PHYTOCHEMICAL AND ANTI-INFLAMMATORY STUDIES OF N-BUTANOL SOLUBLE FRACTION OF THE METHANOLIC EXTRACT OF THE AERIAL PARTS OF *INDIGOFERA HIRSUTA* LINN (LEGUMINOSAE)

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MARCH, 2013

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By

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M. Sc/Pharm-Sci/04528/2008-2009

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

MARCH, 2013

Declaration

I declare that the work in this Thesis entitled **'**PHYTOCHEMICAL AND ANTI-INFLAMMATORY STUDIES OF N-BUTANOL SOLUBLE FRACTION OF THE METHANOLIC EXTRACT OF THE AERIAL PARTS OF *INDIGOFERA HIRSUTA* LINN (LEGUMINOSAE)' has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. 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.

Labbo Sani ABBAS	_____	_____
Name of Student	Signature	Date

Certification

This thesis entitled 'PHYTOCHEMICAL AND ANTI-INFLAMMATORY STUDIES OF N-BUTANOL SOLUBLE FRACTION OF THE METHANOLIC EXTRACT OF THE AERIAL PARTS OF *INDIGOFERA HIRSUTA* LINN (LEGUMINOSAE)' by Labbo Sani ABBAS meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Abstract

Indigofera hirsuta Linn. (Leguminosae) is a well-known plant used in African traditional medicine as an anti-inflammatory, antibacterial, antiulcer, anti-venom and anticonvulsant. The air-dried aerial parts of *I. hirsuta* was extracted with methanol using a soxhlet extractor and fractionated into ethyl acetate (EtOAc), n-butanol (n-BuOH) and aqueous fractions (AF). The n-butanol soluble fraction when subjected to preliminary phytochemical screening revealed the presence of flavonoids, phenolic compounds, saponins and tannins.

Repeated gel filtration of n-butanol soluble fraction over a Sephadex LH-20 column eluted with methanol followed by preparative thin-layer Chromatography resulted in the isolation of two flavonol glycosides (Kaempferol-3-O-β-D-glucopyranoside and Kaempferol-7-O-β-D-glucopyranoside). The structures of these compounds were elucidated using chemical tests, spectroscopic techniques (UV, FTIR, 1D and 2D-NMR), and by comparison with reference spectral data.

The n-butanol soluble fraction was found to have intraperitoneal LD_{50} of 1264.91 mg/kg in rats. Anti-inflammatory study using carrageenan-induced paw oedema in rats indicated that, the fraction significantly ($p<0.05$) inhibited the paw oedema at doses of 150 and 300 mg/kg. The percentage anti-inflammatory activity at the peak hour of the highest dose tested (300 mg/kg) was higher than that of ketoprofen (10 mg/kg), the standard anti-inflammatory agent used.

The result of this research suggests that the n-butanol soluble fraction of the methanolic extract of the aerial parts of *Indigofera hirsuta* contains compounds with anti-inflammatory activity.

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List of Abbreviations

^{o}C	degree Celcius
cm	centimeter
cm^{1}	Wave number unit
g	gram
g/kg	gram per kilogram
Hz	Hertz
mg/ml	miligram per milliliter
ml	milliliter
nm	nanometer
ppm	parts per million
UV	ultraviolet spectroscopy
IR	infrared spectroscopy
λ_{max}	maximum absorption wavelength
V_{max}	maximum absorption wavenumber
TLC	thin-layer chromatography
PTLC	preparative thin-layer chromatography
^{1}H-NMR	proton nuclear magnetic resonance spectroscophy
δ	chemical shift in ppm
s	singlet
d	doublet
dd	double doublet
t	triplet
m	multiplet
mg/kg	milligram per kilogram

