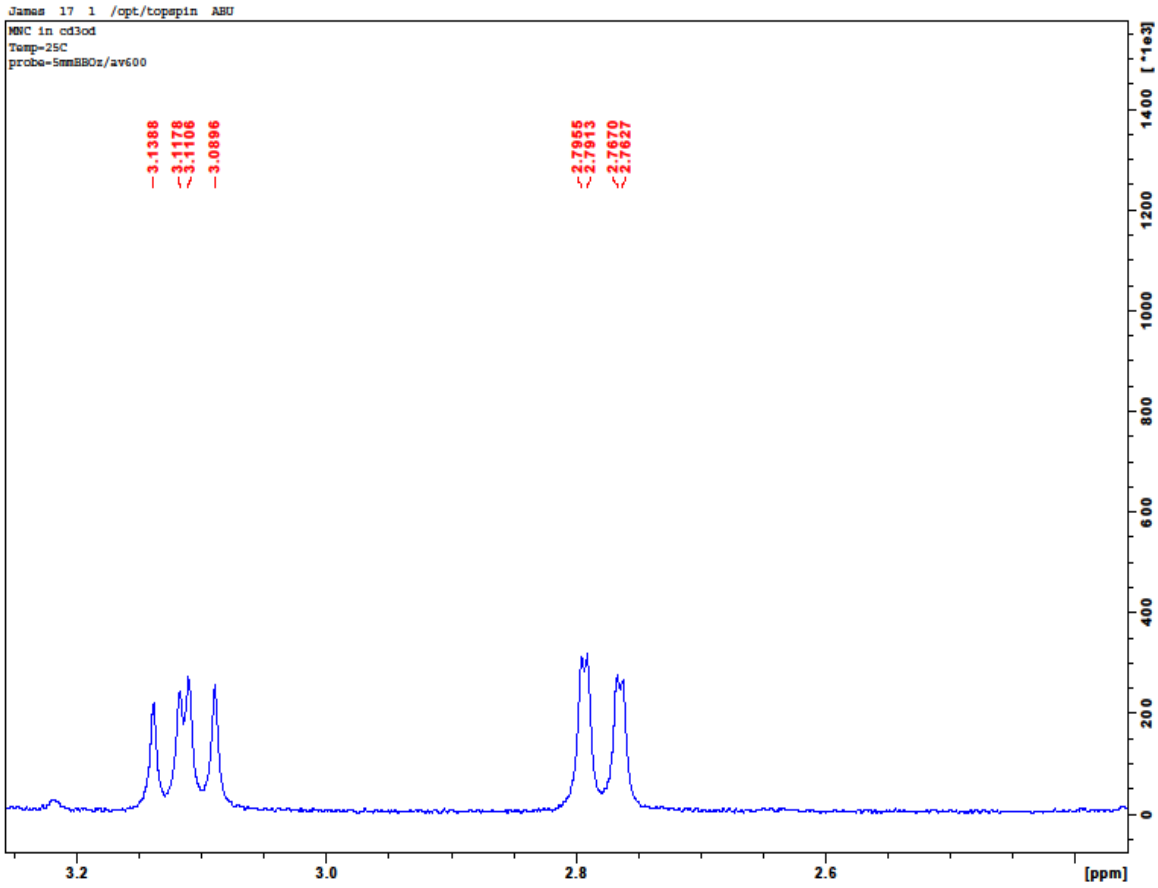


Figure 4.1c: ^{1}H NMR Spectrum of compound MNC (expanded -2)

4.5.1.2 ^{13}C – NMR spectrum Analysis of compound MNC

^{13}C – NMR spectrum of compound MNC shows the following resonances at 7 8.5, 22.7, 42.6, 78.6, 94.2, 95.5, 95.6, 96, 99.5, 101.4, 102.4, 103.2, 116.6, 117.9, 119.2, 121.8, 123.9, 127.6, 133.4, 144.0, 154.8, 158.08, 161.0, 161.1, 163.08, 164.1, 166.7, 166.8, 169.5, 178.8, 182.1, 195.4 (Figure 4.2), as shown in Table 4.8

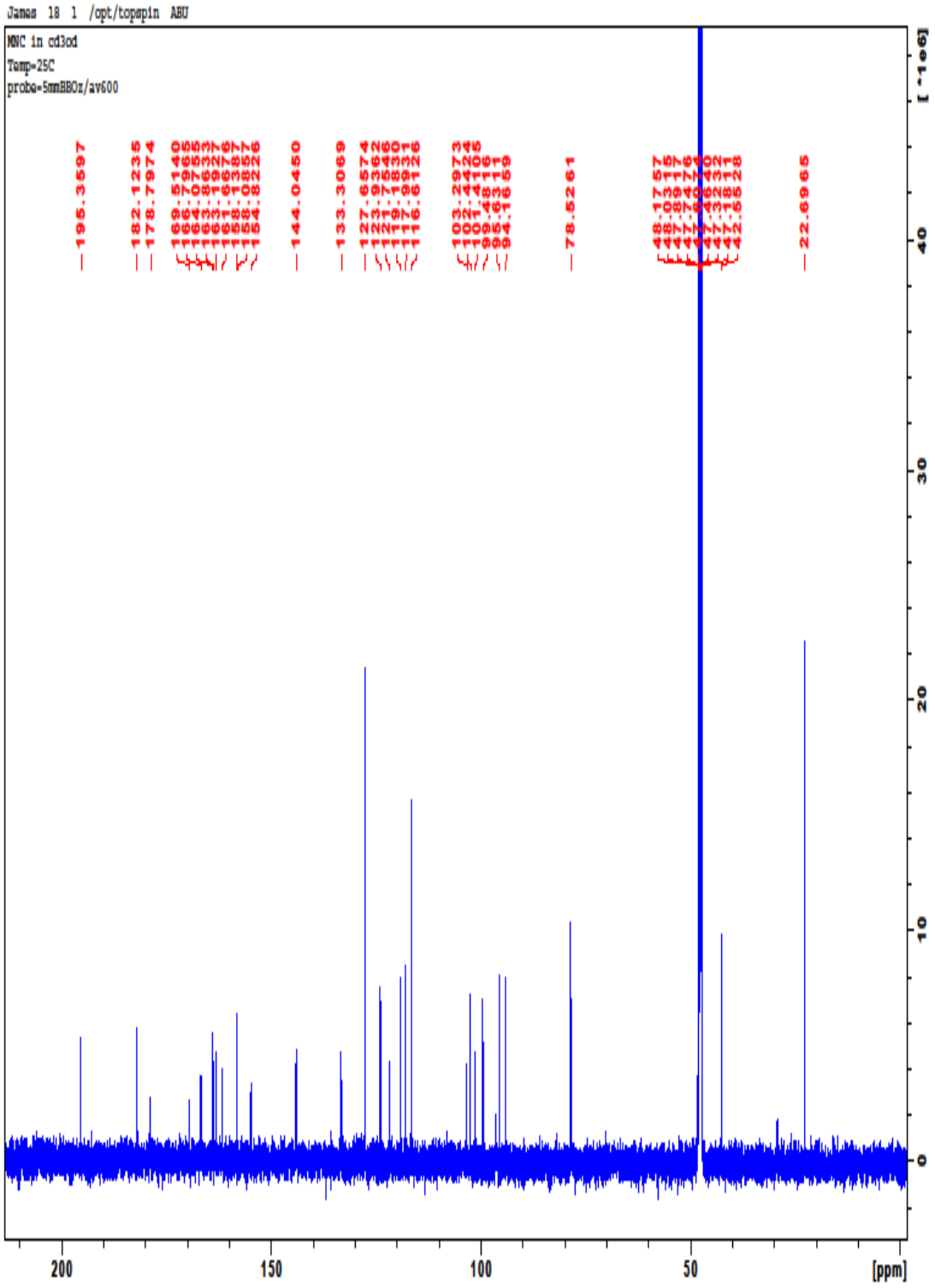


Figure 4.2: ^{13}C Spectrum of compound MNC

4.5.1.3 DEPT spectrum Analysis of MNC

The DEPT spectrum analysis of compound MNC shows the prescence of twenty two carbon atoms, which includes one methyl carbon atom, one methylene carbon atom, twenty methine carbon atoms and three quaternary carbon atom (of c < 127ppm), (Figure 4.3).

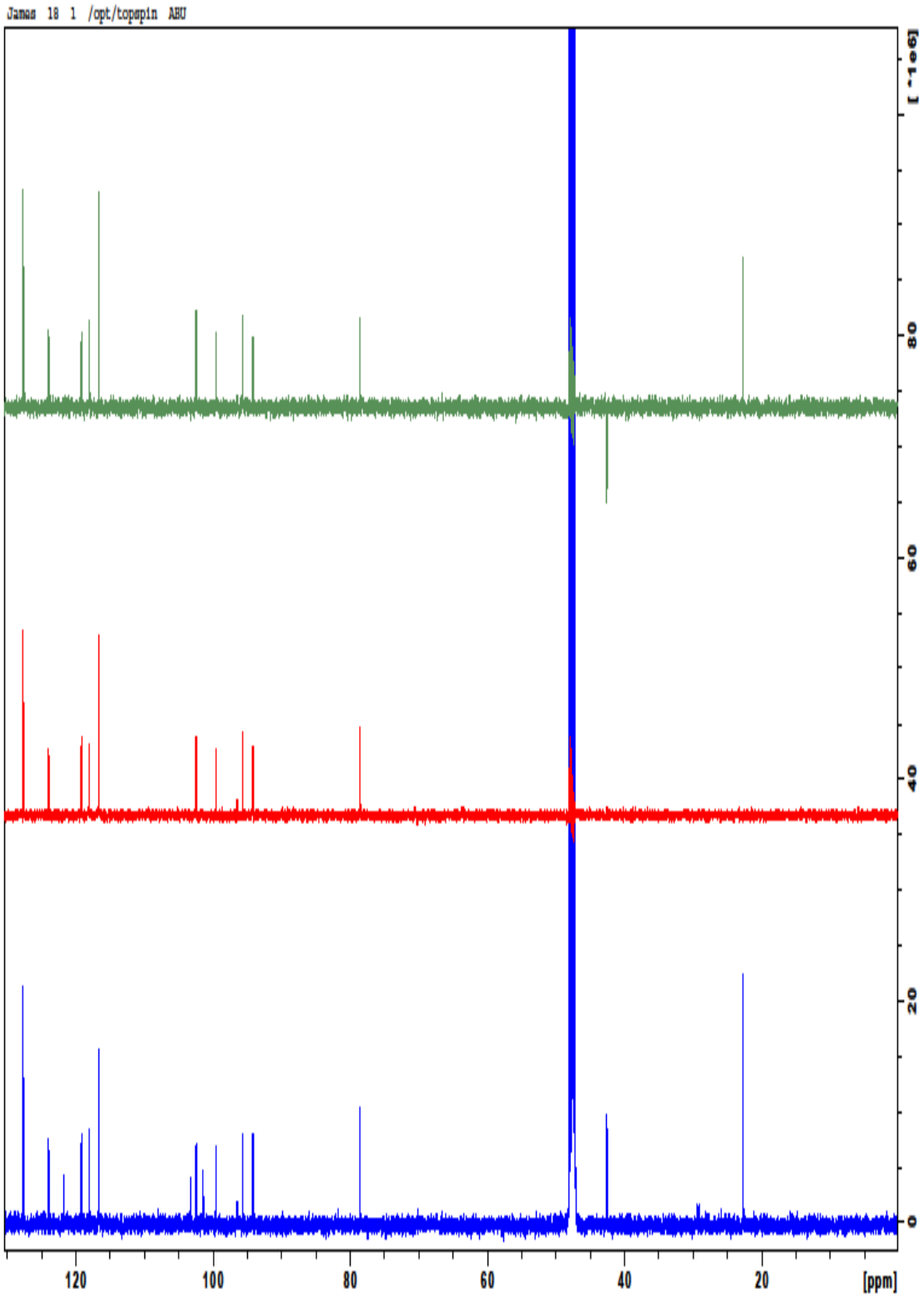


Figure 4.3 DEPT spectrum of compound MNC.

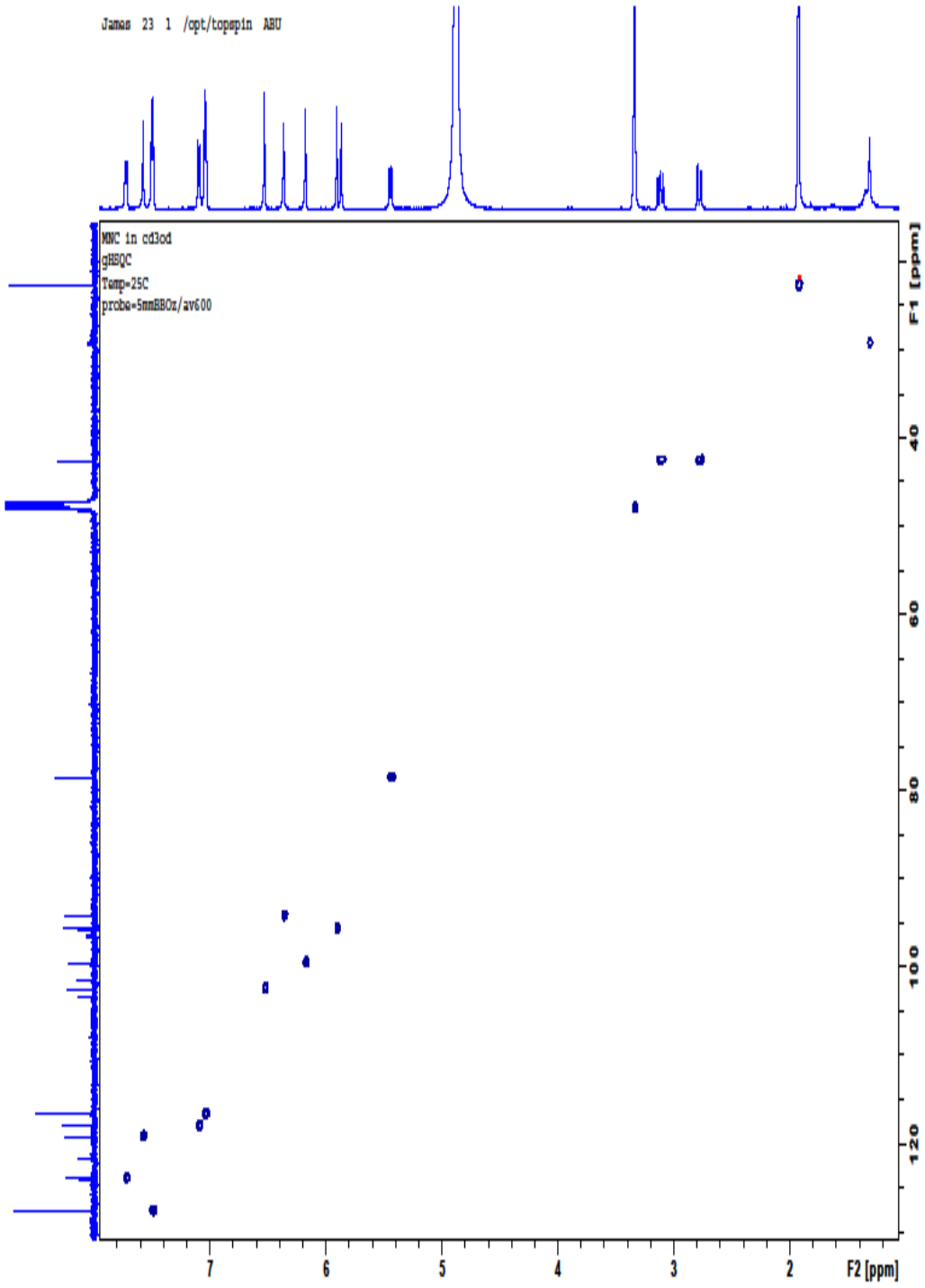


Figure 4.4 HSQC Spectrum of compound MNC

4.5.1.5 COSY Spectrum Data of Compound MNC

The COSY spectrum of compound MNF shows the (^{1}H-^{1}H) COSY correlations of protons situated in the same environment (Figure 4.6).

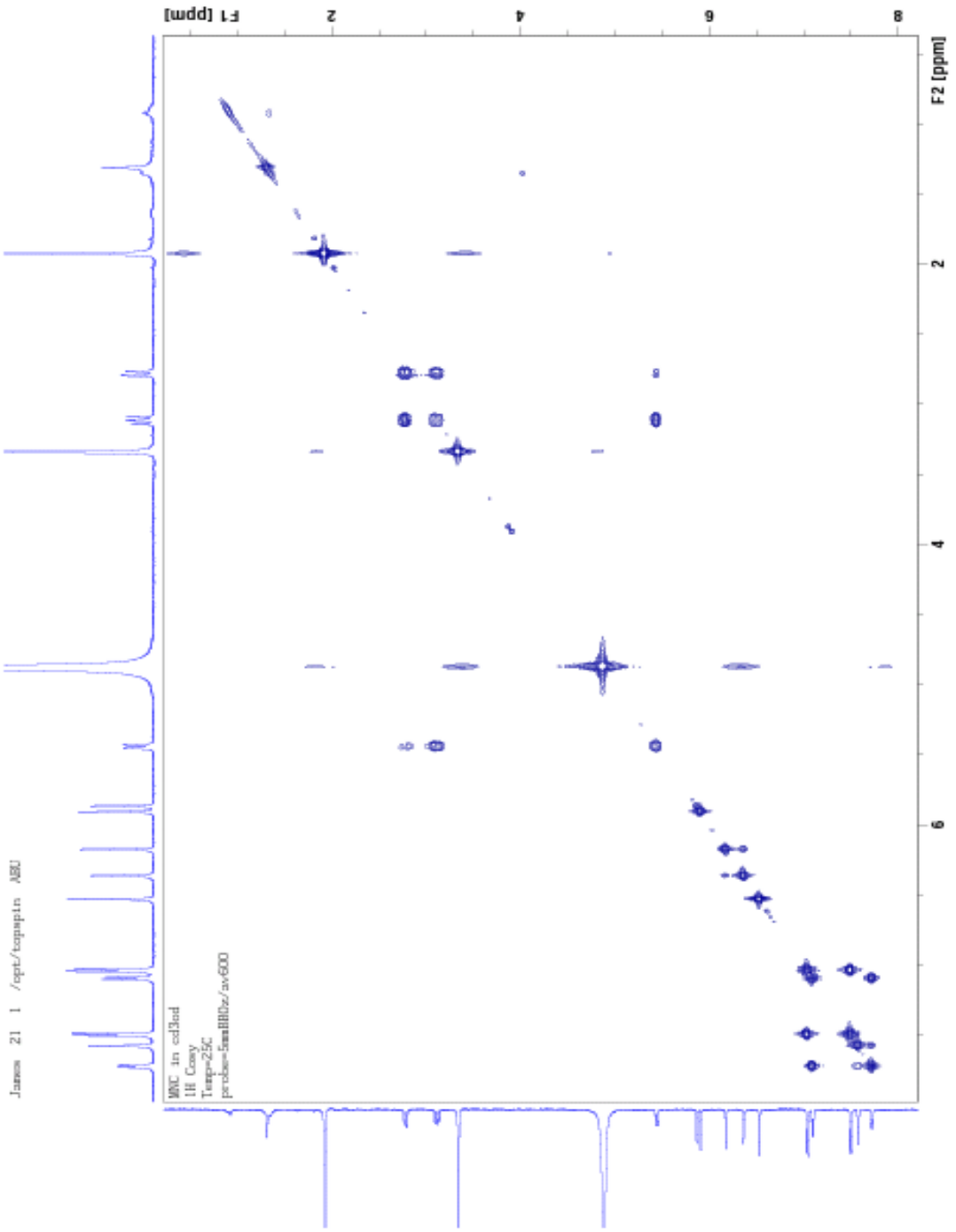


Fig. 4.5: COSY Spectrum of compound MNC

Table 4.8: ID and 2D NMR Spectra Summary for Compound MNC in CD_3OD (600MHz).

	Position	□ Hppm	□ C(ppm)	DEPT
	2	5.44	78.5	CH
	3	2.78/23.11	42.6	CH_2
	4	-	195.4	C=O
	5	-	158.08	C
	6	6.17	99.4	CH
	7	-	166.7	C-O
	8	6.36	94.2	CH
	9	-	161.7	C
I	10	-	96	C
	1’	-	133.4	C
	2’	7.49	127.7	CH
	3’	7.04	116.6	CH
	4’	-	158.13	C-O
	5’	7.04	116.6	CH
	6’	7.49	127.7	CH
	2	-	163.2	C
	3	6.53	102.4	CH
	4	-	178.7	C=O
	5	-	166.7	C-O
	6	6.17	99.4	CH
	7	-	161.7	C-O
	8	6.36	94.2	CH
	9	-	164.1	C
II	10	-	101.4	C
	1’	-	121.8	C
	2’	7.57	119.1	CH
	3’	-	144.0	C-O
	4’	-	154.08	C-O
	5’	7.72	123.9	CH
	6’	7.09	117.9	CH
	2	-	163.8	C
	3	6.53	102.4	CH
	4	-	182.1	C=O
	5	-	166.7	C-O
	6	5.90	95.6	CH
	7	-	161.7	C-O
	8	5.87	95.6	CH
	9	-	164.1	C
III	10	-	103.2	C
	1’	-	121.8	C
	2’	7.72	123.9	CH
	3’	-	144.0	C-O
	4’	-	154.82	C-O
	5’	7.49	127.7	CH
	6’	7.09	117.9	CH
	1’’	-	169.5	-C=O
	2”	1.8	22.7	CH_3