MHz	mega hertz
%	percentage
Hr	hour
R_f value	Retardation factor
β	beta
D	dextrose
DEPT	Distortionless Enhancement Polarisation Transfer
Glc	Glucopyranose
CD_3OD	deuterated methanol
^{13}C-NMR	Carbon-13 nuclear magnetic resonace spectroscopy
HMBC	Heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
COSY	correlation spectroscopy
LD_{50}	median lethal dose
NSAIDS	Non-steroidal anti-inflammatory drugs
PG	prostaglandin
KOH	potassium hydroxide
COX	Cyclooygenase
LOX	lipooxygenase
EtOAc	ethyl acetate
$CHCl_3$	chloroform
MeOH	methanol
MMP - 2	metalloproteinase - 2
MMP - 9	metalloproteinase - 9

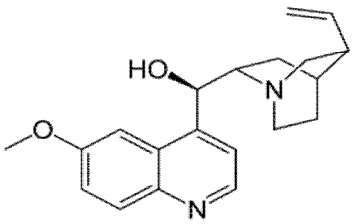
CHAPTER ONE

1.0 INTRODUCTION

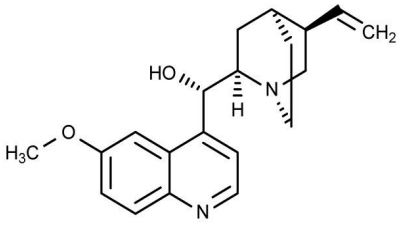
The use of plants in the treatment of various ailments predates modern civilization and medicine (Adiomoelja, 2000). Medicinal herbs exhibit valuable bioactivities, including, antioxidant, anti-inflammatory, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral activities (Kumar *et al.,* 2011*)*. Medicinal plants have become the main target of chemists, biochemists, and pharmacists in the development and discovery of new drugs (Samart, 2007).

Plants play a significant role in the discovery of leads for the development of drugs. Natural product-derived compounds represent great structural diversity, which is not commonly seen in synthetic compounds (Jachak and Saklani, 2007). Of the 1184 new chemical compounds reported during 1981 to 2006, 60% were obtained from plants (Gautam and Jachak, 2009).

Plants have been used as sources of potent therapeutic agents including: quinine (i) and quinidine (ii) two alkaloids isolated from the bark of *cinchona* plant that have been used for ages in the management of malaria (Borris, 1996).



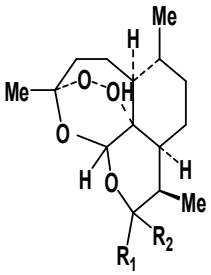
(i)

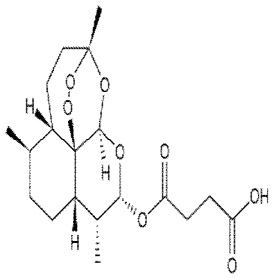


(ii)

Artemisinin (iii), first isolated from the leaves *of Artemisia annua* plant has been used by Chinese herbalists for more than a thousand years in the treatment of many illnesses, including skin diseases and malaria; together with its derivatives such as Artemether (iv),

Dihydroartemisinin (v) and artesunate (vi) reportedly possess the most rapid action of all current drugs against falciparum malaria (White, 1997).



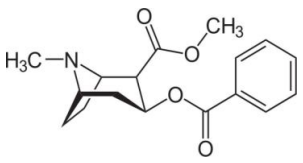


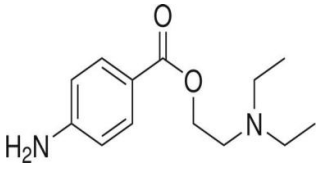
(iii) R_1, R_2 = O, Artemisinin (vi)

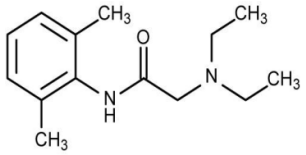
(iv) R_1 = H, R_2 = OMe, Artemether

(v) R_1 = H, R_2 = OH, Dihydroartemisinin

The coca plant, *Erythroxylum coca* is a source of the drug cocaine (vii) and has served as lead for the synthesis of local anaesthetic agents such as procaine (viii) and lidocaine (ix).