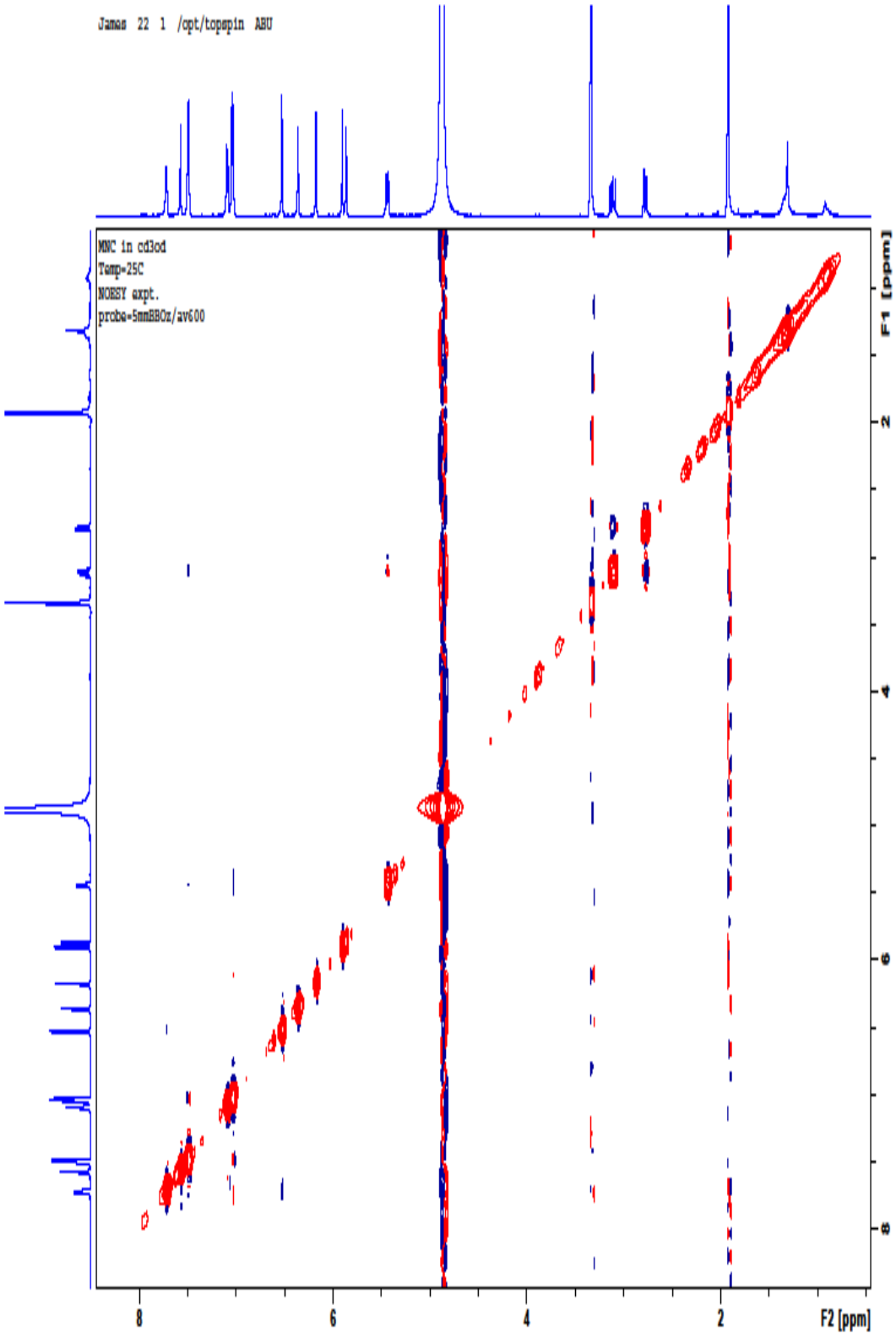


Figure 4.6: NOESY Spectrum of MNC

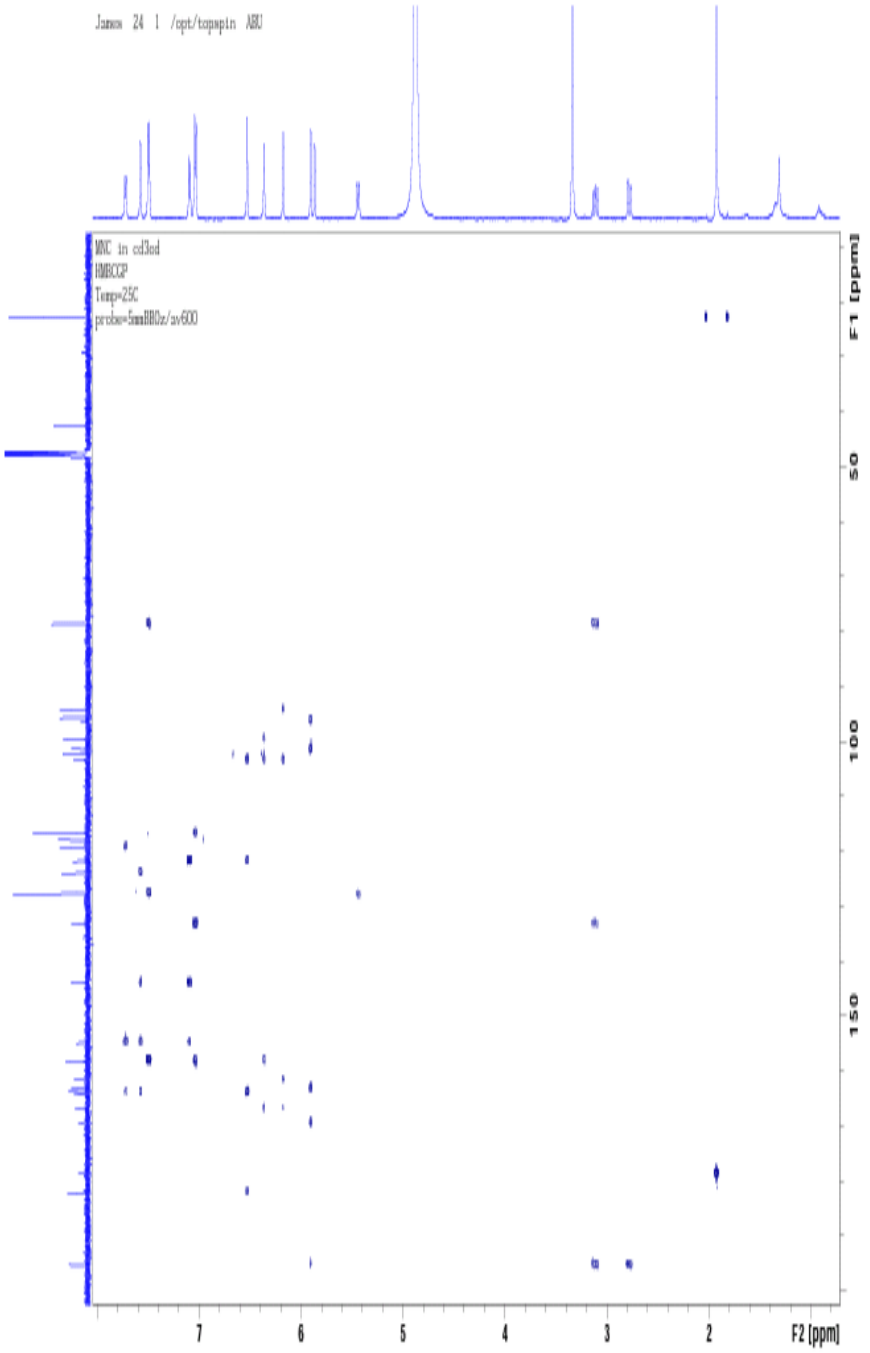
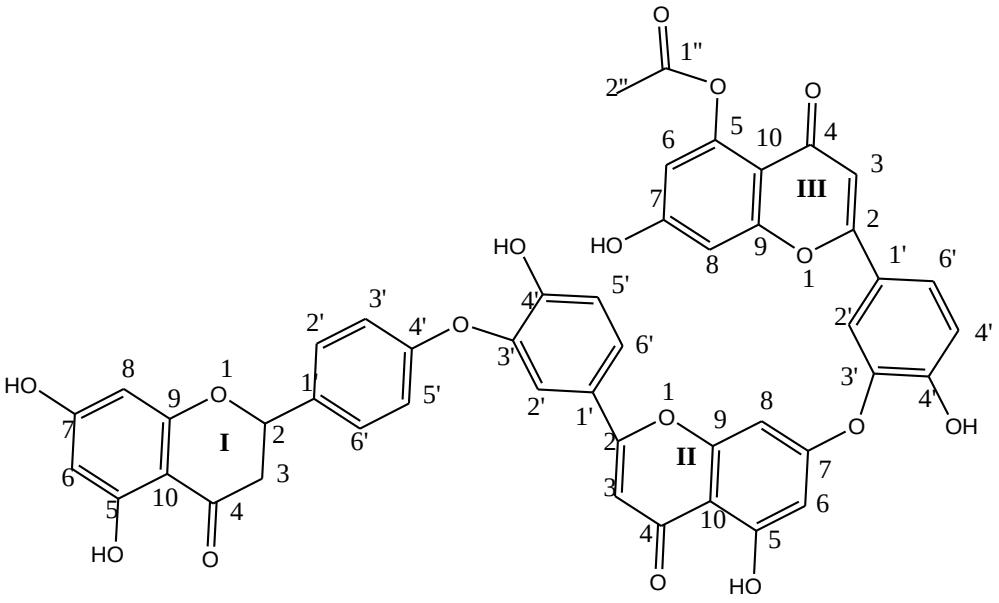


Figure 4.7: HMBC Spectrum of MNC

4.5.6 Structure of Compound MNC



4.6 Compound BND

Compound BND was isolated from Methanol fraction (E) through Column Chromatography as Number E_3 (Fraction 21-26). It was isolated as a brown amorphous solid.

4.6.1 Melting Point of compound BND

The melting point of BND was found to be 175-177^{0}C.

4.6.2 The TLC profile of compound BND.

The TLC profile of compound BND, using chloroform: ethyl acetate: methanol (4:8:2) as solvent system. The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110^{o}C for 5min.

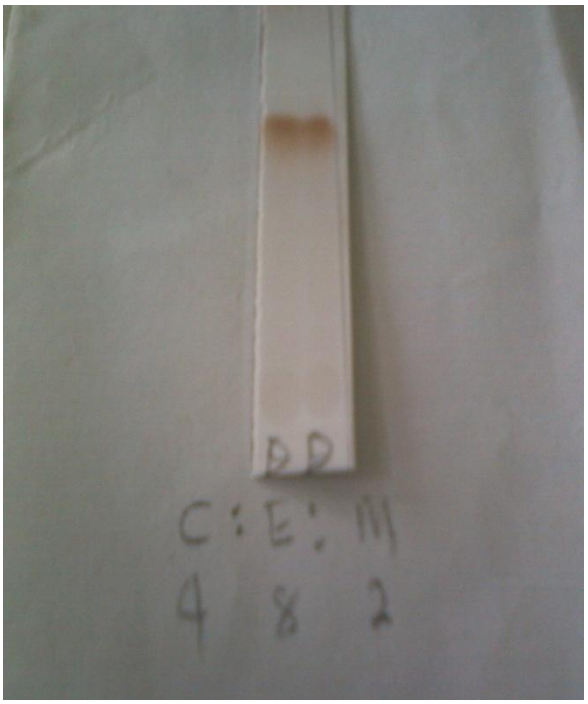


Plate 4.3: The TLC profile of compound BND.

4.4.3 Some properties of BND

The following observations were made on the properties of BND

Table 4.9 : Some Properties of BND

Property	**Observation**
Quantity	15mg
Appearance	brown amorphous solid
Solvent of solubility	methanol
M.P	$175 – 177^0$C
RF value	0.623
Solvent system	Chloroform: Ethyl acetate: Methanol (4:8:2)
Chemical test	negative to shinoda's test, but positive to other flavonoids test

The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110^0C for 5min.

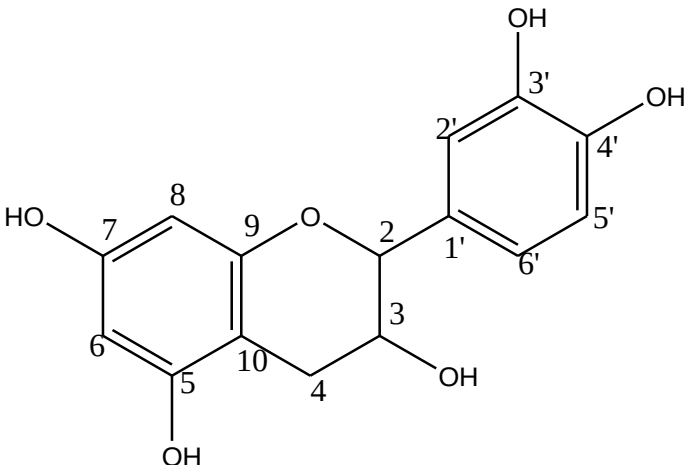
4.4.4 Spectral Analysis of Compound BND

4.4.4.1 Proton Nuclear Magnetic Resonance of Compound BND

The ^{1}H NMR spectrum of compound BND shows the following resonances (Table 4.8, Figure 4.8a-c).

Table 4.10: ^{1}H NMR and chemical shift of Compound BND

□ H(ppm)	Position, chemical shift (Hertz)
4.84	1H,d,H-2
4.2	IH, ddd, H-3
2.77	1H,dd,H-4a
2.87	1H, dd,H-4b
5.98	1H, d, J=2.0Hz, H-6
5.95	1H,d,J=2.0Hz, H-8
6.79	1H, d, J=8.04Hz, H-5’
6.82	1H, dd, J= 2.04Hz, 8.04 Hz, H-6’
7.01	1H, d, J=2.04Hz, H-2’



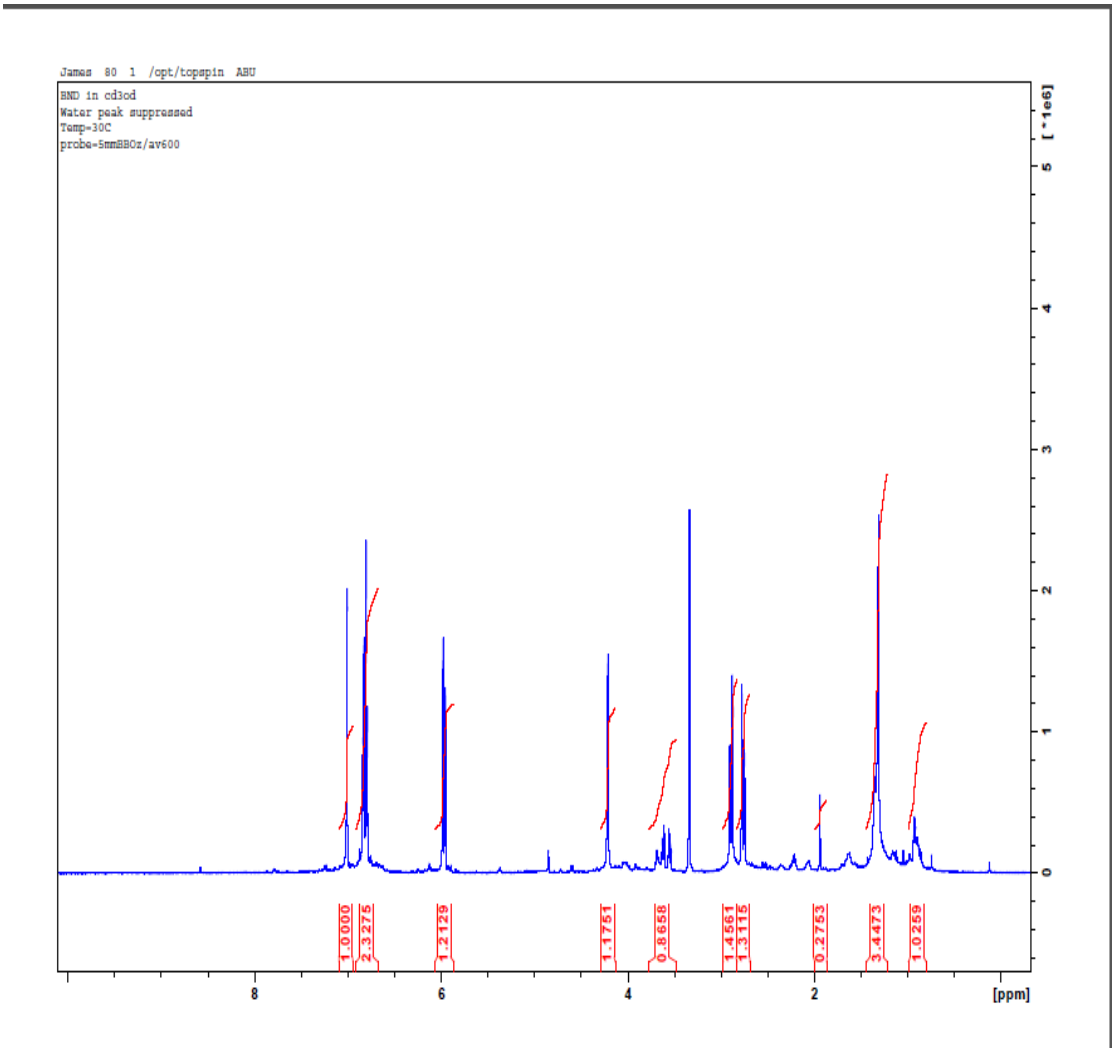
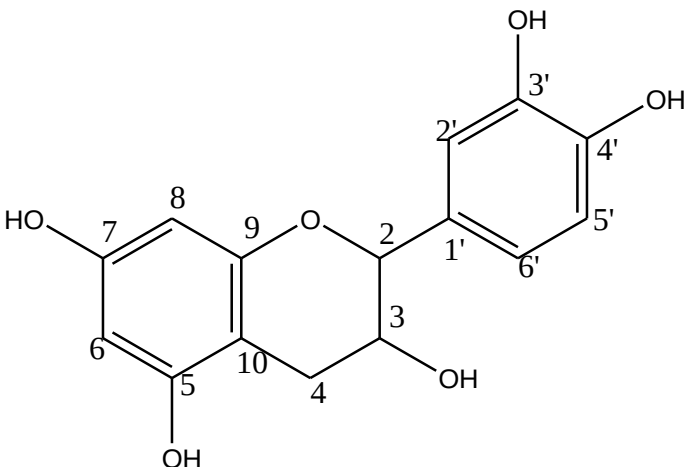


Figure 4.8a: Proton NMR spectrum of compound BND



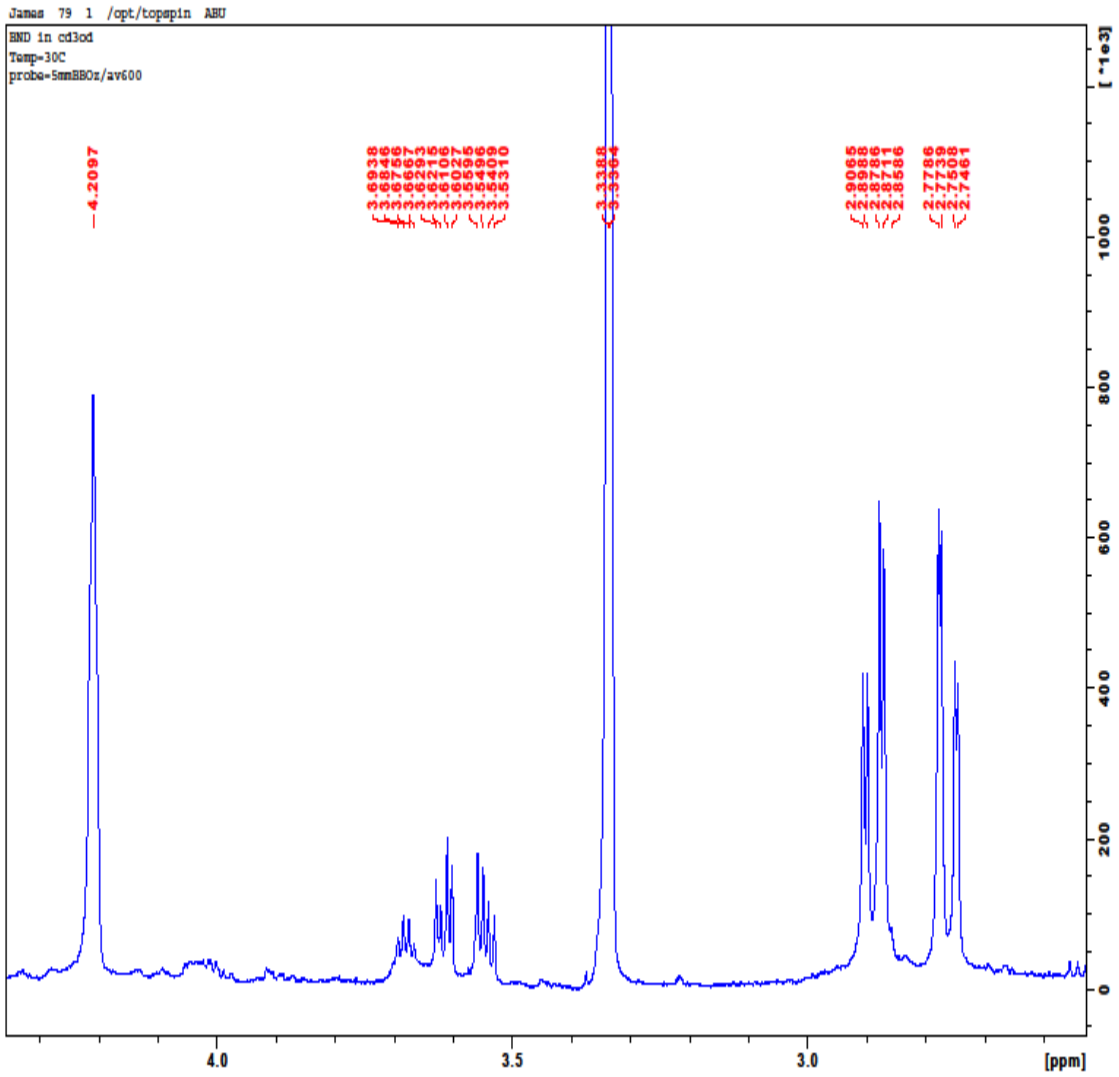
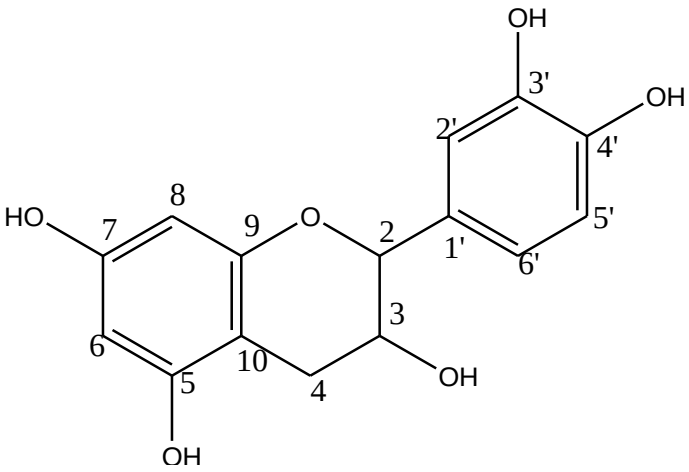


Figure 4.8b: Proton NMR spectrum of compound BND (expanded).



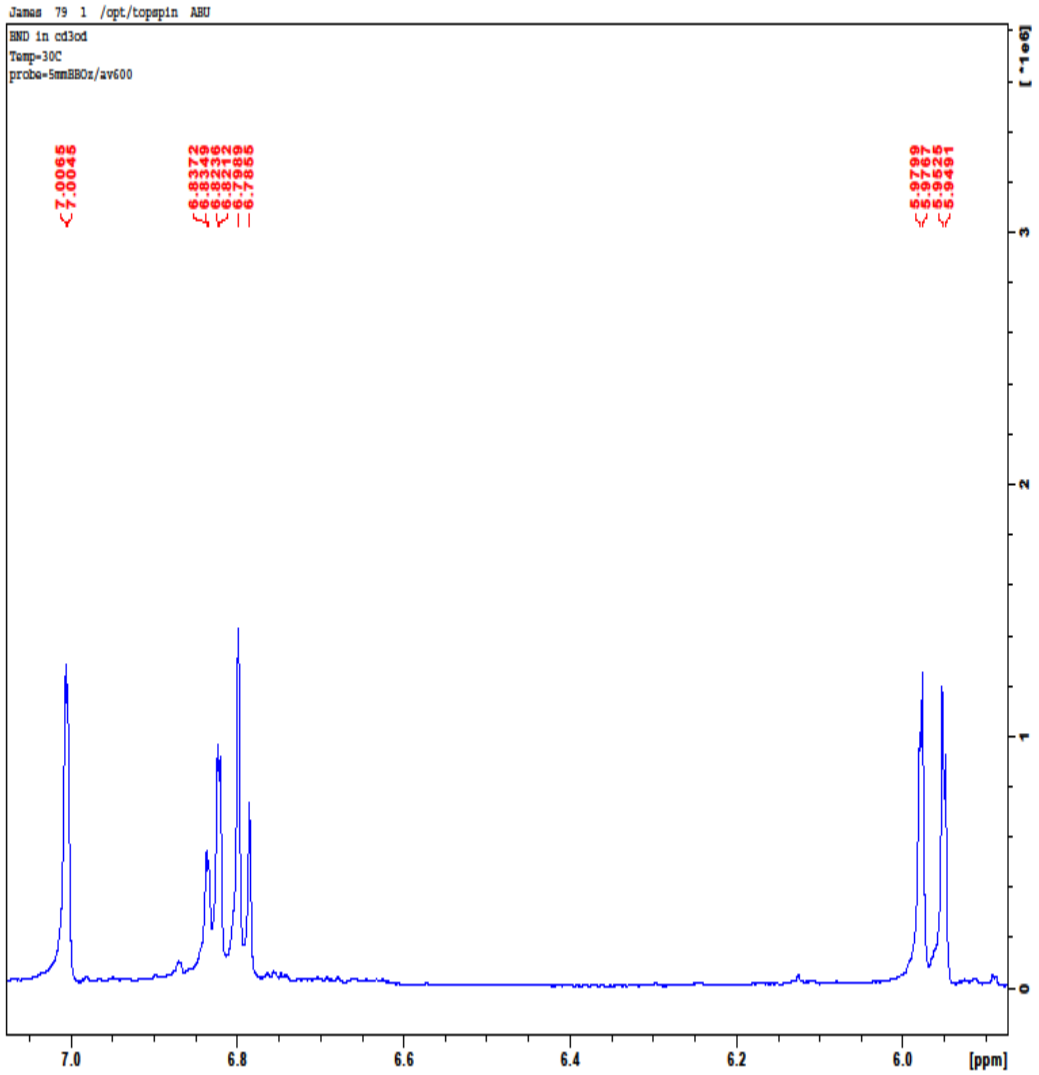


Figure 4.8c: Proton NMR spectrum of compound BND (expanded).