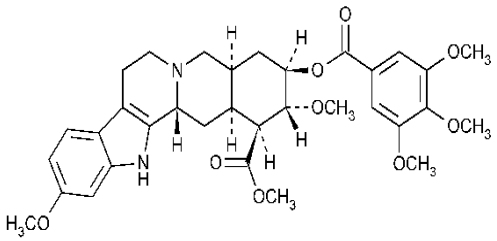






(vii) (viii) (ix)

Reserpine (x) from *Rauwolfia serpentina* is used for the treatment of mental illness in Indian ayurveda medicine (Evans, 2002).



(x)

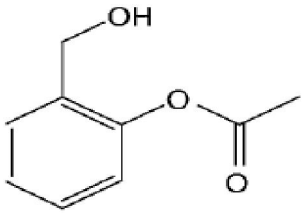
Only about 15% of the more than 250,000 plant species have been investigated phytochemically (Fabricant and Farnsworth, 2001). Moreover, about 80% of the populations of the developing countries rely on ethno-medical practices, most of which are herbal preparations (Farnsworth *et al*., 1985) for their primary healthcare needs. The World Health Organisation (WHO) encouraged the standardization and integration of useful practices into the conventional system through systematic researches to validate activity claims of the herbal preparations **(**Farnsworth, 1984; Sofowora, 1993).

Lack of equipment and resources coupled with the complex nature of the secondary metabolites existing in insufficient quantities in plants (Rates, 2001), remains a major challenge in isolation, characterization and bioassay of compound(s) required for development and clinical use. However, the surge in the use of plant-derived medicaments and nutritionals even in the developed countries of Europe and America (Jachak and Saklani, 2007), the rising costs of prescription drugs and the search for drug treatment in newer and chronic diseases such as hypertension, HIV/AIDS and cancer necessitates interdisciplinary research efforts in higher plants (Newmann and Cragg, 2007).

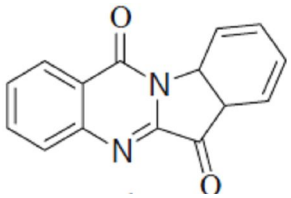
1.1 Inflammation and Anti-Inflammatory Compounds from Plants

Inflammation is a dynamic process that is elicited in response to mechanical injuries, burns, microbial infections, and other noxious stimuli that may threaten the well-being of the host (Gautam and Jachak, 2009). The classical key features of inflammation are redness, swelling and pain. This process involves change in blood flow, increased vascular permeability, destruction of tissues via the activation and migration of leucocytes with synthesis of reactive oxygen derivatives and other local inflammatory mediators, such as prostaglandins (PGs), leukotrienes, platelet-activating factors induced by phospholipase A_2, cyclooxygenases (COXs), and lipoxygenases (Wiart, 2006).

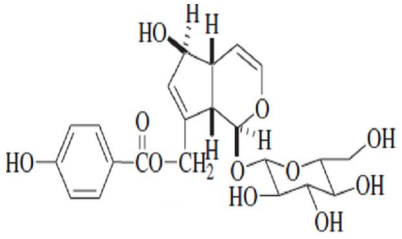
Different classes of compounds from plants have been reported to possess anti-inflammatory activities, these include: salicylic acid (Aspirin) (xi) isolated from the roots of *Glycyrrhiza glabra* (liquorice) and globally used as a non-steroidal anti-inflammatory drug NSAIDs (Wiart, 2006). Tryptanthrin (xii) an indoloquinazoline alkaloid, isolated from dichloromethane crude extract of *Isatis tinctoria* leaves was thought to exhibit its anti-inflammatory effect through COX-2 inhibition (Danz *et al.*, 2001).



(xi)



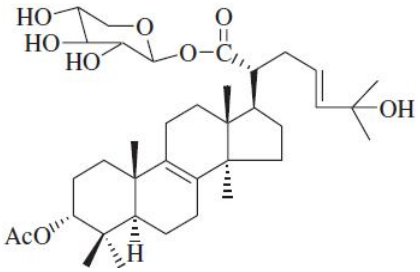
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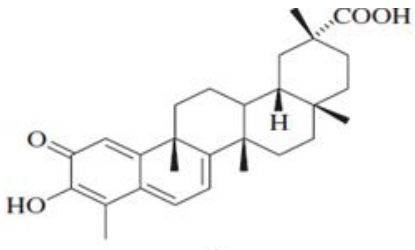
(xiii)

An iridoid glycoside, Agnuside (xiii) isolated from *Vitex peduncularis* also showed inhibition of COX-2 (Suksamrarn *et al.*, 2002).

A new lanostane triterpene glycoside (xiv) isolated from *Fomitopsis pinicola* fruits exhibited more potent inhibitory effect on COX-2 enzyme than indomethacin (IC_{50} 0.60 μM) and a weak inhibitory effect on COX-1 (Yoshikawa *et al.,* 2005). A novel triterpene, celastrol (xv), showed effective anti-inflammatory activity in animal models of arthritis, and alzheimer‘s disease (Corson and Crews, 2007).

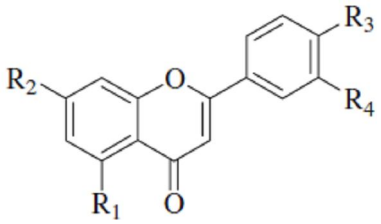


(xiv)

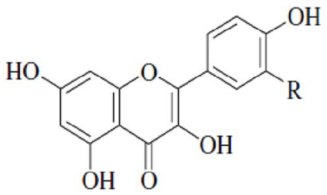


(xv)

The flavonoids luteolin (xvi), kaempferol (xvii) and quercetin (xviii) have anti-inflammatory effect; they were reported to exhibit a significant inhibitory effect on MMP-9 and MMP-2. (Ende and Gebhardt, 2004; Ziyan *et al.,* 2007).



R_1= R_2= R_3= R_4=OH (xvi)



R = H (xvii)
R = OH (xviii)

1.2 Statement of Research Problem

Clinical studies revealed that chronic inflammation is the major risk factor for various types of disease like cancer (Jachak, 2006). It has been estimated that the underlying infections and inflammatory reactions are linked to 15–20% of all cancer deaths (Mantovani and Pierotti, 2008). Inflammation can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, osteoarthritis, multiple sclerosis and inflammatory bowel diseases (Smith, 2005). These diseases are debilitating and becoming increasingly common in our aging society. Rheumatoid arthritis and osteoarthritis are the major inflammatory diseases affecting people worldwide (Lawrence, 2007). Rheumatoid arthritis is an inflammatory condition that usually affects multiple joints and is more prevalent among women in developed countries. Osteoarthritis is characterized by loss of joint cartilage that leads to pain and loss of function primarily in the knees and hips; it affects 9.6% of men and 18% of women who are more than 60 years of age (Gautam and Jachak, 2009). Increases in life expectancy and aging populations are expected to make osteoarthritis the fourth leading cause of disability by the year 2020 (Woolf and Pfleger, 2003).

The two main classes of drugs employed in the management of inflammatory disorders; corticosteroids and NSAIDs do not result in complete cure. Moreover, severe side effects including obesity, hypertension, osteoporosis and increased susceptibility to infections remain the major challenges of corticosteroid therapy while gastrointestinal ulcerations, bleeding and platelet dysfunction are some of the serious side effects of NSAIDs drugs (Jachak, 2006).

The use of biological therapies also presented another set of challenges - difficulty of predicting in vivo adverse responses based on in vitro assays and more risks of cardiac complications (Strand *et al.,* 2007). In view of the above, there is need for a permanent search and development of new effective and safer drugs, and higher plants represent a potential source of novel anti-inflammatory prototypes (Jachak and Saklani, 2007).