4.4.4.2 Carbon – 13 NMR analysis of Compound BND

The ^{13}C NMR spectrum of compound BND shows the following resonances (Figure 4.9, Table 4.9 and Table 4.10).

Table 4.11: ^{13}C NMR Analysis of Compound BND

□ C(ppm)	Position
78.5	C-2
66.1	C-3
27.9/28.3	C-4
155.9	C-5
95.1	C-6
156.3	C-7
94.6	C-8
156.6	C-9
98.8	C-10
130.9	C-1’
113.9	C-2’
144.4	C-3’
144.6	C-4’
114.6	C-5’
118.1	C-6’

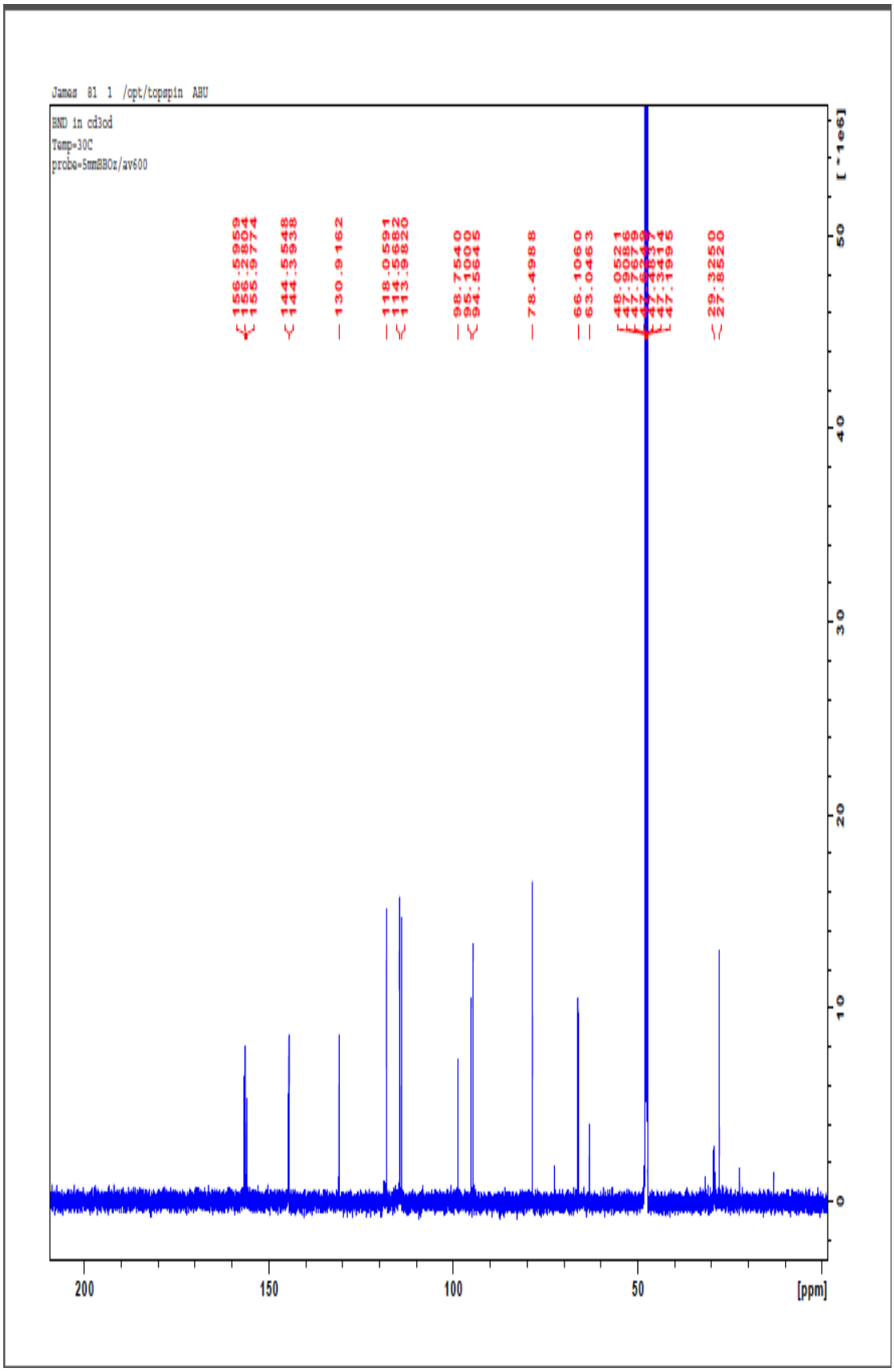


Figure 4.9: ^{13}C Spectrum of compound BND

4.4.4.3 DEPT Spectrum Analysis of BND

The DEPT spectrum analysis exhibited eighteen carbon atoms. This shows the presence of one methylene carbon atom and seven methine carbon atoms (Figure 4.10).

4.4.4.3 HSQC Spectrum Analysis of BND

The HSQC spectrum of compound BND was used to to assign the protons to their respective carbon atoms (Figure 4.11 and Table 4.10).

4.4.4.4 COSY Spectroscopic analysis of BND

The COSY spectrum of compound BND shows the $^{1}H - {}^{1}H$ correlation of protons situated in the same chemical environment (Figure 4.12).

Table 4.12: ^{13}C –NMR spectrum data of compound BND

^{13}C –NMR spectrum data of compound BND, shows the presence of 15 carbon atoms signals.

Position	DEPT	HSQC(^{1}Hppm)	^{13}C(ppm)
2	CH	4.8	78.5
3	CH	4.2	66.1
4	CH_2	2.7/2.8	27.9
5	C	-	155.9
6	CH	5.98	95.1
7	C	-	156.3
8	CH	5.95	94.6
9	C	-	156.6
10	C	-	98.8
1’	C	-	130.9
2’	CH	7.01	113.9
3’	C	-	144.4
4’	C	-	144.6
5’	CH	6.79	114.6
6’	CH	6.83	118.1

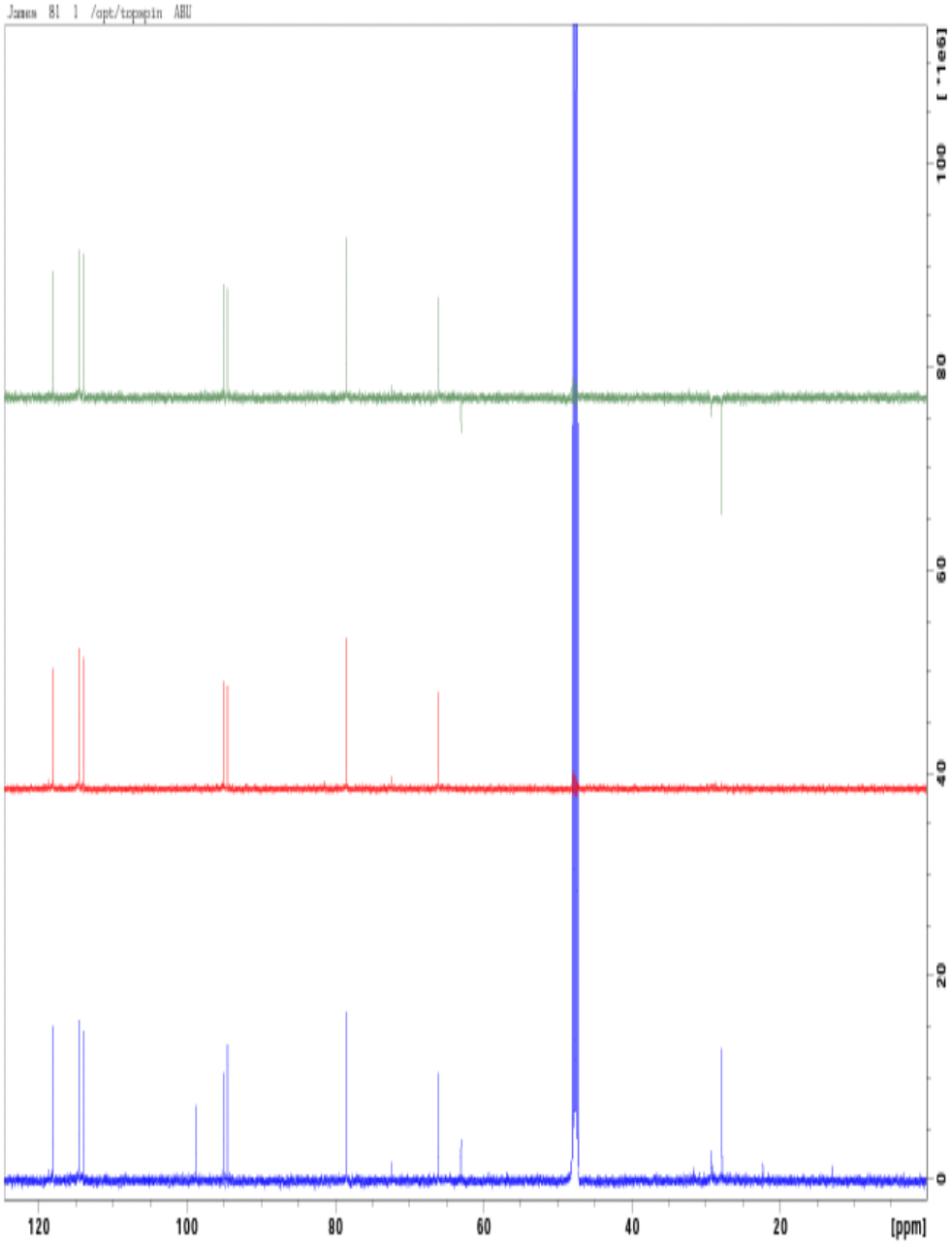


Figure 4.10: DEPT Spectrum of Compound BND

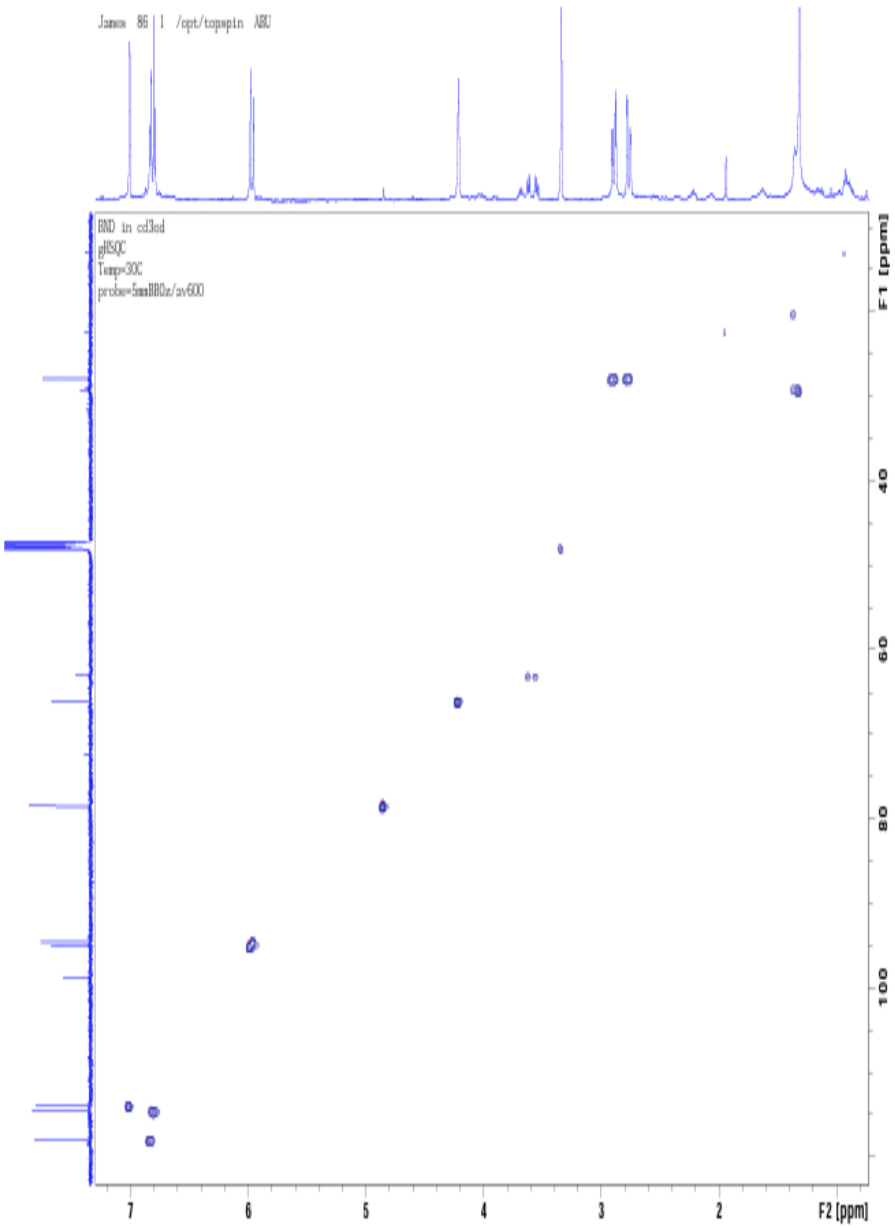


Figure 4.11: HSQC Spectrum of compound BND

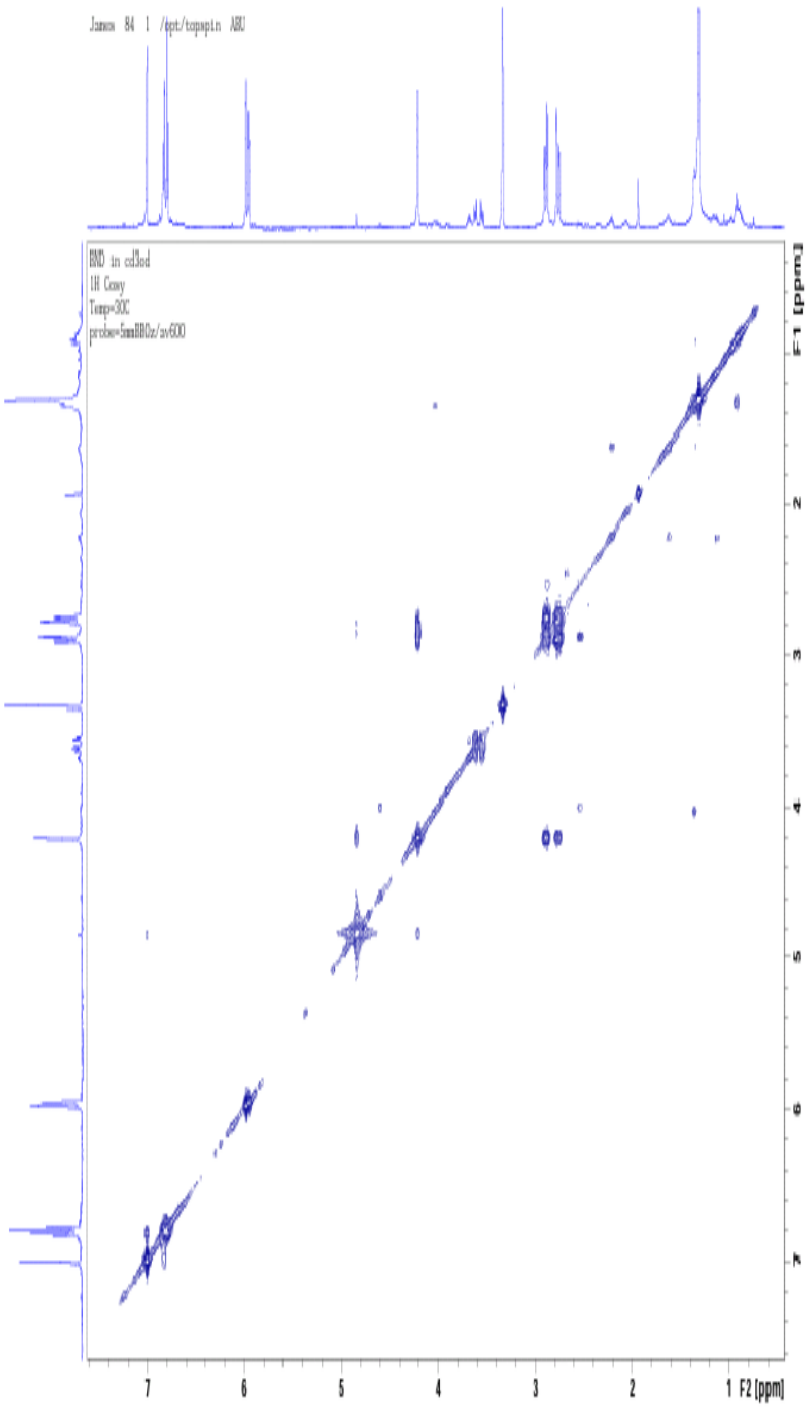


Figure 4.12: COSY Spectrum of Compound BND.

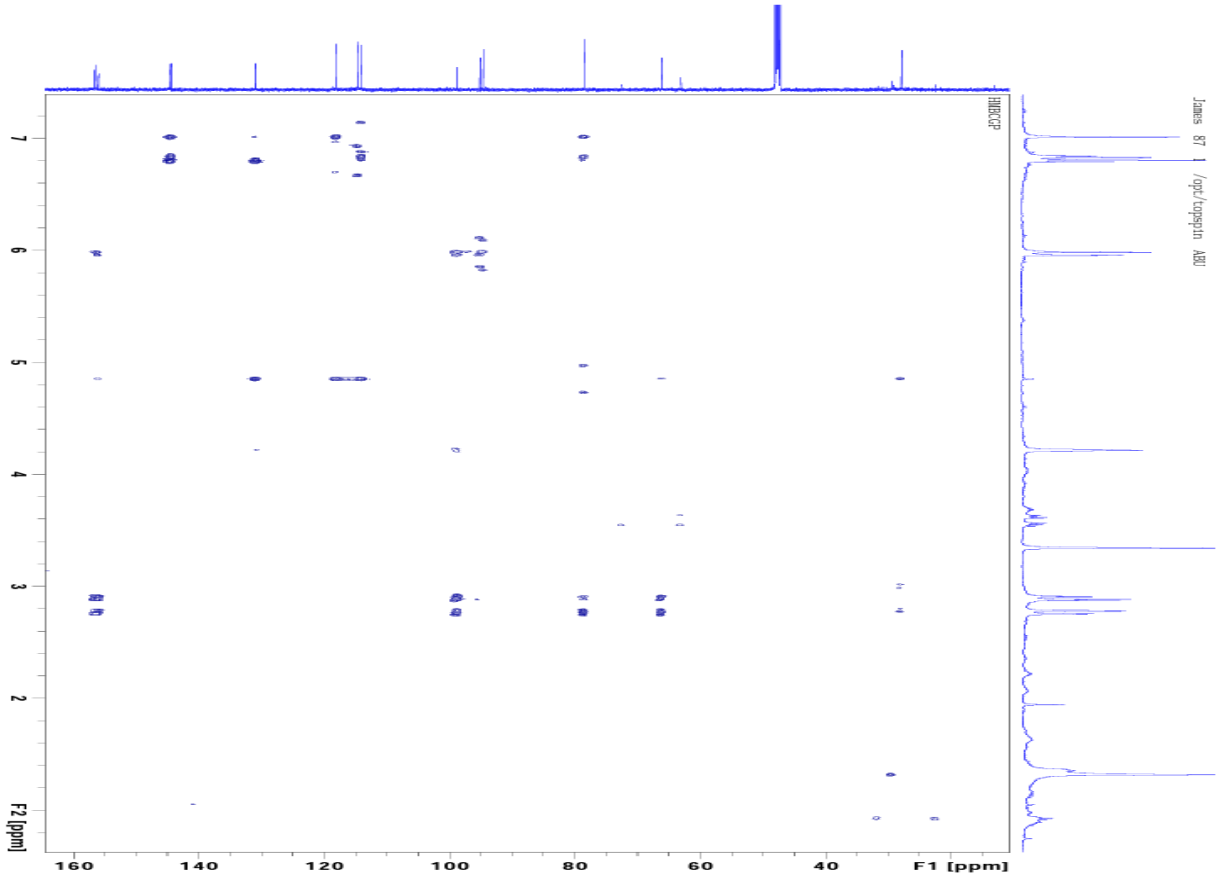
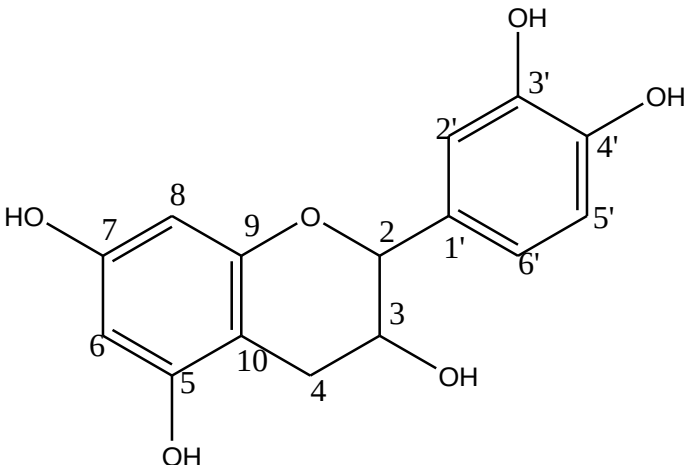


Figure 4.13: HMBC spectrum of Compound BND

4.4.4.5 Structure **of Compound BND**



2 – (3', 4' – Dihydroxy – phenyl – chroman – 3,5,7 – triol (Catechin).

4.5 Compound BNF

Compound BNF was isolated using Gel Filteration Technique with Methanol as the eluting solvent. It was isolated as a yellow powdery solid.

4.5.1 Some properties of BNF

The following observations were made on the properties of BNF

Table 4.12 : Some Properties of BNF

Property	**Observation**
Quantity	4.8mg
Appearance	yellow solid
Solvent of solubility	methanol
M.P	329-331^{0}C
RF value	0.811
Solvent system	Chloroform: Ethyl acetate: Methanol (4:8:2)
Chemical test	Positive to shinoda's test.

The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110^{0}C for 5min.

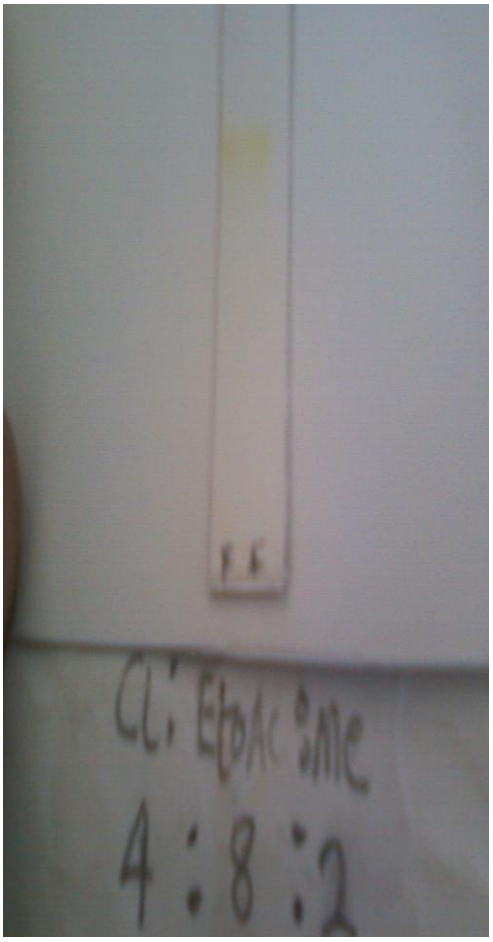


Plate 4.4: TLC profile of compound BNF

4.5.2 Spectral Analysis of Compound BNF

4.5.2.1 Proton NMR Analysis of Compound BNF

The ^{1}H NMR spectrum of compound BNF shows the following resonances as shown in the table below (Figure 4.15 and Table 4.12).

Table 4.13 : Proton NMR Analysis of Compound BNF

^{1}H(ppm)	Position, J-value (Hertz)
6.42	1H, S, I-C-3
6.18	1H, S, I-C-6
6.42	1H, S, I-C-8
7.67	1H, S, I-C-2')
7.06	1H, d, J= 8.5, I-C-5'
7.76	1H, d, J= 8.5, I-C-6'
4.10	1H, S, I-C-1"
1.34	3H,S, I-C-2"
6.64	1H, S, II-C-3
6.16	1H, S, II-C-6
6.38	1H, S,II-C-8
7.96	2H, d, J= 8.3, II-C-2'/6'
7.12	2H, d, J= 8.3, II-C-3'/5'
1.99	2H, S, II-C-1"
3.68	1H, d, J=6, II-C-2"
3.55	2H, d II-C-3"

4.5.2 ^{13}C NMR Analysis of BNF

The ^{13}C NMR Spectrum of compound BNF shows the presence of the following resonances (c) in ppm;

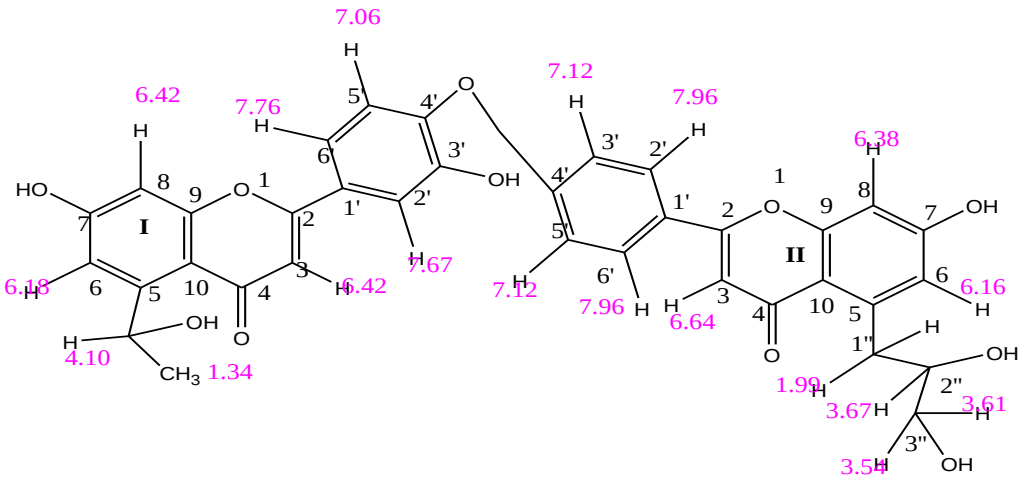
22.3, 29.5, 63.0, 68.2, 72.5, 94.3, 94.5, 99.6, 99.8, 103.2, 116.3(2C), 120.2, 124.76, 124.83, 127.8(2C), 143.4, 158.1, 163.8, 164.04, 178.9, 182.1. (Figure 4.16 and Table 4.13).

4.5.3 DEPT Spectroscopic analysis of BNF

The DEPT spectroscopic analysis of compound BNF revealed eighteen carbon atoms. This shows the presence of a single methyl carbon atom at c 22.75, two methylene carbon atoms at c 29.45 and 63.02 and fifteen methine carbon atoms at c 68.16, 72.46, 94.31, 94.47, 99.6, 99.8, 102, 103.2, 116.3(2C), 118, 120.2,124.8, and 127.8 (2C), (fig. 4.17).

4.5.4 HSQC Analysis of compound BNF

The HSQC spectrum analysis of compound BNF was used to assign the protons to their respective carbon atoms (Figure 4.17 and Table 4.14).



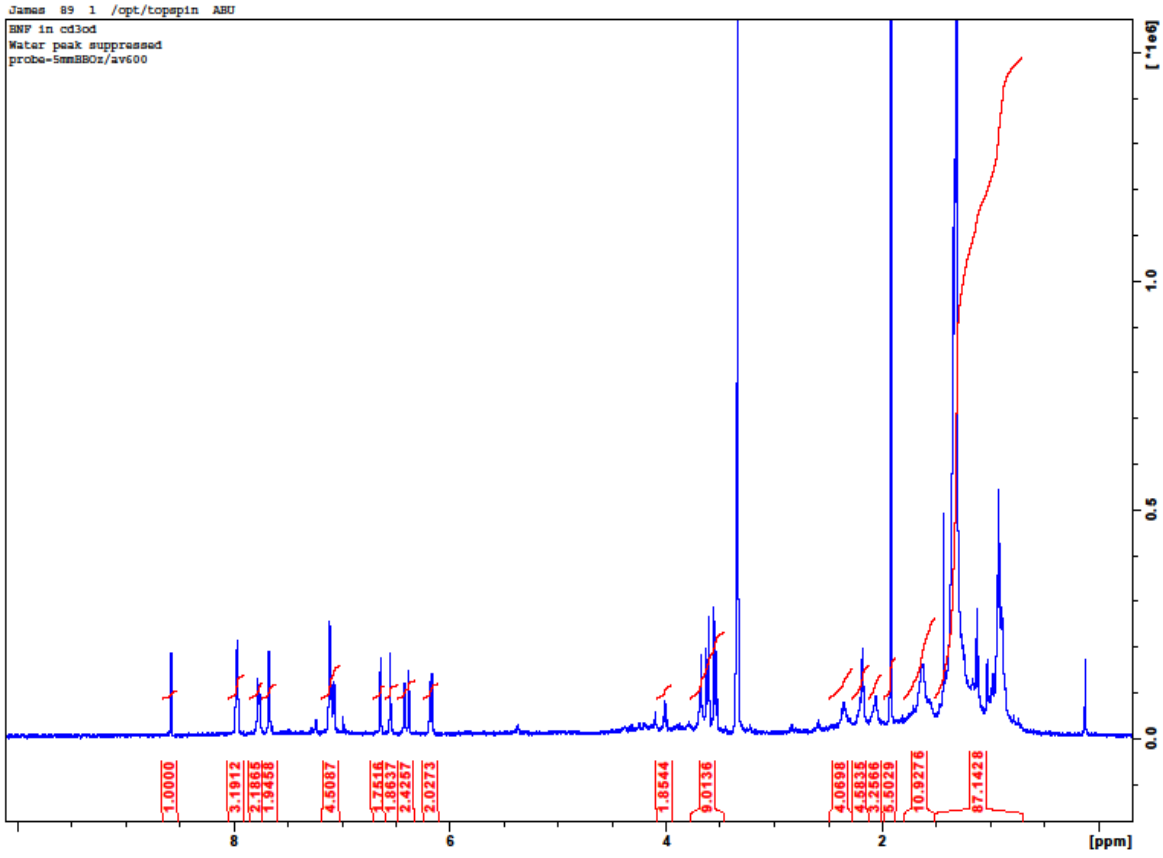


Figure 4.14a: Proton NMR of Compound BNF

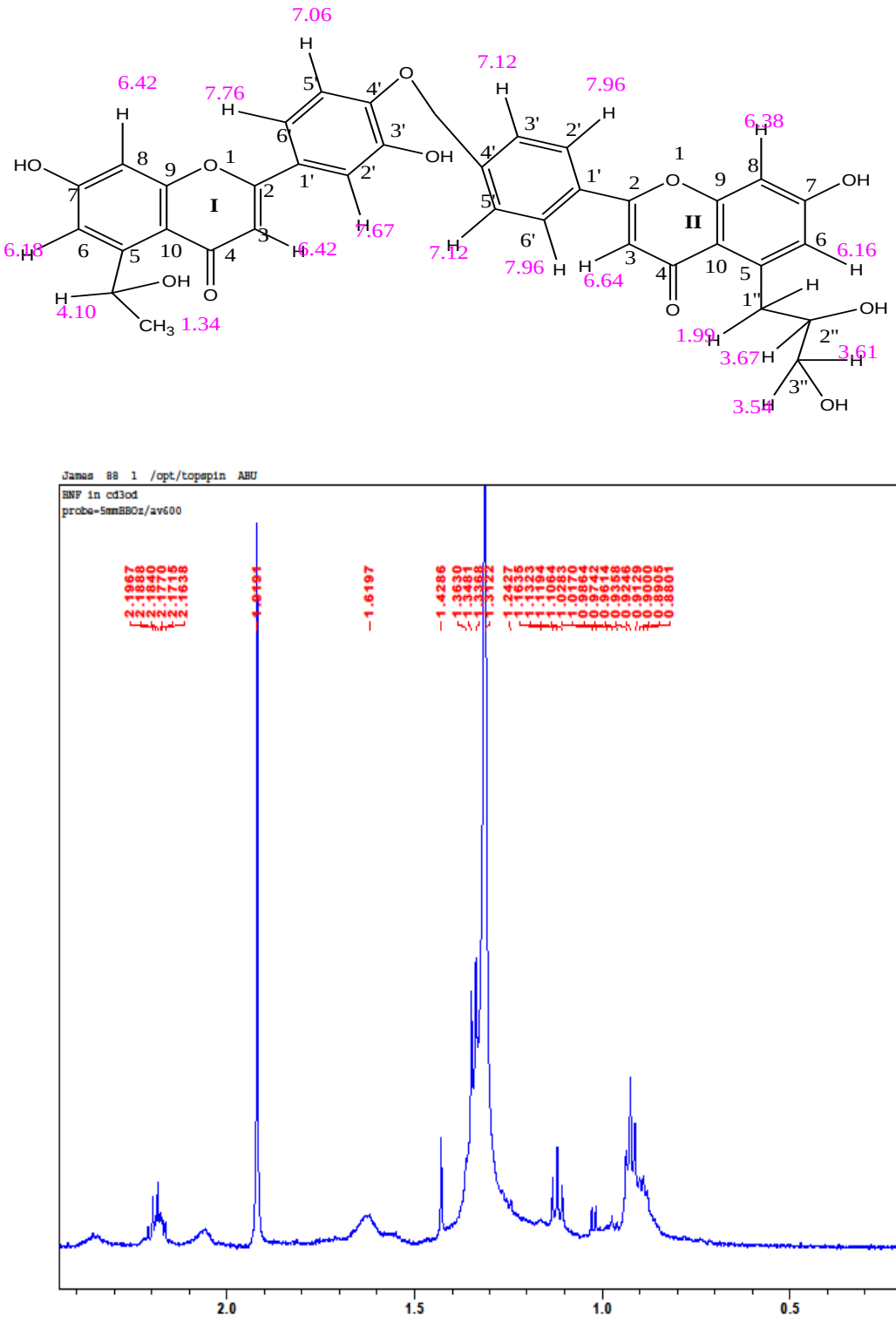
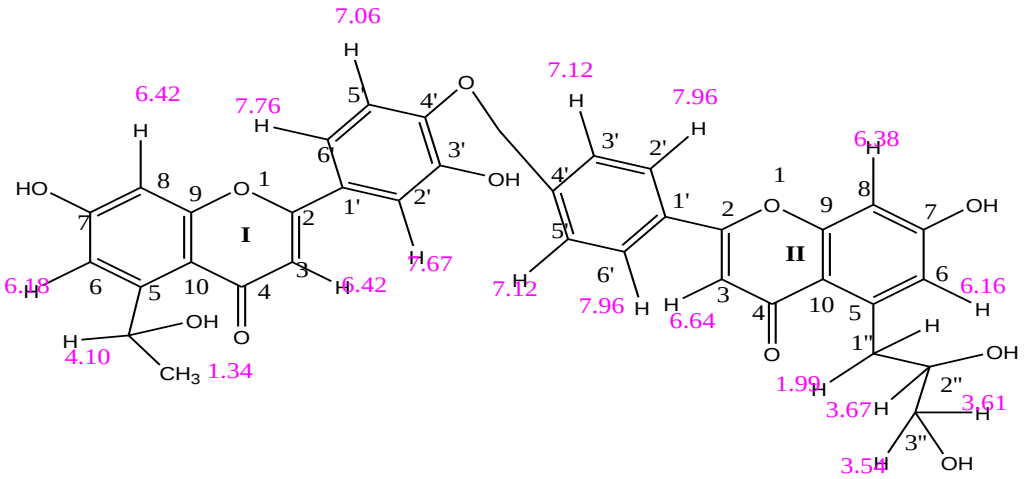
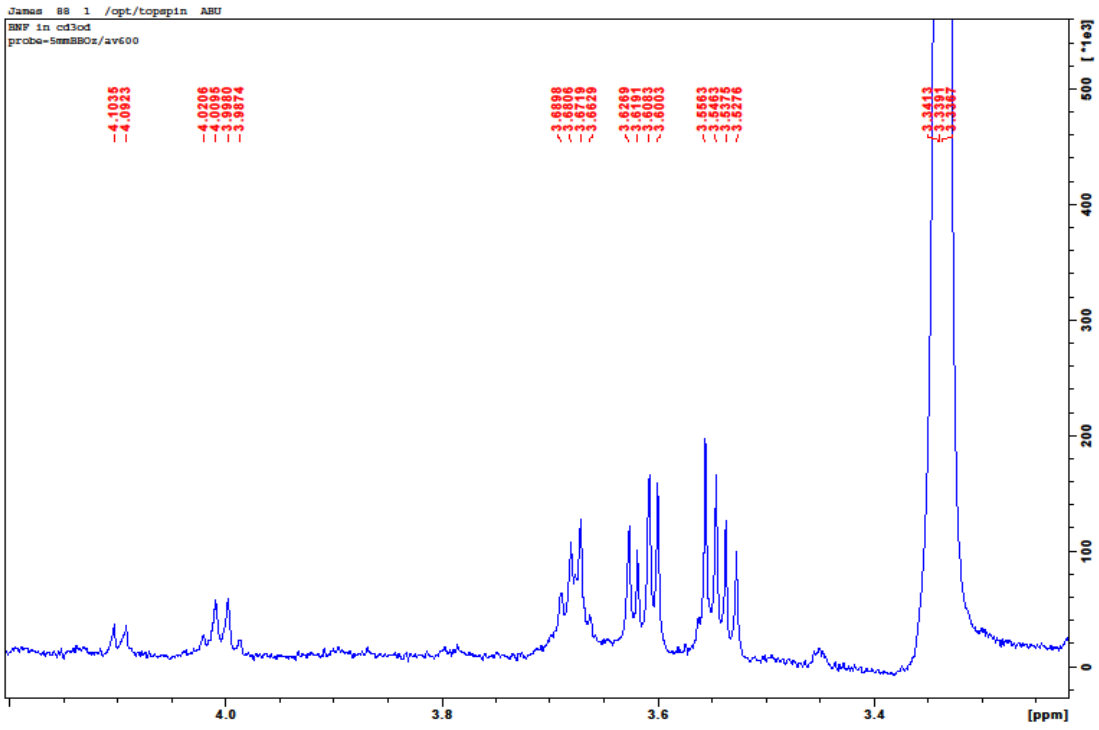
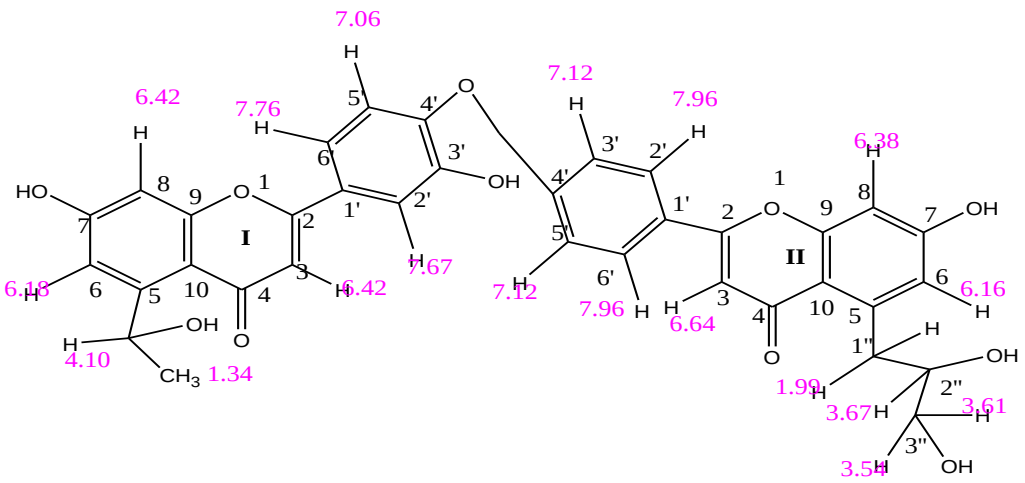


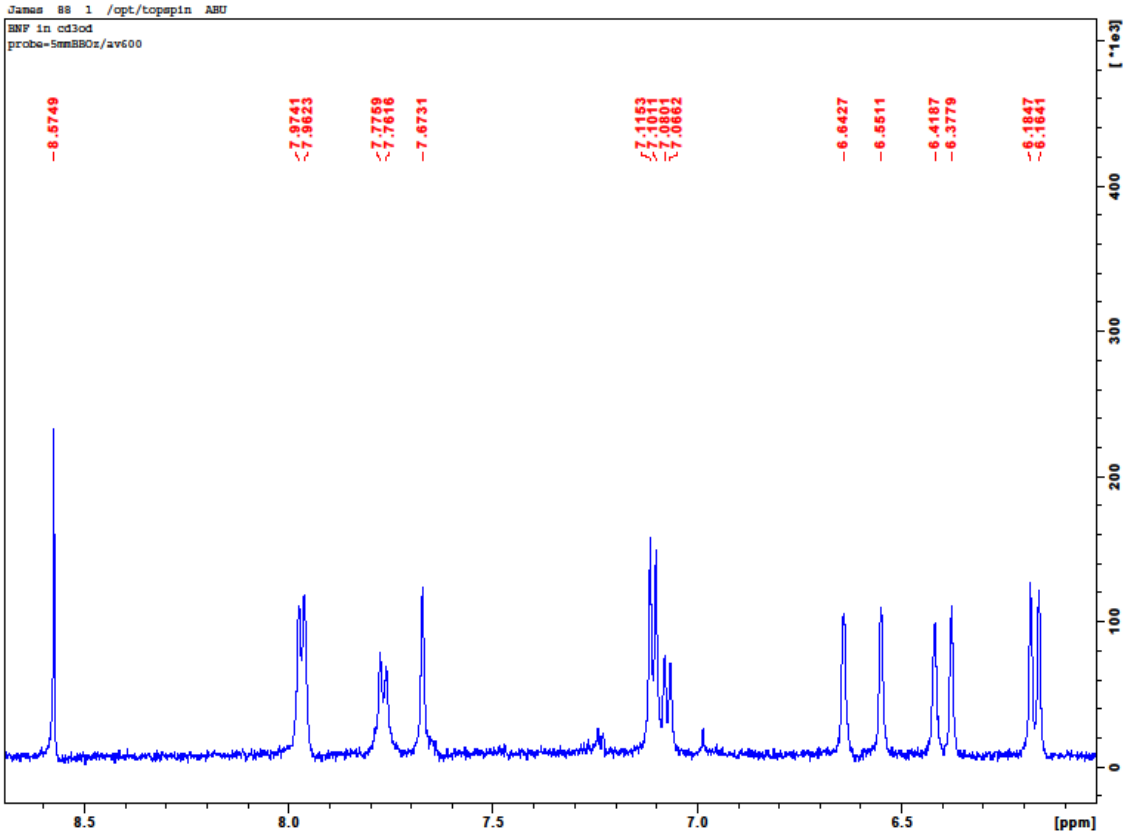
Figure 4.14b: Proton NMR of Compound BNF (expanded).





Fiure 4.14c : Proton NMR of Compound BNF (expanded).





Fiure 4.14d Proton NMR of Compound BNF (expanded).

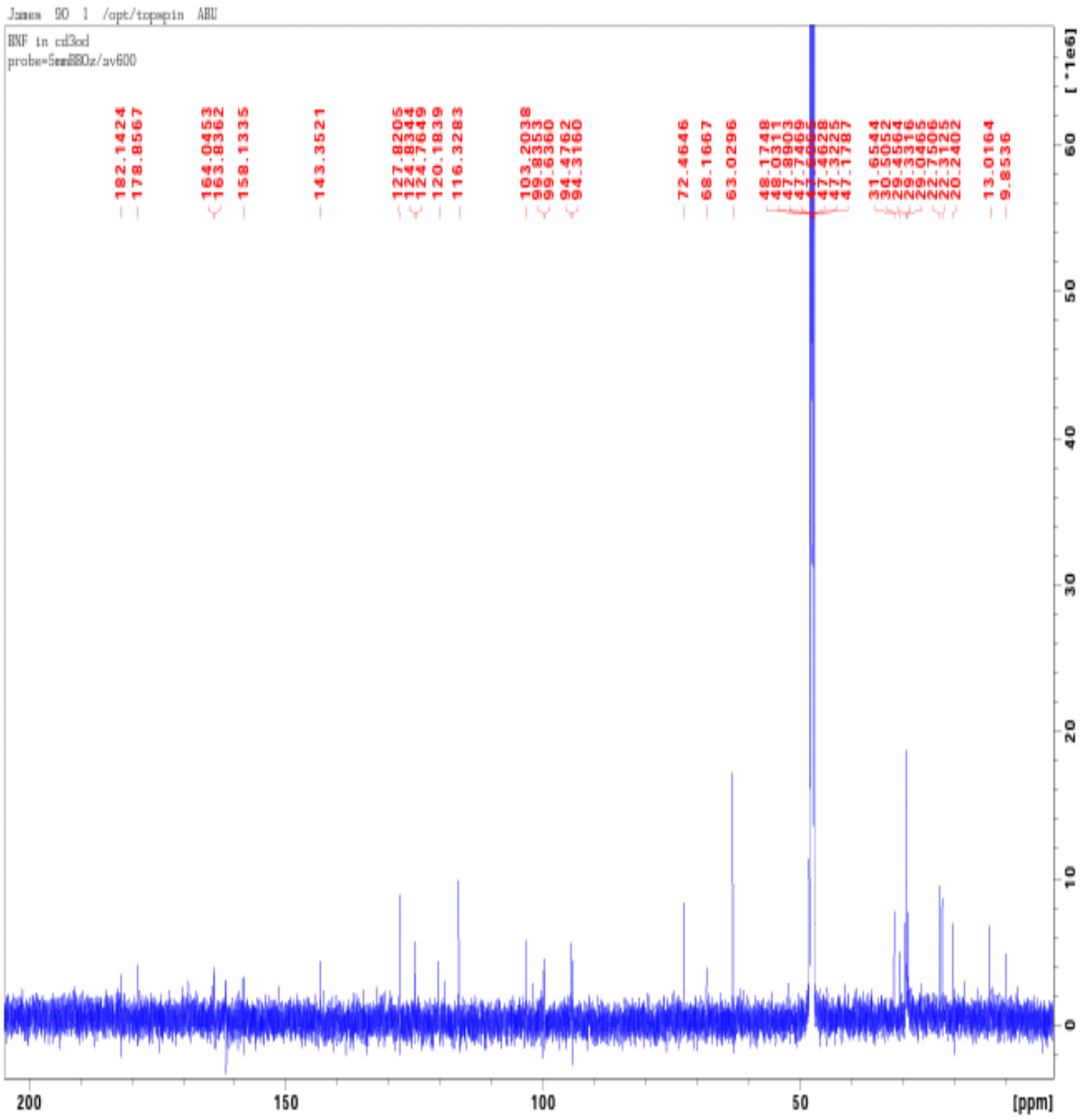


Fig. 4.15: Carbon –13 NMR spectrum of Compound BNF

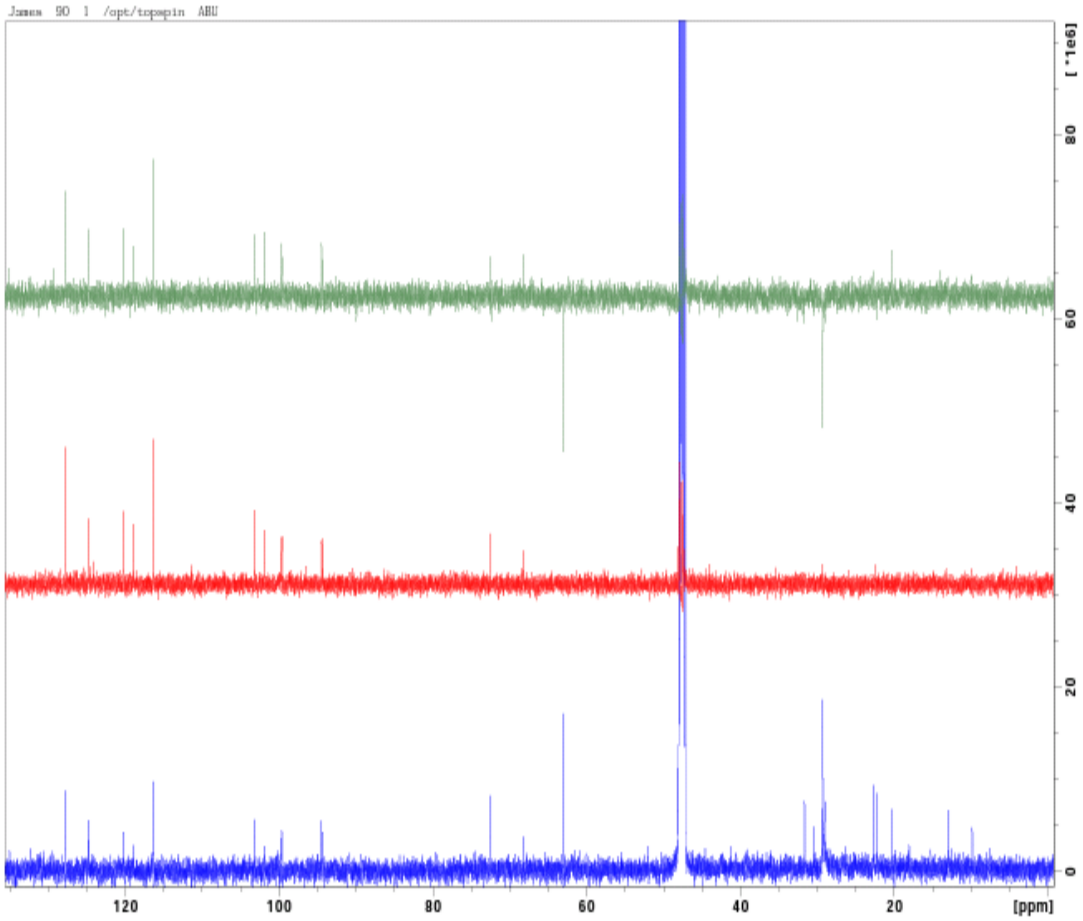


Fig. 4.16: DEPT Spectrum of Compound BNF

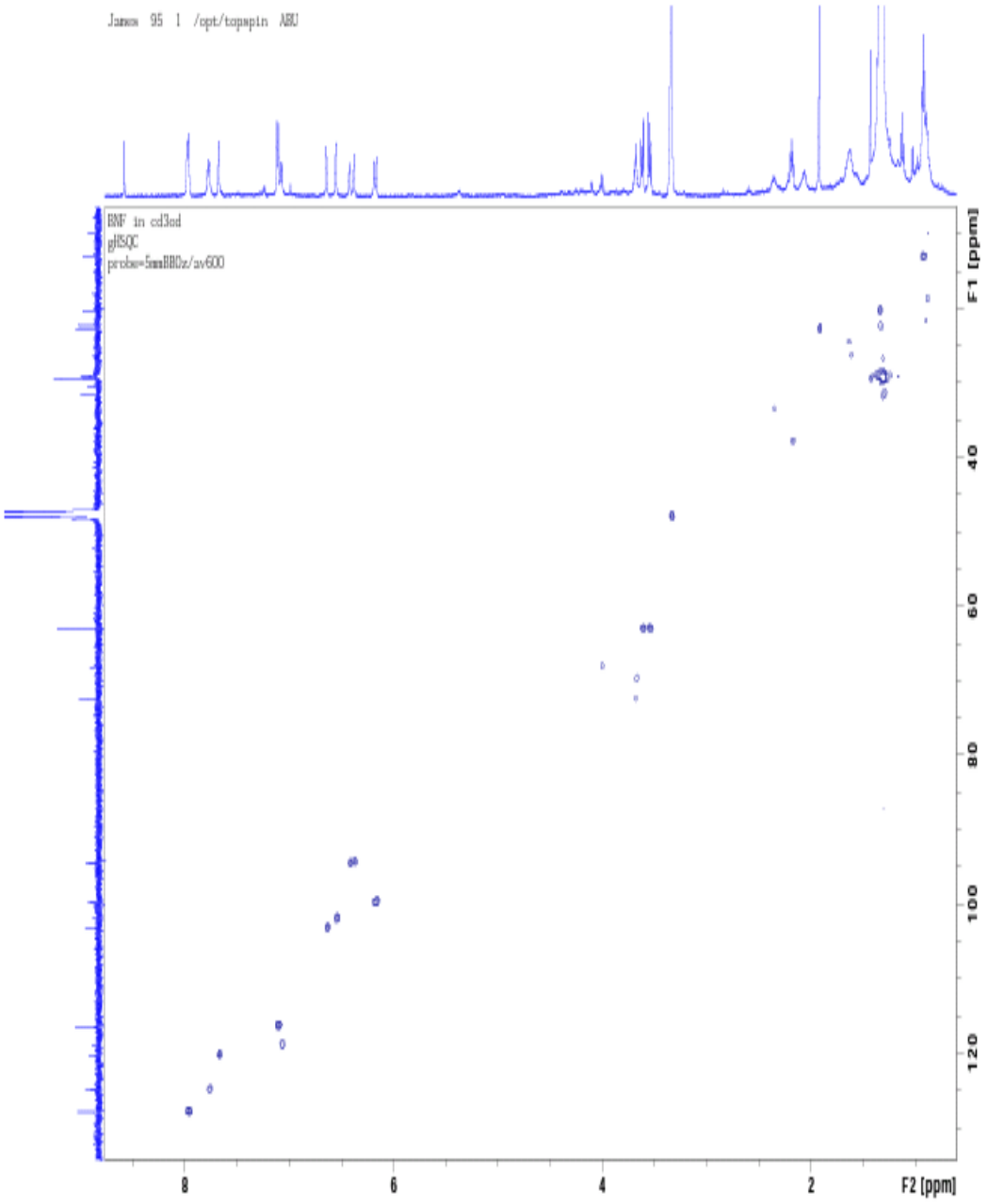


Fig. 4.17: HSQC Spectrum of Compound BNF

4.5.5 The COSY spectrum analysis of BNF

The COSY spectrum of compound BNF shows the ^{1}H-^{1}H correlations of protons situated in the same environment (Figure 4.19).

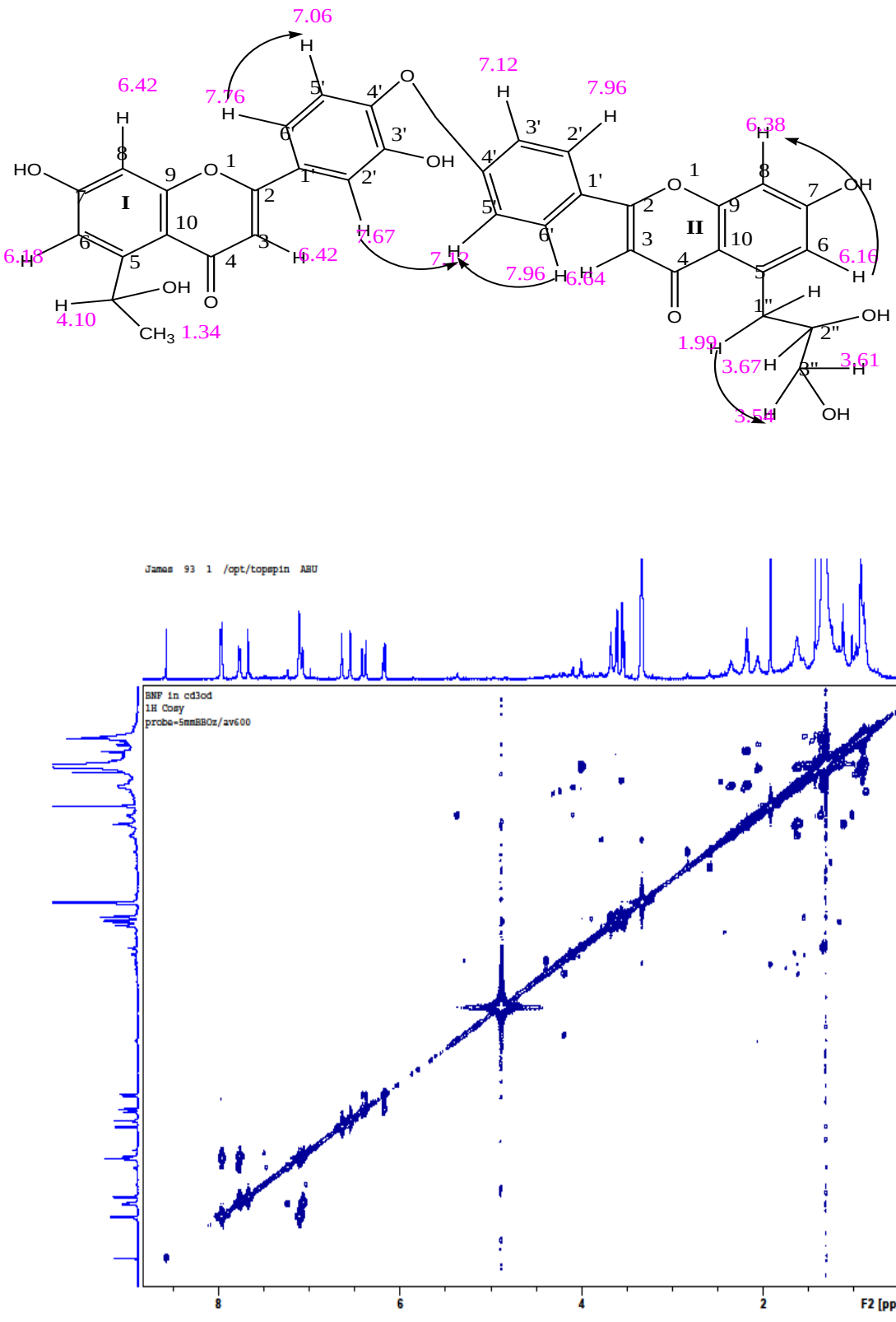
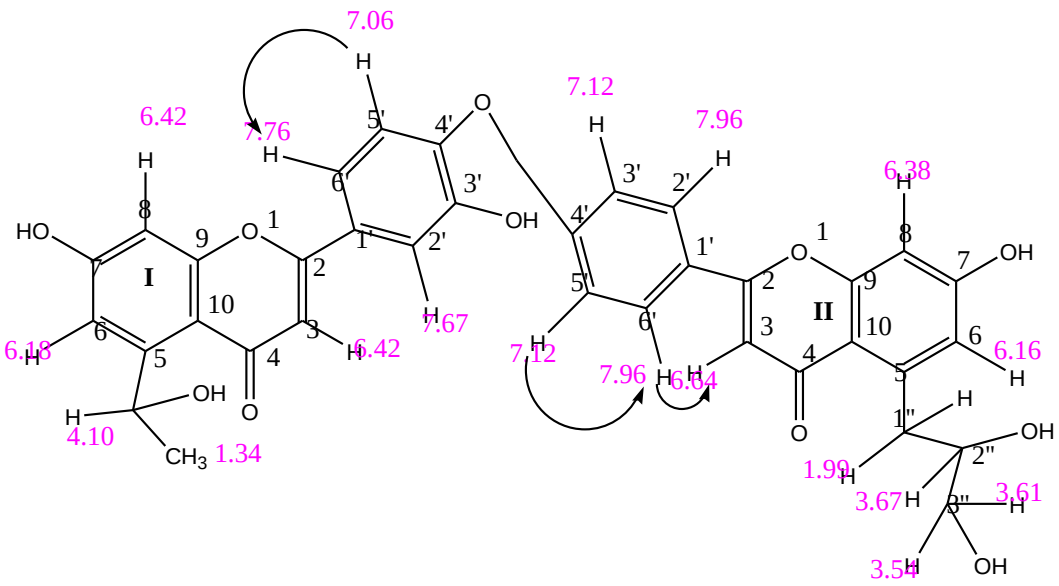


Fig. 4.19: COSY spectrum of BNF

4.5.6 NOESY Spectroscopic analysis of BNF

The NOESY spectrum of compound BNF shows the $^{1}H - {}^{1}H$ correlation of protons situated in the same chemical environment by two to three bonds (Figure 4.20).



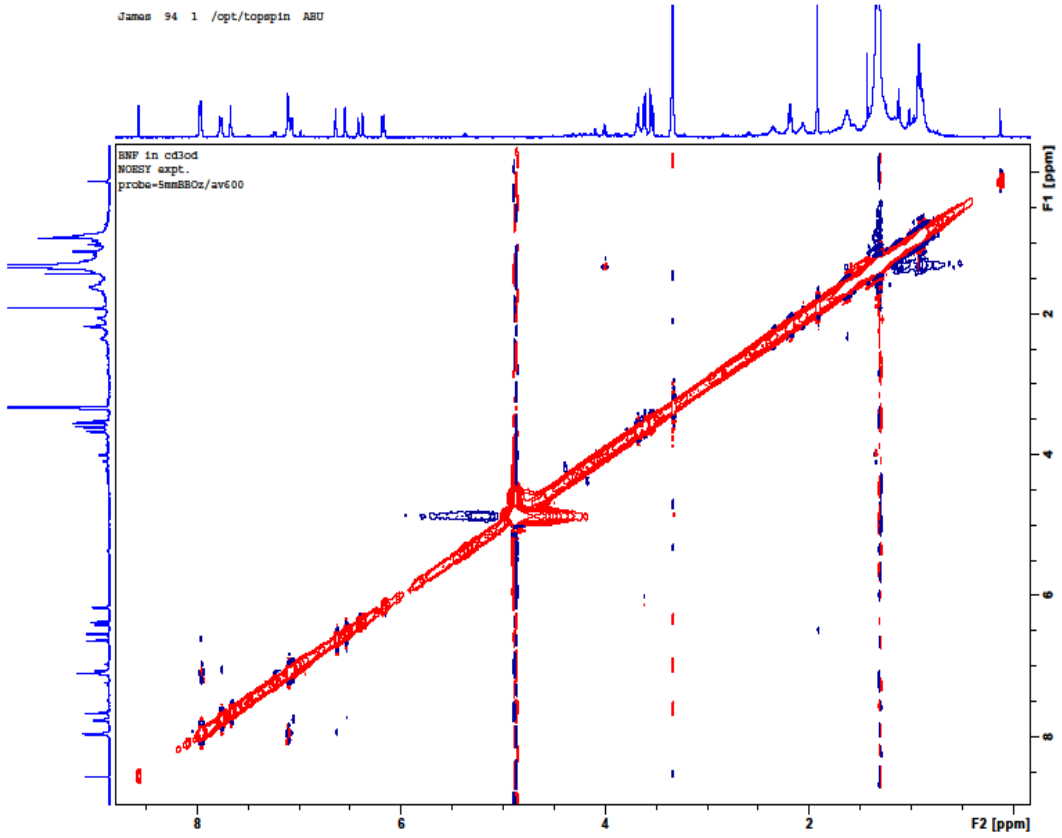


Figure 4.19: NOESY Spectrum of compound BNF

4.5.6 HMBC analysis of BNF (Figure 4.19)

The C-13, DEPT and HMBC analysis of compound BNF is shown in the table below. (Table 4.13 and figure 4.21).

Table 4.13 HMBC OF BNF

	Position	□ Hppm	□ C(ppm)	DEPT
	2	-	158.1	-
	3	6.64	103.2	CH
	4	-	182.1	C=O
	5	-	164.0	C
	6	6.18	99.8	CH
	7	-	163.8	C-O
	8	6.42	94.3	CH
	9	-	163.8	C
I	10	-	102	C
	1’	-	124.83	C
	2’	7.67	120.2	CH
	3’	-	143.4	C-O
	4’	-	143.4	C-O
	5’	7.06	118	CH
	6’	7.77	124.76	CH
	1’’	4.10	68.12	CH
	2’’	1.34	22.75	CH_3
	2	-	163.8	C
	3	6.55	102	C
	4	-	178.9	C=O
	5	-	158.1	C
	6	6.16	99.6	CH
	7	-	163.8	C
	8	6.38	94.2	CH
	9	-	164.0	C
II	10	-		C
	1’	-	124.83	C
	2’	7.96	127.8	CH
	3’	7.12	116.3	CH
	4’	-	158.1	C-O
	5’	7.12	116.3	CH
	6’	7.96	127.8	CH
	1’’	1.99	29.4	CH_2
	2’’	3.68	72.46	CH
	3’’	3.55	63.2	CH_2

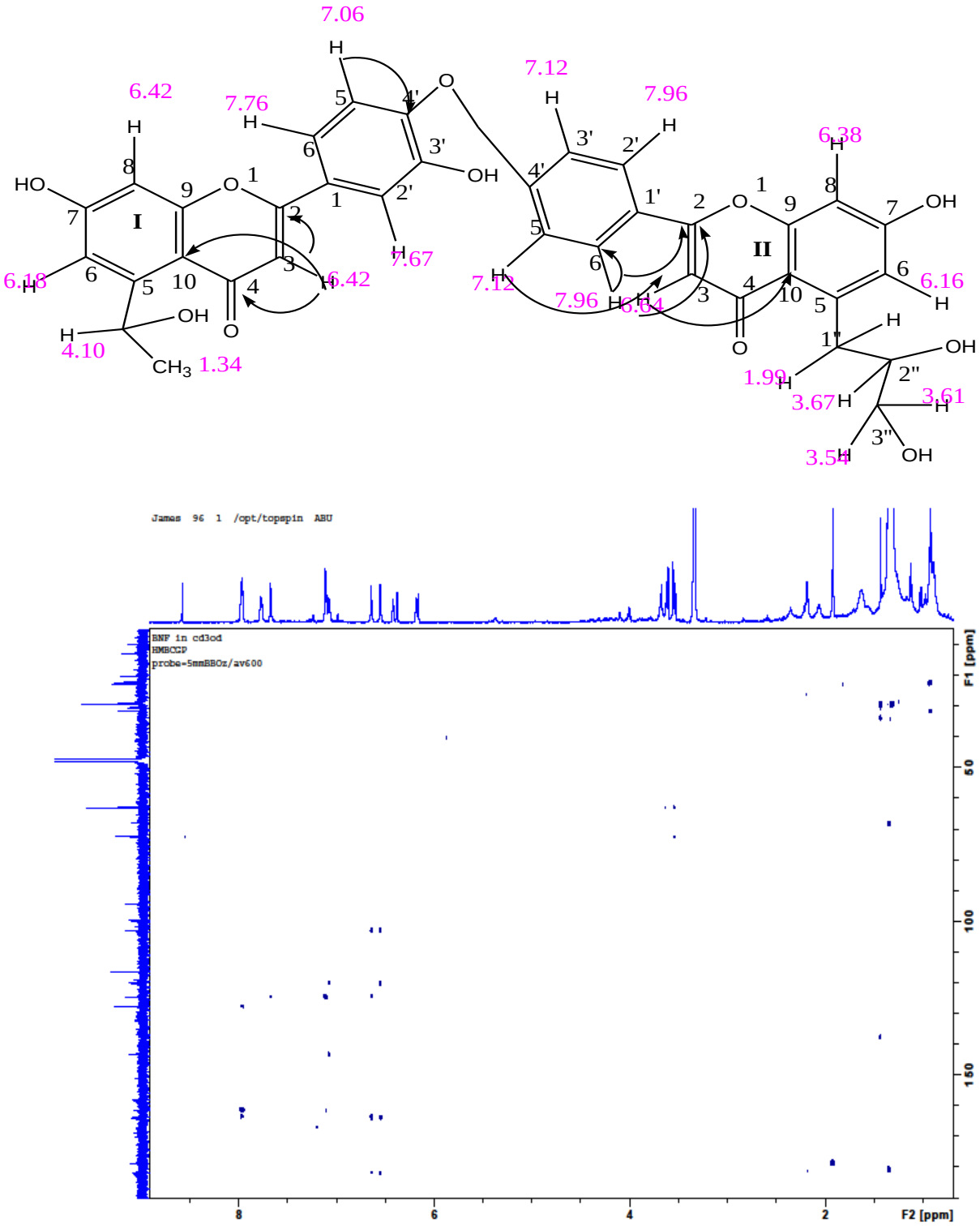
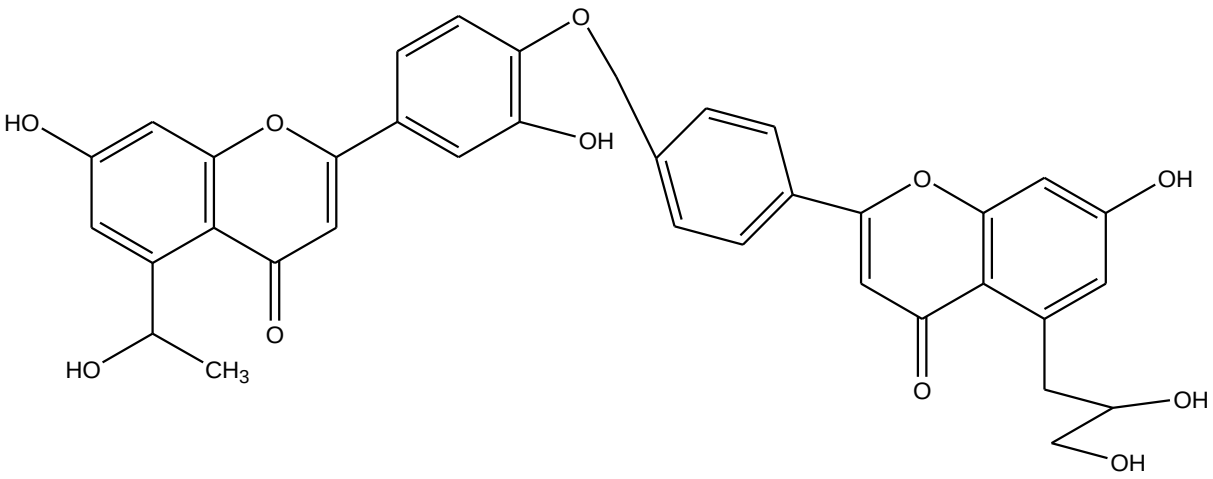


Fig. 4.20: HMBC Spectrum of Compound BNF

4.5.5 Structure of Compound BNF



4.6 RESULT OF PHARMACOLOGICAL STUDIES.

4.6.1 Result of acute toxicity study (LD_{50})

Table 4.15: Result of acute toxicity study (LD_{50})

Group	Dose of extract (mg/kg)	No of mice	No of Dead
1	140	6	0
2	225	6	0
3	370	6	0
4	600	6	6

LD_{50} = √highest LD x √lowest non LD x 30%

= √600 x √370 x 30 /100

= 86mg/kg

The LD_{50} values of *Ochna rhizomatosa* acetone extract in albino mice was found to be 86 mg/kg.

4.6.2 Antimalarial activities of the crude acetone extract.

4.6.2.1 Result of blood schizonticidal activity of acetone extract in suppressive and curative test.

The results obtained for each experimental procedure are as presented in the Tables 4.16 and 4.17.

Table 4.16: Showing the effect of suppressive test on *Plasmodium beighei* parasite.

Treatment	Dose (mg/kg)	Mean ± SEM of parasitaemia	%Parasitaemia Inhibition	Significant Difference (t- test)
N/S	0.2*	3.75 ± 0.09		
Extract	21.5	2.25 ± 0.84	40	p>0.05
Extract	43	2.25 ± 0.09	40	p>0.5
Extract	86	2.25 ± 0.41	40	p>0.05
Chloroquine	5	1.17 ± 0.29	68.8	P<0.05

Key: * = ml

4.6.2.2 Result of blood schizonticidal activity of acetone extract in curative test.

Table 4.17: Showing the effect of curative test on *Plasmodium beighei* parasites.

Treatment	Dose (mg/kg)	Mean ± SEM of parasitaemia	% Parasitaemia Inhibition	Significant difference (t- test)
N.S	0.2*	3.75 ± 0.55	_	_
Extract	21.5	2.20 ± 0.55	41.33	p>0.05
Extract	43	2.16 ± 0.58	42.40	p>0.05
Extract	86	2.00 ± 1.15	46.66	p>0.05
Chloroquine	5	1.17 ± 0.29	68.8	P<0.05

Key: * = ml

4.6.3 Result of antimicrobial studies of crude extract and compound MNC

Table 4.18: Zone of inhibition of the extract and compound MNC against the test microorganisms.

Test organism	Crude (60mg/ml)	MNC (100µg/ml)	Ciprofloxacin (100mg/ml)	Fluconazole (5mg/ml)
Staphylococus aureus	23	27	37	
Streptococcus pyogenes	0	0	35	
Corynebacterium ulcerans	0	0	35	
Escherichia coli	25	31	35	
Strigella dysenteriae	29	32	37	
Salmonella typhi	24	30	41	
Proteus mirabilis	0	0	0	
Pseudomonas aeruginosa	0	0	0	
Klebsiella pneumomiae	27	34	39	
Candida albicans	26	30	0	35
Candida brusei	0	0	0	34
Candida tropicalis	28	29	0	32

4.6.1.3 Antimicrobial activities of the crude acetone extract and compound MNC.

Table 4.19: Minimum Inhibition Concentration (MIC) of acetone extract and compound MNC

Test organism	Extract (mg/ml)					MNC (µg/ml)				
	60	30	15	7.5	3.75	100	50	25	12.5	6.25
S. aureus	-	-	*	+	++	-	-		*	+
E. coli	-	-	*	+	++	-	-	-	*	+
S. dysentriae	-	-	-	*	+	-	-	-	*	+
S. typhi	-	-	*	+	++	-	-	-	*	+
K. pneumoniae	-	-	-	*	+	-	-	-	*	+
C. albicans	-	-	*	+	++	-	-	-	*	+
C. tropicalis	-	-	-	*	+		-	-	*	+

KEY: -- = No turbidity (No growth), * = MIC,

\+ = turbid (light growth), ++ = moderate turbidity,

+++ = high turbidity

Table 4.20: Minimum Bacticidal / Fungicidal concentration of the acetone extracts

and compound MNC against the test microbes.

Test organism	Extract(mg/ml)					MNC (µg/ml)				
	60	30	15	7.5	3.75	100	50	25	12.5	6.25
S. aureus	-	+	++	+	+++	-	*	+	++	+++
E. coli	-	*	+	++	+++	-	-	-	*	+
S. dysentriae	-	-	*	+	++	-	-	*	+	++
S. typhi	*	+	++	+++	++++	-	-	-	*	+
K. pneumonia	-	-	-	*	+	-	-	-	*	+
C. albicans	-	-	*	+	++	-	-	-	*	+
C. tropicalis	-	-	-	*	+	-	-	-	*	+

Key:

-- = No turbidity(No growth)

* = Minimum inhibitory concentration

\+ = Turbid (light growth)

++ = Moderate turbidity

+++ = High turbidity

CHAPTER FIVE

5.0 DISCUSSION

The plant material (500g) yielded 51.15g of the acetone extract, equivalent to 10.23% which was a dark brown gummy mass. The mass gave dark green colour with alcoholic ferric chloride, orange colour with Zn –hydrochloric acid test. This clearly indicates that this extract contains flavones nucleus. Preliminary phytochemical examination of the extract of the leaves of *Ochna rhizomatosa* indicates the presence of flavonoids, glycosides, tannins, saponins steroids and carbohydrates. However alkaloids are found to be absent in this specie (Table 4.1). The extract was subjected to chromatographic procedures, which led to the isolation of three compounds coded MNC, BND and BNF. The three compounds were subjected to spectroscopic analysis.

5.1 Isolated Compounds

5.1.1 Compound MNC

Compound MNC was isolated as yellow amorphous solid weighing 25mg and gave a positive result for flavonoids.

Compound MNC was isolated in its aglycone form as the absence of glycoside derivative is confirmed by the complete absence of glycosyl signals in the chemical shift range of 4-5ppm.

The presence of a *p*-substituted aromatic ring, a trisubstituted aromatic ring and two tetrasubstituted aromatic rings were established by careful examination of ^{1}H NMR (Figure 4.1) and the ^{1}H - ^{1}H COSY (Figure 4.5) spectra of MNC. This compound was clearly a triflavonoid which consisted of one flavonone unit and two flavone units. The ^{1}H NMR spectrum (Figure 4.1) showed the presence of one ABX spin system of a flavanone moiety at δ_H 5.44 (dd, *J*=2.1, 12.6 Hz) and 3.12 (dd, *J*=12.6, 17.1 Hz), 2.78 (dd, *J*=2.1, 17.1 Hz) for I- H-2 and 3, respectively.

Strong indication of a triflavonoid was in the ^{13}C NMR spectrum (Figure 4.2), which showed the presence of four carbonyl carbon signals, indicated by the peaks at δ_C 195.4, 182.12, 178.79 and 169.5 ppm with the last (169.5 ppm) indicating carbonyl signal of an ester group. The carbonyl carbon signal of 195.4 ppm which is much downfield is characteristic of C-4 of a flavonone unit, whereas the other two (182.1 and 178.7) are characteristics of C-4 of flavone moieties. Another indication of a triflavonoid is the quaternary carbon signals δ_C 96, 101.4 and 103.2 ppm, each of which are characteristics of C-10 of flavonid unit. There is one singlet proton at δ_H 6.53 in the ^1H NMR spectrum which is characteristic of H-3 of flavones. In the HSQC spectrum this signal correlated with carbon resonating at δC 102.8 ppm. The flavanone moiety can be linked to the flavone units either at I- C-3' or I-C-4' and the two will give a similar splitting pattern in their ^1H NMR spectra. HMBC (Figure 4.7) correlations between I-H-2'/6' (δ_H = 7.49 ppm) and I-C-2 unit and the correlation between I-H-2 (δ_H = 5.44 ppm) and I-C-2'/6' implied that the *p*-substituted aromatic system was linked to I-C-2 of the flavonone unit. This is also comfirmed from the long range ^1H -^1H NOESY (Figure 4.6) spectrum showed correlation between the proton at δ_H 5.44 ppm (I-C-2) and 7.49 (1-C-2').

The C-13 also showed peaks at δ_C 94.6 and 99.4 ppm typical of C-6, and C-8 for 5, 7 – dihydroxyl substituted flavonoids which usually give a difference of >4 between the resonance of C-6 and and C-8 of the flavonoid units. The HSQC (^1H-^{13}C) spectrum showed a signal at δ_C 95.6 ppm integrating for two protons at δ_H 5.87 and 5.90 ppm. The HMBC showed correlation between one of this protons to a second carbon of the δ_C = 95.6 ppm and to a carbonyl carbon of the ester (δ_C = 169.5 ppm). The methyl carbon of the ester group also showed long range correlation (HMBC) to one of the C-4 carbonyl carbons (δ_C 182.1). This suggest that the δ_C of 95.6 ppm

carbons are at III - C- 6 and III- C-8 while the esther functional group is between III- C – 4 and III- C – 6 (III- C – 5).

Furthermore, 9 peaks appearing in the δ_C between 165 to 140 ppm resonance region accounting for oxygen-linked quaternary aromatic carbons (6 peaks) and the two β-carbons of α, β-unsaturated pyran ring system. This number is actually short of the expected number of quaternary carbon signals for the three flavonoid units due to the expected equivalent chemical shifts of most of the quartenary carbon atoms, owing to similar chemical environments. The DEPT 135 (Figure 4.3) spectrum showed the presence of 14 methine carbons within the range of 5.8 -8 ppm which is in accordance with the proposed structure.

The position of the *p*-hydroxyphenyl ring was derived from the long-range HMBC (Figure 4.7) correlations between II-H-3 at δH 6.53 and II-C-1' at δC 121.8 ppm, confirming that *p*-hydroxyphenyl ring is linked to the benzopyran moiety at II-C-2'.

An ether linkage between the flavonone and the two flavone units was proposed due to the fact that there is an absence of long-range correlations between the protons in the *p*-substituted and trisubstituted aromatic rings (the B rings) indicated that the two rings are not C-C linked.

The ^{1}H NMR, ^{13}C NMR and 2D NMR (HSQC, HMBC, COSY and DEPT) data showed that compound MNC is a triflavonoid with ether linkage consisting of a naringenin and two luteolin units.

5.1.2 Compound BND

This was isolated as a brown powdery solid. The NMR spectra of compound BND (Figure 4.7 – 4.13), showed that the ^{1}H NMR spectrum of compound BND displayed two ortho coupled doublets at δ_H 6.83 ppm (J = 8.0Hz, 1H and δH 6.79 ppm (J=8.0Hz, 1H) respectively for aromatic protons H-5' and H-6' on ring B. A singlet

signal at d 7.01 ppm (1H, s) showed proton-2' has isolated proton, hence position 3' and 4' are substituted. The 1H NMR also shows C-4 with a typical 4 peak pattern of two doublets of doublets (H-4a, δH = 2.85, H-4b, δ_H = 2.77 ppm) with a characteristic coupling constant. In the ^{13} C NMR, signals appeared at δ_C 27 .9, 66.1, 78.5, 94.6,114.6, 114.0 ,118.1 due to C -4, C -3, C -2, C-8, C -2', C -5', C-6', carbon respectively. Other aromatic carbons showed peaks at δC = 98.8, 130.9, 144.4, 144.6, 156.3, 156.6, 155.9 ppm. The HMBC displayed several correlations between the H-atoms and carbon atoms. The spectra data are consistent with that of a Catechin (see appendix).

5.1.3 Compound BNF

The presence of a *p*-substituted aromatic ring, a trisubstituted aromatic ring and two tetrasubstituted aromatic rings were established by careful examination of ^{1}H NMR (Figure 4.16) and the ^{1}H - ^{1}H COSY (Figure 4.20) spectra of BNF. This compound was clearly a biflavonoid which consisted of two flavone units. This is due to the presence of singlet protons at δ_H 6.64 and δ_H 6.55 ppm in the 1H NMR spectrum which is characteristic of H-3 of flavones. In the HSQC spectrum this signal correlated with carbons resonating at δ_C 103.2 and 102.0 ppm respectively.

More indication of a biflavonoid was in the ^{13}C NMR spectrum (Figure 4.17), which showed the presence of two carbonyl carbons indicated by the peaks at δC 182.14 and 178.86 ppm.

The DEPT 135 (Figure 4.18) spectrum showed the presence of 15 methine carbon atoms (out of which 13 are within the range of 6-8 ppm), two methylene (δ_H 3.55 and 1.42 ppm) and one methyl (δ_H 1.34 ppm) which is in accordance with the proposed structure.

The peaks at δ_C 94.3, 94.5 and 99.6, 99.8 ppm in ^{13}C NMR spectrum for I-C- 6, II-C-6 and I-C-8, II-C-8 respectively, are typical for 5,7-disubstituted flavone derivatives which usually give a difference >4 between the resonance of C-6 and C-8.

The position of the *p*-hydroxyphenyl ring was derived from the long-range HMBC (Figure 4.22) correlations between II- H-3 at δ_H 6.64 ppm to II-C-1' at δ_C 124.8 ppm, confirming that *p*-hydroxyphenyl ring is linked to the benzopyran moiety at II-C-2. Furthermore, the HMBC correlations between II-H-2', 6' (δ_H 7.96 ppm) and II-C-2 (δ_C 163.8 ppm) also supported this derivation. The trisubstituted aromatic ring was attached to I- C-2 of the second benzopyran moiety based on the correlations between H-3 (δ_H 6.42 ppm) and C-2' (δ_C 118 ppm).

An ether linkage between the two flavone units was proposed due to the fact that there is an absence of long-range correlations between the protons in the *p*-substituted and trisubstituted aromatic rings (the B rings) indicating that the two rings are not C-C linked.

Also, the down-field resonance of the I-C-3' signal as compared to the unsubstituted flavones, suggests that I-C-3' and I-C-4' are involved in the interflavonoid linkage.

The COSY spectrum showed correlation between the methine proton at δ_H 4.10 ppm and methyl proton at 1.34 ppm as well as a correlation between δ_H 3.54 ppm and 1.43 ppm. The methyl carbon at 1.34 (δ_C 22.75 ppm), also showed long range correlation to the methine carbon at δ_C 68.16 ppm as well as to the carbonyl carbon at δ_C 182.1 ppm, leading to their assignment as I-C-1'' (δ_C 68.12 ppm) and I-C-2'' (δ_C 22.75 ppm).The methylene proton at 1.43 ppm (δ_C 29.45 ppm) showed long range HMBC correlation to the carbonyl carbon at δ_C 178.8 ppm (II-C-4), leading to its assignment as II-C-2''. The methine protons at δ_H 4.10 ppm (δ_C 68.13 ppm) and δ_H 3.68 (δ_C 72.46 ppm) are both possibly hydroxyl.

Therefore, BNF was identified as a new Ochnaflavone derivative. This was confirmed because the spectra data for compound BNF were similar to the data in the first isolation of ochnaflavone (Rao *et al.*, 1997), except for the substituents at I-C-5 and II-C-5 (see appendix).

5.2 PHARMACOLOGICAL ACTIVITIES

5.2.1 Antimalarial

The acute toxicity studies showed that the mice were observed to showed general CNS depression, restlessness and subsequently death.

A total of 25% of the animals survived after 28days of inoculation.

The blood schizoticidal activity of various doses of O. rhizomatosa leaf extract, Chloroquine and normal saline shows that the average percentage chemosuppression of O. rhizomatosa at the highest dose administered was found to be 40%. This 40% activity was found to be insignificant when compared with the positive standard drug (chemosuppresion is 68.8%) using students t- test. The `p' value greater than 0.05 was insignificant while the result of *Plasmodium berghei* on curative test shows that the average percentage chemosuppression of *O. rhizomatosa* at the highest dose administered was found to be 46.66%. This 46.66% activity was also found to be insignificant when compared with the positive standard drug (chemosuppresion is 68.8%) using students t- test, where a `p' value greater than 0.05 is insignificant.

5.2.2 The Anti-microbial Screening.

Both the acetone extract and the pure compound (MNC) showed broad spectra of activity against the tested organisms. The extract and the compound MNC both have activity against the bacteria *E.aureus, E.coli, S.dysenterae, S.typhi* and *K.pneumoniae* as well as the fungi *C.albicans* and *C.tropicalis*. It is an indication that both the extract and the pure compound (MNC) can be good antibiotic and antifungi.

Both the crude and the pure compound also had good activity at the concentration tested with mean zone of inhibition diameter >18mm (23-34mm). (Tania *et al.*, 2000). The zone of inhibition observed with both the crude and compound MNC were slightly lower than those of the standard drugs used (ciprofloxacin and fluconazole). The minimum inhibitory concentration (MIC), values were really low for the compound (MNC). It was 12.5µg / ml for all the organisms that tested sensitive. The low MIC value suggests that the compound possesses good antimicrobial activity, considering that compounds with MIC values less than 100µg / ml are regarded as having strong antimicrobial activity (Tang *et al.*, 2003). Ochnaflavone has a broad range of biological/pharmacological activities including anti-inflammatory, anticancer, anti-HIV and anti-atherogenic activities (Suh *et al.*, 2006).

The traditional use of *Ochna rhizomatosa* in wound healing and malarial treatment can be said to have scientific basis, since both the crude extract and one of the isolated compounds are sensitive to the bacteria implicated in wound infections except for *P.aeruginosa*.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the acetone leaf extract of *Ochna rhizomatosa r*evealed the presence of Flavonoids, Tannins, Steroids, Saponins, Carbohydrates and Glycosides. Alkaloids were however found to be absent. Column chromatography led to the isolation of three compounds with flavonoidal nucleus, a triflavonoid, a biflavonoid (an ochnaflavone derivative) and a Catechin with these codes MNC, BNF and BND respectively.

Result of the antimalarial activities of the leaf acetone extract showed that the extract has antimalarial property of which the activity was found to be insignificant when compared with the standard drug (Chloroquine) using student's t- test.

In addition, the extract as well as one of the isolated compounds showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Strigella dysentriae, Salmonella typhi, Klebsiella pneumonae, Candida albican and Candida tropicalis*.

6.2 Conclusion

The Ochna genus is reported to contain this group of compounds in abundance. Catechin has been previously isolated from other species in the genus. Triflavonoid ester derivative (MNC) and the ochnaflavone derivative (BNF) are being reported for the first time in the plant and in this genus. This is also the first report on the isolation of these compounds from *Ochna rhizomatosa.*

Based on the findings in this study, it could be concluded that the use of *Ochna rhizomatosa a*s an antimalarial and conditions related to bacterial infections in ethnomedicine has scientific basis.

6.3 Recommendations (suggested further studies):

- Further studies should be carried out to isolate other classes of compound in this plant.
- There is need to work on other parts of this plant other than the leaf part.
- More pharmacological work should be done to validate the use of the plant for the treatment of jaundice, wound and as an anti- helminthiasis.

REFERENCE

Abdullahi, M.I., Iliya, I., Haruna A.K., Sule M.I., Musa, A.M., Abdullahi , M.S.(2010), Antimicrobial flavonoids diglycoside from the leaves of Ochna species. *African Journal Pharmaceutical Pharmacol*., 4, 083.

Abourashed, E., El-Alfy, A., Khan, I., Walker, L., (2003): Ephedra in perspective - a current review. *Phytother Reserch* 17, 703-712.

Adebayo J.O., Yakubu M.T., Egwim E.C., Owoyele B.V., B.U. Enaibe B.U. (2003): Effect of ethanolic extract of *Khaya senegalensis* on some biochemical parameters of rat kidney. *Journal of Ethnopharmacology*, 88, pp. 69–72.

Agbedahunsi J.M., Elujiuba A.A., Makinde J.M. and Ododa A.M.J (1998): Antimalarial activity of *Khaya grandifoliola* stem-bark. *Pharmaceutical Biology,* 36, 8-12.

Agra, M.F., Franca, P.F., and Barbosa- Filho, J.M(2007). Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. *Journal of Pharmacology*. 17: 114-140.

Agrawal, P.K. (1989) : Carbon – 13 NMR of flavonoids. *Amsterdam , Elsevier*, 564.

Ahmed, S. and Razaq, S. (1971): A New Approach to the Synthesis of Symmetrical Biflavones, *Tetrahedron Letters* ,4. 633.

Ajaiyeoba**, E** (2002). Phytochemical and antibacterial properties of *Parkia biglobosa* and *P. bicolor* leaf extracts. *African Journal of Biomedicinal Reearch* **5**:125-129.

Akereke O. (1990): Medicinal plants and primary health care. An agenda for action in essential drugs monitor. 10, p8-9. Ds ; Conservation of medicinal plants ; Cambridge University press London (UK) P.3-22.

Akinde, B. E. and O. O. Odeyemi (1987)**.** Extraction and Microbiological Evaluation of the oils from the leaves of *Acalypha wilkesiana*. *Nigerian Medicininal Journal* 17:163-165.

Akinyemi, K. O., Oladapo, O., Okwara, C. E., Ibe, C. C and Fasure, A. K (2005). Screening of crude extracts of some medicinal plants used in South-West Nigerian Unorthodox medicine for anti-methicilin resistant *Staphylococcus aureus*. *Complete Alternative Medine.* **5:6** doi:10.1186/1472-6883-5-6.

Al-wadh, A.N., Julich,W.D., Kusmik, C., Lendequist U. (2001). Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. *Journal Ethnopharmacol*. 74: 173-179.

Amin, A., Mousa, M.,(2007). Merits of anti-cancer plants from Arabian Gulf region. *Cancer Therapy*. 5, 55-56.

Ansari**,** F.R.(1981): Some reaction with the Micheal adducts of Chalcones with benzyl Ketones. *Indian Journal of chem*istry, 208, 724-5.

Anuradha, V., Srinivas, P,V., Rao, R.R., Manjutha, K ., Purohit, M.G., Rao, J.M.(2006):Isolation and synthesis of analgesic and anti-inflammatory compounds from *Ochna squarros. Bioorganic Medicine Chemistry,* 14, 6820.

Aperia, A., (2007). New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *Journal International Medicine,* 261, 44-52.

Attaran A.(2004): Rescuing malaria treatment,or not? *Lancet,* 364: 1922 – 1923.

Aynechi, Y.(1971): Desoxypodophyllotoxin, the cytotoxic principle of *Callistris columellaris*. *Journal of Pharmaceutical Sci*ence, 60,121-2.

Ayoola, G.A., H.A.B. Coker, S.A. Adesegun, A.A. Adepoju-Bello, K. Obaweya, E.C. and Ezennia T.O. Atangbayila (2008). Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in South Western Nigeria. *Tropical Journal of Pharmaceutical Research,* 7: 1019-1024.

Balick M.J., Elizabetsky E., S.A. Laird S.A., (1996): Medicinal Resources of the Tropical Rain Forest, Columbia University Press, New York.

Balunas, M. J., Kinghorn, A. D., 2005. Drug discovery from medicinal plants. *Life Science*. 78, 431 - 441.

Baker, W., Finch, A.C.M., Ollis, W.D. and Robinson, K.W. (1963): The Structures of the naturally occuring biflavonyls. *Journal Chemistry Society,* 14, 77.

Beckmann, S., Geiger, H. and Degroot W.(1971): Biflavone und 2, 3-Dihydrobiflavone aus Metasequoia *Glypto Stroboides*. *Phytochemistry* 10, 2465.

Betti, J.L (2011), Contribution of the knowledge of non – wood forest products of the far North region of Cameroon: Medicinal plants sold in the Kousseri market. *Journal of Ecology and the Natural Environment* Vol. 3(7), p. 241-254.

Birch, A J. and Hextall, P. (1953): Flavonoid basic metabolites. *Australian Journal Chemistry*. 8, 263.

Bork, P.M., Barcher, S., Schmitz, M.I., Kaspers, U., Heinrich, M.(1999): Hypericin as a non- antioxidant inhibitor of NF-kB. *Planta Medicine,* 65,297.

Bouquet, A. (1969): 'Feticheurs et Medecines Traditionnnelles du Congo (Bras Zzaville); ORSTOM, Paris, 1969, p.178.

Bumah V.V., E.U. Essien E.U., Agbedahunsi J.M., O.U. Ekah O.U.(2005): Effects

of *Khaya grandifoliola* (Meliaceae) on some biochemical parameters in rats. *Journal of Ethnopharmacology,* 102 (2005), pp. 446–449.

Burrows, B.F. and Ollis, W.D. (1960):The number of naturally flavones known at present. *Process Chemical Society,* 17, 7.

Brain, K.R. and Turner, T.D. (1975): " The Practical Evaluation of Pharmaceutics" Wright Scientechnica, Bristol, U.K pp140-144, 152-154.

Bregoff, H.M., Roberts, E. and Dewiche, C.C. (1953):A new technique for the identification and chromatographic separation of phospholipids and sulpholipids. *Journal of Biological Chem*istry. 205, 565.

British Pharmacopoeia (1993): vol II market towers 1 nine elms lane London, U.K pp A157. Appendix XIM.

Burkill H.M (1985):The usefull plant of West tropical Africa. Complete set of volume 1, Royal Botanical Gardens, Kew. U.K.

Carlsson, A., (2001). A paradigm shift in brain research. *Science* 294, 1021.

Chan, O. T. M., Yang, L. X., 2000. The immunological effects of taxanes. Cancer Immunol. *Immunother*. 49, 181-185.

Carvalho M.G., Minghou X., Xiuguang, He, Azevedo, J.L.T. and Carvalho M.G(200): Flexibility using results of CFDAND simplified heat transfer model for pulverized coal – fired boilers. *International Journal of Energy Research,* 24, 13, 25, 1161–1169

Chambers F.H (2006): General principle of antimicrobial therapy in the pharmacological basis of therapeutics Bruton, L.L., LazoJ.S and Parker, L.L.(Eds). M Graw-Hill publishers, New York U.S.A. p1095.

Chandhouri, S.K., Fekadu, F., Munsukh, C.W., Monroe E.W., John, C.T., Christpher, W.W. B. and Douglas K. (1996): Range forb lectins. *Phytochemistry* 41, 6, 1625 - 1627.

Chandramouli, N., Murti, V.V.S., Natarajan, S. and Sechadri, T.R. (1971): Structure of Heveaflavone. *Indian Journal of Chemitry* 9, 895.

Chandramouli, N., Natarajan, S., Murti, V.V.S. and Sechadri, T.R.(1972):Synthesis of a range of polyhydroxy 8- aryl flavones. *Indian Journal of Chemisry,* 10, 1115.

Chang, C.T., Cham, T.S., Ueng, T., Choong, S. T. and Chen, F.C. (1969): Compounds derived from acetic acid. *Journal Formosen Sci*ence, 14, I.

Chexal, K.K., Handa, B.K. and Rahman, W. (1970): Some Optically Active Biflavones from *Podocarpus Gracilior*. *Chemistry and Indusrty* 1, 28.

Cowan M.M., (1999): Plants products as antimicrobial agents. *Clinical Microbiology. 12, 4: 564 – 582.*

Cragg, G. M., Newman, D. J., 2009. Nature: a vital source of leads for anticancer drug development. *Phytochemistry, Rev* 8, 313-331.

Dallimore, W. and Bruce, A. (1948): A Handbook of Conifereae and Ginkgoaceae, Third Edition, Edward Arnold London, UK, 80.

Daly, J. W., (2005). Nicotinic agonists, antagonists, and modulators from natural sources. *Cell Molecular Neurobiol*ogy 25, 513-552.

Dapper, D.V., Aziagba B.N. and Ebong O.O. (2007): Antiplasmodial effects of the Aqueous Extract of *Phyllantus amarus* Schumach and Thonn Against *PlasmodiumBerghei* in Swiss Albino mice. *Nigerian Journal of Physiological Science.* 22(1-2): 19-25.

Dastur, J. (1964): Usefull plant of India and Pakistan. A popular handbook of trees and plant of industrial economic. D.B. Taraporevela, pp 185.

Deshpande, V.H., parthasarathy, P.C and Venkataraman, K. (1968):A geranyl substituted flavonol from *Macaranga vedeliana*. *Tetrahedron letter*, 1715.

De Boer, J. H., Kool, A., Broberg, A., Mziray, W. R., Hedberg, I., Levenfors, J. J.,(2005). Anti-fungal and anti-bacterial activity of some herbal remedies from Tanzania. *Journal of Ethnopharmacol*. 96, 461-469.

Dibia , B.C., Igbigbi, P.S., Dapper, D.V., (2002), Preliminary study on the effect of Halofantrine Hydrochloride on the tests of mature wistar rats. *Journal of Applied Sciences and Environmental Management,* 6(1): 45- 48.

Dossaji, S.F., Bell, E.A.and Wallace (1973). C-glycosylflavones in *Gnetum gnemon*. *Phytochemistry*. 12, 371.

Dossaji, S.F., Mabry, T.J., Bell, E.A. (1975):Biflavonoids of the *Cycadales*. *Biochemistry of Cyst Ecology,* 2: 171.

Duffy P.E, Mutabingwa T.K. (2004): Drug combinations for malaria: Time to a act? *Lanceoletter*.; 363:3 – 4.

Dur-volsteed, F.T and Roux, D.G. (1971): Chalcones, aurones and Dihydrochalcones. *Tetrahedron Letters*. 1, 1647.

Edeoga, H. O., Okwu, D. E., Mbaebie, B. O., (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*. 4, 685-688.

Elizabetsky, E., Amador, T.A., Albuqueirque, R.R., Nunes, D.S and Carvalio, A(1995). Analgelsic activity of psychotia coterata (Wild ex R and 3) muet. Arg. Alkaloids. *Journal of Ethnppharmacology*. 48: 77-85.

Estevam C.S., Oliveira, F.M., Conserva, L.M., Lima, l.f., Barros, E.C.P., Barros, S.C.P., Rocha, E.M.M. and Andrade, E.H.A 2005. Preliminary screening of constituents of O*ratea nitida Aubi* (Ochnaceae), for invivo antimalarial activity. *Brazilian Journal of Pharmacology. 195-198.*

Fansworth, N.R. (1984): Screening of medicinal plants used by the Carifina of Eastern Nicaragua for bioactive compounds. *Economic Botany*, 38 (1984), pp. 464–489

Fansworth, N. R., Akerele, R. O., Bingel, A. S., Soejarto, D. D., Guo, Z., (1985). Medicinal plants in teraphy. *Bulletin of World health organization*, 63(6): 965-981.

Fansworth, N.R., (1996), NAPRALET program of Collaborative research in the Pharmaceutical Science. Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois, Chicago, 833 South Wood street Chicago, Illinois. U.S.A . p.1046-1064.

FDA (2010). New risk management plan and patient medication guide for qualaquin (quinine sulfate)". Food and Drug Administration. Drug Safety Communication.

Fennell, C. W., Lindsey, K. L., McGaw, L. J., Sparg, S. G., Stafford, G. I., Elgorashi, E. E., Grace, O. M., van Staden, J., (2004). Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmaco*l. 94, 205-217.

Fidock D.A, Rosenthal P.J.,Croft S.L., Brun R and Nwaka S. (2004). Antimalarial drug Discovery. Efficasy models for compound screening. *Nature Reviews,* 3 : 509 - 519.

Fink, K. and Fink, R.M. (1949):Use of paper chromatography in the identification of volatile fatty acids. *Process Experimental Biology of Medicine*. 70, 654.

Galea, V., Preda, N., Nitelea, I. and Ariesan, M. (1968): Flavones excluding flavonols and glycosides of both flavones and flavonoids. *Elsevier*. Volume 29, Issue 4.

Ganesan, A., 2008. The impact of natural products upon modern drug discovery. *Current Opinion in Structural Biology*. 12, 306-317.

Gao, J., Wymore, R. S., Wang, Y., Gaudette, G. R., Krukenkamp, I. B., Cohen, I. S., Mathias, R. T., (2002). Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. *Journal of General Physiology,* 119, 297-312.

Gbodossou, E. (2005). Efficacy of Metrafaids in the treatment of persons living with HIV/AIDS. Book of abstract, International conference on HIV/AIDS and STI in Africa (ICASA) Abuja Nigeria. P.57.

Geiger, H. and Seeger, T., (2000). Triflavones and a biflavone from the moss *Rhizogonium distichum, Naturforsch.*, 55, 870.

Geiger, H., (1995). Cyclobartramiatriluteolin, a unique triflavonoid from *Bartramia Stricta. Phytochemistry*, 39, 465.

Geiger, H. and Degroot-pfeider, W. (1971): 2, 3 – Dihydrobiflavone in *Cycas revoluta* (Cycadaceae). *Phytochemistry* 10, 1936.

Geissmann, T.A. (1962): The Chemistry of Flavonoid compounds Pergomon press Oxford London, Preface pp. 7.

Gessler, M.C., Mysuya, D.E., Nkunya, M.H.H., Mwasumbi, L.B., Schar, A., Heinrich, M., Tanenr, M., 1995. Traditional healers in Tanzania: the treatment of malaria with plant remedies. *Journal of Ethnopharmacology* 48, 131–144.

Gibbons, S., Udo, E.E., (2000): Anti-bacterial properties and cannabis research a-z. *Phytother Research.* 14, 139.

Gonzalez C E, Venzon D, Lee S, Mueller B U, Pizzo P A, Walsh T J (1996): Risk factors for fungemia in children infected with human immunodeficiency virus: a case-control study. *Clinical Infectious Diseases*. 23:515–521.

Graybill J R. (1988): Systemic fungal infections: diagnosis and treatment. *Infectious Disease Clinics of North America. 805–825.*

Guerra, F., (1977). The introduction of cinchona in the treatment of malaria. *Journal of Tropical Medicine.* 80, 135-140.

Gunasekar, D., Jayaprakasam, B. and Damu, A.G. (1998): Polyphenol Commun. 98, XIXth International Conference on Polyphenols, Lille ,France.1998, p.175.

Gupta, R. C., (2009). Handbook of Toxicology of Chemical Warfare Agents. *Elsevier Inc.* 240, 2, 124-131.

Gurib-Fakim, A.,(2006). Medicinal plants: traditions of yesterday and drugs of today. *Molecular Aspects of Medicine*. 27, 1-10.

Hackman, R. M., Havel, P. J., Schwartz, H. J., Rutledge, J. C., Watnik, M. R., Noceti, E. M., Stohs, S. J., Stern, J. S., Keen, C. L., (2006). Multinutrient supplement containing ephedra and caffeine causes weight loss and improves metabolic risk factors in obese women: a randomized controlled trial. *International Journal of Obesetrics*. 30, 1545-1556.

Hahn, H.,(1995). The first biaurone, a triflavone and biflavonoids from two

Aulacomnium species. *Phytochemistry*, 40, 573.

Halberstein, R. A., (2005). Medicinal plants: historical and cross-cultural usage patterns. *Ann Epidemiol.* 15, 686-699.

Handa, B.K., Chexal, K.K., Mah, T. and Rahman, W.(1971a): Some Observations on Partial Demethylation of Biflavonyls. *Journal of Indian Chemical Soc*iety. 48, 177.

Harborne, T. B., Mabry, T.J. and Mabry, H. (1975): The Flavonoids. Chapman and Hall Ltd London U.K. Pp289-297, 321-430, 693-792, 972-1042.

Harborne, T. B. and Baxter, H. (1993). Phytochemical dictionary. A hand book of bioactive compounds from plants publish by Taylor and Francis London U.K, pp 410, 390-395.

Hartwell, J. L; and Abbott, B. J.. (1969): Antineoplastic Principles in plants, Recent Development in the field. *Advanced Pharmacol Chemother*. 7, 117.

Hegnauer, R.,(1969): Studies showed that purslane contains high amounts of noradrenaline, which is known to stimulate the adrenal glands and antioxidants. *Journal of the American College of Nutrition.* 11(4): 374.

Hillis, W.E and Isoi. K. (1965):Flavonoids glycosides and the chemosystematics of *Eucalyptus camaldulensis. Phytochemistry.* 4,541.

Horhammer, L., Wagner, H. and Reinhardt, H (1966) : Dalpaniculin, a C glycosylisoflavone from Dalbergia paniculata seeds. *Botanical Management Tokyo*. 79, 510.

Hooper D.C.(2001): Emerging mechanisms of fluoroquinolone resistance. *Emerging Infectious Diseases.* 2001; 7:337–341.

Horowitz, R.M. (1957):A Chalcone glycoside from the seeds of *Bauhinia purpurea*. *Journal of Organic Chemistry.* 22, 1733.

Hye, M,A., Taher, M.A.,Ali, M.Y. and Zaman, S (2009): Isolation of (+)Catechin from Acacia Catechin (Cutch Tree) by a Convenient method. *Journal of Scientific Research* 1(2), 300-308.

Ibekwe V.I, Nnayere NF, Akujobi CO (2001). Studies on Antimicrobial Activity and Phytochemical Qualities of Extracts of Orange peels. *International Journal of Environmental Health and Human Development.* 2(1): 41-46.

Ichino C., Kiyohara H., Soonthornchareonnon, N., Chuakul, W., Ishiyama, A., Sekiguchi, H., Namatame, K., Otoguro, K., Omura, H., Yamada H.(2006):Antimalarial activity of biflavonoids from *Ochna integerrima*. *Planta Medicine*. 72, 611.

Ilyas, M., Usmani, J.N., Bhatnager, S. P., Rahman, W. and Pelter, A. (1968): The first

Optically Active Biflavones. *Tetrahedron letters*, 5515.

Imai, S., Kanai, Y., Nohara, A., Otsuka, H. and Sanno, Y. (1970):The 95 flavones. *Journal of Pharmaceutical Society. Japan*. 70. 25716.

Isah A.B., Ibrahim Y.K., E.O. Iwalewa E.O.(2003): Evaluation of the antimalarial Properties and standardization of tablets of *Azadirachta indica* (Meliaceae) in mice. *Phytotherapy Research.* 17 (2003), pp. 807–810.

Jackson, B., Locksley, H.D., Scheinmann, F., Wolstenholwe, W.A., (1971): Extractives from Guttifereae, The Isolation and Structure of 4 Novel Biflavones form The Heartwood of *Garcinia buchalalii*. *Journal of Chemical Society*. 37, 91.

Jacouljevic, I. M., Seay, L.D. and Shaffer, R.W. (1964): Method of analysis for some *Vinca rosea* alkaloids. *Journal of Pharmaceutical Sci*ences. 53, 553.

Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O. (2005): Resistance of *Plasmodium falciparum* field isolates to invitro artemether and point mutations of the serca-type Pfatpase 6. *Lanceoletter.* 366, 1960–1963.

Jayakrishna G. and Reddy, M.K (2003): A new biflavonol glycoside from *Solanum melongena*. *Asian Journal of Natural Product Research*. 5, 83-87.

Jayaprakasam, B., Damu, A.G., Rao, K.V., Gunasekar, D., Blond, A., Boko, B.(2000): Involvement of T-cell immunoregulation by Ochnaflavone in therapeutic effect on fungal arthritis due to *Candida albicans*. *Journal of Natural Product*, 63, 507.

Johns, S.R., Russel, J.H. and Heffernan, M.L. (1965):Lottanongine, an unprecedented flavonoidal indole alkaloid from the roots of Thai medicinal plants, *Trigonostemon reidoides*. *Tetrahedron letters*,1987.

Kaewamatawong, K., Likhitwitayawuid, K., Ruangrungsi, H., Takayama, H., Kitajima, M., Aimi, N.(2002):Novel biflavonoids from the stem bark of *Ochna integerrima. Journal of Naural Product*, 65, 1027.

Kamil M., Khan N.A., Ilyas M., Rahman W.(1983): Biflavones of the subfamily Cupressoideae, Cupressaceae. *Indian Journal of Chemistry, Sect. B.* 22, 608.

Kaneta, M. and Sugiyama,N. (1972):The synthesis of new flavones with a H-Benzyl [1,3- b: 3, 4-b] dipyran - 4, 10 - dione skeleton and the light resistance of these compounds. *Buletin of Chemical Society. Japan* 45, 528.

Khadzhai, Y. I., Obolentsera, G.V. and Serdyak, A.D. (1969): *Famakol Toksikol*, 32, 451. Khan, N.U. and Ansari, W.H.O (1970): *Tetrahedron*, 264, 1221-2.

Khalivulla S.I., Reddy n.p., Reddy B.A.K., Reddy R.V.N., Gunasekar D.,Blond A. and Bodo B. (2008): A new biflavonone from *Ochna lanceolata*. *Natural*

product communication. 3, 9, 1487-1490.

Khan, N.U., Ilyas, M., Rahman, W., Mashima, T., Okigawa, M. and Kawano, N. (1972): Biflavones from the Leaves of *Araucaria bidwilliihooker. Tetrahedron* 22, 5689 - 5695.

Kim HP[1], Park H, Son KH, Chang HW, Kang SS (2008): Biochemical pharmacology of biflavonoids: implications for anti-inflammatory action. *Archive of Pharmacology Research* 31(3), 265-273.

Kirtikar K.R., Basu B.D (1980): Indian Medicinal Plants, Periodical Book Agency, NEW Delhi, 1980, p. 515.

Knight, D.J., Peters, W. (1980): The activity of N-benzoloxy dihydrotriazines. I. The activity of clociguaami (BRL 50216) against rodent malaria and studies on its mode of action. *Journal of Annals of Tropical Medicine and Parasitology*, Vol. 74 No. 4 pp. 393-404.

Koehn, F. E., Carter, G. T., (2005). The evolving role of natural products in drug discovery. *National Revolution in Drug Discovery*. 4, 206-220.

Konoshima, M., Ikeshiro, Y., Nishinaga, A., Matuura T., Kubota, T. and Sakamoto, H. (1969): The Constituent of the flavonoids from *Garcinia spicata. Tetrahedron letters.* 121.

Konoshima, M. and Ikeshiro, Y. (1970): Fukugi side, the First Biflavonoid Glycoside from *Garcinia spicata*. *Tetrahedron Letters* 1717.

Kracke, N. A., (2004). Ouabain (g-Strophanthin) "milk for the ageing heart". Sanum-Post magazine Institut GmbH, 27318, Hoya, Germany.

Kremsner P.G, Krishna S. (2004): Antimalarial combinations. *Lanceoletter*. 364: 285 – 294.

Kritchersky, D. and Kirk, M.L. (1952): Detection of steroids in paper chromatography. *Biochemistry Biophysiology*, 35, 346.

Laliberte, R., Campbell, D. and Bruderlein, F. (1967):Physiology and functions of flavonoids. *Canadian Journal of Pharmaceutical Sciences,* 2, 37.

Li , R., Konyon, G.L., Cohen, F.E., Chen, X., Gong, B., Dominguez, J.N., Davidson, E., Kuzban, G., Mill, R.E., Nuzam, E.O.(1995). In vitro Antimalarial Activity of Chalcones and Their Derivatives. *Journal of Medicinal Chemistry.* 38: 5031-5037.

Lie-Chwen Lin , Yuh-Chi Kuo , and Cheng-Jen Chou, (2000): Cytotoxic Biflavonoids from *Selaginella delicatula. Journal of Natural Product,* 63 (5)**,** pp 627 – 630.

Likhitwitayawuid, K., Rungserichai, R., Ruangrungsi , N., Phadungcharoen,

T.(2001):The absolute configuration of (+) – isoshinanolone and in situ LC – CD analysis of its stereoisomers from crude extracts. *Phytochemistry,* 56, 353.

Lin RD, Chin YP, Lee MH (2005): Antimicrobial activities in combination with natural flavonoids against clinical extended spectrum b-lactamase producing *Klebsiella pneumoniae*. *Phytother Research,* 19: 612-617.

Lisboa, B.P. (1984): Application of thin layer chromatography on purification, Separation and quantitative determination of steroid metabolilites. *Journal of Chromatography*.16,136.

Lorke, D. (1983): A new approach to acute toxicity testing. *Journal of Toxicology*. 54: 75 – 287.

Magassouba, F. B., Diallo, A., Kouyat´e, M., Mara, F., Mara, O., Bangoura, O., Camara, A., Traor´e, S., Diallo, A. K., Zaoro, M., Lamah, K., Diallo, S., Camara, G., Traor´e, S., K´eita, A., Camara, M. K., Barry, R., K´eita, S., Oular´e, K., Barry, M. S., Donzo, M., Camara, K., Tot´e, K., Vanden Berghe, D., Tott´e, J., Pieters, L., Vlietinck, A. J., Bald´e, A. M., (2007). Ethnobotanical survey and antibacterial activity of some plants used in Guinean traditional medicine. *Journal of Ethnopharmacol.* 114, 44-53.

Majekodunmi S.O., Adegoke O.A., O.A. Odeku O.A.,(2008): Formulation of the extract of the stem bark of *Alstonia boonei* as tablet dosage form. *Tropical Journal of Pharmaceutical Research,* 7 (2008), pp. 987–994.

Makhafola T.J., (2009): Chemical and biological characterization of antibacterial compounds present in *Ochna pretoriensis* (Ochnacae) leaf extracts, Theses. Paraclinical sciences, University of Pretoria.

Makinde J.M., Awe S.O., Salako L.A.(1994): Seasonal variation in the antimalarial activity of *Morinda lucida* on *Plasmodium berghei* in mice. *Fitoterapia,* 65, pp. 124–130.

Maureer-Grimmes, B., Macbeth, D.L., Hallitian, B, and Delph, S. (1996):Antimicrobial activity of medicinal plants of the Scrophulaciaceae and Acanthaceae. *International Journal of Pharmacognosy*. 34: 243-248.

Meenakshi, S., Hain, D.C., Dorokar, M.P. and Sharma R.P (2001): Antibacterial Activity of *Ailanthus axcelsa* (Roxb). *Journal of Phytotherapy Research.* 15: 165 - 166.

Messanga, B.B., Tih, R.G., Sondengam, B.L., Martin, M.T., Blond, A., Brouard, J.P.,Bodo, B.(1998):Biflavonoids from *Ochna lanceolata*. *Planta Medicine*. 64, 760.

Messanga ,B.B., Kimbu, S.F., Sondengam, B.I.,(2001): Two new fatty acid glycosides from the root back of *Ochna calodendron. Fitoterapia,* 72, 732.

Miura, H., Kihara, T. and Kawano, N. (1968): New Bisflavones from Podocarpus and

Chamaecyparis plants. *Tetrahedron letters.* 2339.

Miura, H. and Kawano, N. (1968a): The distribution of Bisflavones in the leaves of Taxodiaceae and Cupressaceae plants. *Journal of Pharmaceutical Society. Japan*. 88, 1459.

Miura, H. and Kawano, N. (1968b): Sesquoiaflavone in the leaves of *Sequoia sempervirens* and *Cunninghamia lanceoplata*. *Journal of Pharmaceutical Society. Japan*. 88, 1489.

Miura, H. and Kawano, N. (1968c): The Partial Demethylation of flavones, Part 4, formation of new Bisflavones. *Chemical Pharmaceutical Bulletin. Tokyo*, 16, 1838.

Miura, H., Kihara, T. and Kawano, N. (1969): Studies on Bisflavones in the leaves of *Podocarpus macrophylla*. *Chemical Pharmaceutical Bulletin.*Tokyo Japan 17, 150.

Miura, H., Kawano, N.and waiss, A.C.Jr. (1970): Cryptomerin A and B, Hinokiflavone methyl Ethers from the leaves of *Cryptomeria japonica*. *Chemical Pharmaceutical Bulletin.* Tokyo Japan, 14, 1404.

Murakami, A., Tanaka, S., Ohigashi, H., Hirota, M., Irie R., Takeda, N., Tatematsu A. and Koshimizu K (1992). Chalcone tetramers, lophirachalcone and alatachalcone, from *lophira alata* as possible anti-tumur promoters. *Biochemistry.* 56 (5):769-72.

Murkerjee, S.K., Sarkar, S.C. and Seshadri, T.R. (1969): Total synthesis of Isorobustin. *Tetrahdron* 25, 1063.

Murnigsih, T.S., Matsuura, H., Takahashi, K., Katakura, K., Suzuki, M., Kobayashi S., Chairul, S., Yoshihara T., (2005). Evaluation of the inhibitory activities of the extracts of the Indonesian traditional medicinal plants against *Plasmodium falciparium* and *Babesia gibsoni*. *Journal of Vetinary Medical Sciences* 67 (8): 829-831.

Muthukumarasamy, S., Mohan, V.R., kumaresan, S.(2003):Screening of certain ethnomedicinal plants for antibacterial activity. *Journal* of *Applied Research on Medicinal* and *Aromatic Plants*. 25, 344.

Molyneux, R.J., Waiss, A. C. Jr. and Haddon, W. F. (1970): Synthesis of flavonoids. *Tetrahedron*. 26, 1409.

Nakazawa, K. (1962): Synthesis of Nuclear – Substituted Flavonoids and Allied Compounds. *Chemistry Pharmaceutical Bulletin* 10, 1032.

Nakazawa, K. (1968): Synthesis of Ring – Substituted flavonoids and Allied compounds, part II. *Chemistry Pharmaceutical Bulletin. Tokyo*, 16, 2503.

Nara, P.L., Harch W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A.,

Fishinger, P.J.(1987): Stimulation of HIV-1 neutralizing antibodies in simian HIV, *AIDS Resistance Human Retroviral. 3, 283.*

Nawab N., Yunus M., Mahdi A.A., and Gupta S., (2011). "Evaluation of anticancer properties of medicinal plants from the Indian sub-continent,"*Molecular and Cellular Pharmacology*, vol. 3, no. 1, pp. 21–29.

Ng P C (1994): Systemic fungal infections in neonates. Aids-related complex *Disease of Childhood*. 71:F130– F135.

NCCLS, (1993), Performance standards Antimicrobial Susceptibility Tests, Approved Standard Fifth Edition, NCCLS Document M2-as Villanova, PA, USA. P.185 - 240.

Newman, D. J., Cragg, G. M., (2007). Natural products as sources of new drugs over the last 25 years. *Journal of Natural Product.* 70, 461-477.

Ngo, M.T., Ngweherd, G.C., Delheodore, A.A., Allouhghi, H., Gangoue-pieboji ., Mbafor J.I., Ghogonu T.R. (2006). Two biflavonoids ourantine A and B, from *Ouratea nigroviolacea. Phytochemistry.* 67 (2424): 2666-2770.

Ndole M.M., (2012): Structure ,synthesis and Biological activities of Biflavonoids isolated from *Ochna serrulata* (Hochst) walp. Theses, School of Chemistry and Physics, College of Agriculture, Engineering and Science, University of KwaZulu-Natal Pietermaritzburg.

Nilson, E. (1973): Unique biflavonoid types from the moss *Dicranoloma robustum, Chemistry Scripta*, 4, 66.

Nsimba S.(2006): How sulphadoxine-pyrimethamine (SP) was perceived in some rural communities after phasing out chloroquine (CQ) as a first-line drug for uncomplicated malaria in Tanzania: lessons to learn towards moving from monotherapy to fixed combination therapy. *Journal of Ethnomedicine.* 2: 5.

Ntiejumokwu, S. and Alemika T.O.E. (1991): Antimicrobial and phytochemical investigation of stem bark of *Boswellia dalziella*. *West African Journal of Pharmacology and Drug Researc*h . 10: 100 – 104.

Nwabuisi C.(2002): Prophylactic effect of multi-herbal extract Agbo-Iba on malaria induced in mice. *East African Medical Journal*, 79, pp. 343–346.

Nweze, E.I., Okafor, J.I., Njoku, O. (2004): Antimicrobial activities of methanolic extracts of *Trema guineensis* (Schumm and Thon) and *Morinda lucida (*Benth) used in Nigerian Herbal Medicinal Practice. *Journal of Biological Research and Biotechnology* 2, 1 , 39 – 46.

Obih P.O. and Makinde J.M. (1985): Effect of Azadirachta indica on *Plasmodium berghei* in mice. *African Journal of Medicine and Medical Science*, 14, pp. 51–54.

Odebiyi O.O. and E.S. Sofowora E.S.(1979): Antimicrobial alkaloids from a Nigerian Chewing stick (*Fagara zanthoxyloides*). *Planta Medica,* 36 (1979), pp. 204–207.

Odugbemi, T. (2006). Outline of Medicinal Plants in Nigeria. 1st Edition, University of Lagos Press, Nigeria, p.77.

Ohta, N. and Yagishita, K. (1970): Isolation technique for flavonoids. *Agricultural Biological Chemistry*. 34, 900.

Okigawa, M., Kawano, N., Aqil, M. and Rahman, W. (1973): Total synthesis of justicidin B and retrojusticidin B using a Tandem Hornor-Emmous- claisen condensation sequence. *Tetrahedron Letters, 22, 2003.*

Oliver, B. (1960): Nigerian useful plant part II. *The Nigeria Field* ,24, 104.

Osadebe, P.O and Ukwueze, S.E. (2004): A comparative study of the phytochemical and antimicrobial properties of the Eastern Nigerian species of Africa Mistletoe (Loranthus micranthus) sourced from different host trees. *Journal of Biological Research and Biotechnology* 2(1): 18 – 23.

Owada, E., Yoshira, M. and Izawa, T. (1969): Pharm. of flavonoids. *Japan Patent* 32784, 69.

Pegnyemb, D.E., Tih R.G., Sondengam, B.L., Blond, A. and Bodo A.(2003): Biflavonoids from *Ochna afzeli*. *Phytochemistry* ,31,2, 219-221.

Pegnyemb, D.E., Tih, R.G., Sondengam, B.L., Blond, A., Bodo, A.(2003): Isolation and structure elucidation of a new isobiflavonoid from *Ochna afzeli. Pharmaceutical Biology*. 41, 92.

Pelter, A., Warren, R., Rahaman, N., Khan, U.N., Ilyas, M. and Rahman, W. (1965): A possible new group of some reactions of metal borides. *Journal of Chromatography*. 20, 572.

Pelter, A., warren, R. Ilyas, M., Usmani, J.N., Bhatriager, S.P., Riv, R.H. and Rahaman, W. (1969a): The Structure of W13, the First Optically Active Biflavone of the Amentoflavone series. *Experimentia* 25, 350.

Pelter, A., Warren, R., Usmani, J.N., Ilyas, M. and Rahman, W. (1969b): Biflavonyls from *Guttifereae garcinia. Tetrahedron Letters*. 25, 4259.

Pelter, A., warren, N., Ilyas, M., Usmani, J.N. and Rahaman, W. (1969c): The Isolation and Characterization of two members of a new series of naturally occurring biflavones. *Experimentia* 25, 351.

Pelter, A., Warren, R., Hameed, N., Khan, N.U., Ilyas, M. and Rahman, W. (1970): Biflavonyl Pigments from *Thuja orientalis* (Cupressaceae). *Phytochemistry* 9, 1897.

Pelter, A., Warren, R., Chexal, K.K., Handa, B.K., Rahman, W., (1971a). Biflavonyls from *Guttifereae* – Garcinia livingstonii. Tetrahedron 27, 1625–1634.

Peter, W. (1965). Drug resistance in *Plasmodium beighei* Vincke and Lips,1948. I. Chloroquine resistance. *Experimental Parasitol.* 17(1): 80-89.

Peter, W., Portus J.H. and Robinson B.L., (1975): The chemotherapy of rodent malaria. The value of *P. berghei* in screening of blood schinzonticidal activity. *Ann Tropical Medical Parastol*, 1975, 69, 171.

Prasad, J.S.and krishnamurty, H.G. (1977): C- glycosylflavones from *Rhynchosia minima*. *Phytochemistry*, 16, 801-3.

Perry, L.M.(1980):'Medicinal Plants of East and Southeast Asia', MIT Press, Massachusetts, 1980, 289.

Peschke, W. (1965): Flavonoid from *Viburrium cotinifolium*. *Phytochemistry* 9, 1897.

Phillipson J.D., and Wright C.W.(1991): Can ethnopharmacology contribute to the development of antimalarial agents? *Journal of Ethnopharmacology*, 32 (1991), pp. 155–171.

Pusztai, R., Beladi, I., Bakai, M., Musci, I. and Kukan, E. (1966): Physiology and functions of flavonoids. *Acta Microbiology journal of Academic Science.* 13, 113.

Rahman, W., Hameed, N. and Ilyas, M. (1972): Peltogynoids and evaluation of their estrogenic activity. *Journal of India Chemical Society.* 49, 917.

Ramawat, K. G., Goyal, S., (2008). Bioactive Molecules and Medicinal Plants. Springer, Heidelberg, New York. 325 – 347.

Ramoutsaki, I. A., Askitopoulou, H., Konsolaki, E., 2002. Pain relief and sedation in Roman Byzantine texts: *Mandragoras officinarum*, *Hyoscyamos niger* and *Atropa belladonna. International Congress Service.* 1242, 43-50.

Rampendahl, C., Tassito, S., Hans G. and Hansdietmar, Z. (1996): The biflavonoids of *Plagiomnium undulatum. Phytochemistry* 41, 6, 1624.

Rangaswani, S. and Sastry, B.V.R. (1955): Constitution of Lanceolatin C and Lanceolatin B. *Current science. (Ind*) 24, 13.

Rao, N.S.P., Row, L.R. and Brown, R.T. (1973):Composition of bhilawanol from *Semecarpus anacardium. Phytochemistry*, 12, 671.

Rao, K.V., Sreeramulu K., Rao C.V., Gunasekar D., Martin M.T., Bodo B.(1997): Shoot organogenesis and somatic embryogenesis from leaf and shoot explants of *Ochna integerrima* (Lour). *Journal of Natural Product*. 60: 632-634.

Reddy, B.A., Reddy N.P., Gunasekar D.,Blond A., Bodo B.(2008): Total synthesis of Ochnaflavone. *Phytochemical Letter* 2008, 1, 27.

Reddy B.A., Lee D., Tih, R.G., Gunasekar D. and Bodo, B (2012): Phytochemical and Biological Studies of *Ochna* Species. *Chemistry and Biodiversity*,9.

Rendle, A. B(1952): 'The Classification of Flowering Plants', Vol. 2 Cambridge University Press, London, U.K. 1952.

Reutrakul, V., Ningnuek , N., Pohmkotr ,M., Yoosook, C., Napaswad, C., Kasisit, J., Santisuk T., Tuchinda, P.(2007):Anti HIV-1 flavonoid glycosides from *Ochna integerrima. Planta Medicine*. 73, 683.

Richard, J.P.C. (1998): Natural Product Isolation, Glaxo welcome. Research and development, Sterenage, Herts, U.K. 1.

Robinson, G.M. and Robinson, R. (1931): Flavonoid glycosides and hydroxycinnamic acid ester of black currents. *Journal of Biochemistry*. 25, 1687.

Robinson, R., (1945). The constitution of strychnine. Communication provisores 1, 28-29.

Ryley, J.F., Peter, W.(1970). The antimalarial activity of some quinolone esters. *Ann Tropical Medical Parasitol* 64 (2): 209-222.

Sardari, S.A., Gholameraza, L.G. and Daneshtalab, M. (1998): Phyto-pharmaceuticals. Part 1: Antifungal activity of selected Iranian and Canadian Plants. *Journal of Pharmaceutical Biology*. 36: 180-188.

Seaquamat, T., (2005). Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. *The Lanceoletter* 366, 717-725.

Seeger, Tl., (1995). Isomeric triluteolins from Bartramia stricta and *Bartramia pomiformis*, *Phytochemistry*, 40, 1531.

Shekelle, P. G., Hardy, M. L., Morton, S. C., Maglione, M., Mojica, W. A., Suttorp, M. J., Rhodes, S. L., Jungvig, L., Gagn, J., (2003). Efficacy and safety of Ephedra and ephedrine for weight Loss and athletic performance. *Journal of American Medical Association.* 289, 1537-1545.

Shengmin, S., Xianfang, C., Nangun, Z., Jin-Woo. And Chi-tang, H.O.(2003): Iridiod Glycoside from the leaves of *Morinda citrifolia*. *Journal of Natural Product.* 66: 799 – 800.

Sheng –Hong, L., Hong, L.Z., Xue – Mei, N., Ping, Y., Hang – Dong, S. and Harry, H.S.F. (2003): *Journal of Natural Product.* 66, 7 pp 1002 – 1005.

Si H, Hu S, Lin Z, Zeng Z.L.(2008): Antimicrobial effect of oregano essential oil

along and in combination with antibiotics against extended spectrum b-lactamase-producing *Scherichia coli*. *Immunology Medicinal Microbiol*ogy. 53:190-194.

Sieradzki K, Wu S.W, Tomasz A. (1999). Inactivation of the methicillin resistance gene in vancomycin-resistant *Staphylococcus aureus*. *Micro Drug Resistant* 5(4): 253 – 257.

Sisowath C, Ferreira P. E, Bustamante LY, Dahlströ̈m S, Mårtensson A, Björkman A, Krishna S, Gil JP(2007):. The role of pfmdr1 in *Plasmodium falciparum* tolerance to artemether-lumefantrine in Africa. *Tropical Medicine in International Health*. 2007; 12: 736 –742.

Sofowora, A. (1982): Medicinal Plant and Traditional Medicine in Africa, New York. U.S.A. 6, 14 142 – 145, 150 – 154, 208 – 213.

Sofowora, A. (2008): medicinal plants and traditional medicine in Africa. 3rd edition, Spectrum books Ltd. Ibadan, Nigeria pp. 23-25.

Stahl, E. (1969): Thin Layer Chromatography. A Lab Handbook 2nd Edition George Allen and Unwin LTD Springer – verlag London U.K pp 7 – 8, 873 –893.

Stauffer, D.A. and mile laboratories (1968): United State Pharmacopeioe. 3, 410, 851.

Suh, S. J., Chung, T. W., Son, M. J., Kim, S. H., Moon, T. C., Son, K. H., Kim, H. P., Chang, H. W., Kim, C. H., (2006). The naturally occurring biflavonoid, ochnaflavone, inhibits LPS-induced iNOS expression, which is mediated by ERK1/2 via NF-kB regulation, in RAW264.7 cell. *Biochemical Biophysiology.* 447, 136-146.

Sutherland CJ, Ord R, Dunyo S, Jawara M, Drakeley C.J, Alexander N, Coleman R, Pinder M, Walraven G, Targett G.A. (2005). Reduction of malaria transmission to *Anopheles* mosquitoes with a six dose regimen of co-artemether. *Plant Medicine*. 2, 92.

Szirmai, E. (1965): Pharmacology studies of flavonoids. Ztsch *Inn Medicine* 20, 755.

Talisuna AO, Bloland P, D'Alessandro U. (2004): History, Dynamics, and Public Health Importance of Malaria Parasite Resistance. *Clinical Microbiology Revison.* 17:235–254.

Tang, S., Bremner P., Kortenkamp, A., Schlage, C., Gray A.I., Gibbons, S., Heinrich, M.(2003): Natural products and their role as inhibitors of the por-inflammatory transcription factor NF-kB. *Planta Medicine*. 69, 247.

Tan, T.G., Pezzuto A.D., Kinghorn A.D.,Hughes S.H.(1991): Extraction of secondary metabolites from plants. *Journal of Natural Product*. 54, 143.

Tania M.A.A, Axréia F.S, Mitzi B, Telma S.M.G, Elza A.S, Artur S.J and Carlos L.Z

(2000). Biological Screening of Brazilian Medicinal Plants. *Mem Inst Oswaldo Cruz, Rio de Janeiro.* 95(3): 367-373.

Trease, E. and Evans, W.C. (2002): Pharmacognosy. Balliere Tindall as edited in 15th edition. 193, 196, 223 – 4, 336 – 7, 544.

Tschesche, R. (1962): Thin layer chromatography of lipids. *Journal of Chromatography*. 5, 217.

Uhlemann A.C, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, Lee A, Brady L, Haynes RK, Krishna S. (2005); A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Natural Structural Molecular Biol*ogy. 12:628–629.

UNICEF, (2004): Malaria: a major cause of child death and poverty in Africa. New York: United Nations Children's Fund (UNICEF).

Vane, J. R., Botting, R. M., (2003). The mechanism of action of aspirin. *Thromb. Research*. 110, 255-258.

Venkataraman, K. (1972): Wood phenolice in the chemotaxanomy of the Moraceae. *Phytochemistry.* 11, 1571.

Vianna, G.S.B., Bandena, M.A.M., Matos, F.T.A., (2003). Analgesic and anti-Inflammatory effects of Chalcones isolated from *Myracrodruon urudeuva* Allemao. *Phytomedicine*. 10(2-3):189-197.

Voigt, H. (1961):The number of naturally occurring flavones known at present. *Chemical Pharmacology*. Fabrik. DBP.1, 270, 567.

Vollokova, A.D., Kostalova S. and Sochorova, F. (2001): Isoquinoline alkaloid from *Mahonia aquifoluim* stem bark is active against Maiseisia species. *Journal of Microbiology.* 46, 107-111.

Vuorela, P., Leinonen, M., Saikku, P., Tammela, P., Rauha, J.-P., Wennberg, T., Vuorela, H., (2004). Natural products in the process of finding new drug candidates. *Curricullum in Medicinal Chemistry.* 11, 1375-1389.

W.H.O (1996). Expert Committee on specification pharmaceutical preparation report, Geneva.WHO technical report services 823. P.44-75.

W.H.O (2002). Traditional Medicine: Planning for Cost-effective Traditional Health Services in the New Century. A Discussion Paper. Centre for Health Development.

W.H.O (2005). National policies on traditional medicine and regulation of herbal medicine. Report of W.H.O global survey. World health organization geneva.

W.H.O (2006). National Policies on Traditional Medicine and Regulation of herbal

Medicine, Reports of WHO global survey. World Health Organization, Geneva.

W.H.O (2008). World malaria report, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

W.H.O (2011). World malaria report. Nigeria Malaria Fact Sheet. United States Embassy in Nigeria.

W.H.O (2014.) World malaria report, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

Woodward, R. B., Brehm, W. J., (1948). The structure of strychnine. Formulation of the neo bases. *Journal of American Chemical Society*. 70, 2107-2115.

Woodward, R. B., Brehm, W. J., Nelson, A. L., (1947). The structure of strychnine. *Journal of American Chemical Society*. 69, 2250-2250.

Woodward, R. B., Cava, M. P., Ollis, W. D., Hunger, A., Daeniker, H. U., Schenker, K., (1954). The total synthesis of strychnine. *Journal of American Chemical Society.* 76, 4749-4751.

Yala D, Merad AS, Mohamedi D, Ouar Korich MN (2001): Classification et mode d'action des antibiotiques. Médecine du. Maghreb, 91:5-12.

Yaching, S., Chung-Ling, L., Shih-Chao, C., Ashraf, T.K., Chinlien, K. and Chin-hsin, W., (2004): Vibsane Diterpenoids from the leaves and flowers of *Viburnum odoratissismum. Journal of Natural Product*. 67: 74 – 77.

Yildirim, A., Turker, A., (2009). In vitro adventitious shoot regeneration of the Medicinal plant meadowsweet (*Filipendula ulmaria* (L.) Maxim). In Vitro Cell. *Developmental Biology.* 45, 135-144.

Young, H.C., Young, H.L., Hosup, Y. and Jin, W. K. (1996). Diglycoside from *Lepisorus ussuriensis. Journal of Natural Product.* 43, 5, p1111-1113.

APPENDIX

Table 1: ^{13}C NMR of BND and a Reference compound (Hye *et al*., 2009)

C – position	Literature ^{13}C (ppm)	BND ^{13}C (ppm)
2	80.0	78.5
3	66.3	66.1
4	27.7	27.9
5	155.3	155.9
6	93.9	95.1
7	156.7	156.3
8	95.1	94.6
9	156.4	156.6
10	99.1	98.8
1’	130.6	130.9
2’	114.5	113.9
3’	144.6	144.4
4’	144.8	144.6
5’	115.1	114.6
6’	118.4	118.1

Literature: Hye *et al*., 2009

Table: 2

^{13}C of BNF and a Reference Compound (Rao *et al.*, 1997)

Position	MNC ^{13}C (ppm)	DEPT	Literature ^{13}C (ppm)
2	163.8	C	163.6
3	103.2	CH	103.9
4	182.1	C(C=O)	182.2
5	164.0	C	161.8
6	99.5	CH	94.6
7	163.8	C	164.9
8	94.2	CH	99.4
9	158.1	C	157.8
10	102	C	104.1
1’	124.83	C	122.5
2’	120.2	C	121.6
3’	143.4	C	142.2
4’	143.4	C	154.1
5’	118	C	118.5
6’	124.76	CH	125.8
1’’	68.12	CH	-
2’’	22.27	CH_3	-
2’’’	163.8	C	163.2
3’’’	102	CH	104.2
4’’’	178.9	C(C=O)	182.1
5’’’	158.1	C	161.8
6’’’	99.6	CH	94.6
7’’’	163.8	C	164.7
8’’’	94.2	C	99.4
9’’’	164.0	C	157.8
10’’’	103.2	C	104.4
1’’’’	124.83	C	124.8
2’’’’	127.7	CH	128.9
3’’’’	116.3	CH	116.6
4’’’’	158.1	C	154.1
5’’’’	116.3	CH	116.6
6’’’’	127.7	CH	128.9
1’’’’’	29.4	CH_2	_
2’’’’’	72.46	CH	_
3’’’’’	63.2	CH_2	_

Literature: Rao *et al.*, (1997).