1.3 Justification of the Study

The plant *Indigofera hirsuta* is among the several species of the genus *Indigofera* found mostly in highlands of northern part of Nigeria that are largely un-investigated. In ethno-medicine, the plant is employed as anti-inflammation of the eyelids, treatment of diabetes, leprosy, tuberculosis, infections, snake bite, and in the management of malaria. These are significant health problems afflicting substantial number of the communities in developing countries, like Nigeria. Non-steroidal anti-inflammatory drugs are the main class of drugs employed in the management of inflammatory disorders and do not result in complete cure. Moreover, gastrointestinal ulcerations, bleeding and platelet dysfunction are some of the serious side effects of NSAIDs drugs (Hassan *et al.,* 2010).

Species of *Indigofera* genus have been extensively employed in many parts of the world including Asia and Africa for the treatment of pains, bacterial infections, inflammatory disorders, dysentery, hepatitis, stomach and urinary complaints (Schmitz and Bacher, 2005).

Phytochemical investigations conducted on some of these species revealed the presence of diverse classes of phenolic compounds, most especially flavonoids which have been shown to possess anti-microbial and anti-inflammatory activities (Newman and Cragg, 2007).

Although research work was carried out on the anti-inflammatory activity of the crude methanolic extract of the aerial parts of *I. hirsuta*, not much has been done in terms of isolation of phytoconstituents and pharmacological studies on the partition fractions of the methanol extract of the plant. Therefore, the aim of the present study is to carry out the isolation of the phytoconstituents from the n-butanol fraction of the methanol extract of the aerial parts of *I. hirsuta* and to carry out anti-inflammatory activity of the fraction.

1.4 Aim and Objectives

1.4.1 General aim of the study

The aim of this study is to isolate and elucidate some of the bioactive compounds present in n-butanol soluble fraction of the methanolic extract of *Indigofera hirsuta* aerial parts and to establish the anti-inflammatory activity of the fraction using laboratory animal models.

1.4.2 Specific objectives of the study

The specific objectives of the study are:

i. To establish the anti-inflammatory potential of the n-butanol soluble fraction using laboratory animal.

ii. To establish and isolate some of the phytochemical constituents present in the fraction using standard phytochemical methods.

iii. To elucidate the structures of the isolated compounds through spectroscopic analysis.

1.5 Statement of Research Hypothesis

Indigofera hirsuta contain bioactive compounds with anti-inflammatory activity that can be isolated and tested using laboratory models.

CHAPTER TWO

2.0 LITERATURE REVIEW

The genus *Indigofera* Linn is a large genus of about 700 species of flowering plants belonging to the sub-family Papilionoideae in the family Leguminosae. The species are native mainly to tropical and sub-tropical regions of the world (Burkill, 1995; Nwachukwu and Mbagwu, 2007). About 60 species have been identified in Nigeria (Soladoye and Lewis, 2003) with over 60% abundance distributed in the Northern region of the country.

2.1 Morphology of Indigofera Species

Indigofera species are creeping prostate, annual, biennial or perennial herb or semi-woody under shrubs, and few can become small trees up to 5-6 ft in height (Dalwitz and Wattson, 2000). The stem may be slender, erect or conspicuously branched with bright green or tinged with red colour. The leaves are mostly openly spaced on the stem around 10cm long and may be roughly or velvety smooth to touch (Dorfling, 2001). The fruits are oval shaped and elongated, 4-angled or flattened usually smooth, reddish brown flowers that look like butterflies and turn into peapods (Soladoye *et al.,* 2010). The fruits are often curved with 2-15 seeds in most species.

2.2 Morphology of *Indigofera hirsuta*

Indigofera hirsuta is an annual herb or sub shrub, up to 1.5 m tall, variable with two varieties in the region: var. *hirsuta* commonly found in tropical Africa and across Asia to China and Australia; var. *pumila* Welw., a small plant found in northern part of Nigeria and in Angola (Burkill, 1995)

2.3 Ethnomedicinal Uses of *Indigofera hirsuta* and Other Species in the Genus

Indigofera hirsuta has several medicinal uses; in Kenya it is used as chest medicine while in Tanganyika the whole plant is prepared as an external application for back ache. In Nigeria, the leaf-sap is taken internally for liver complaints and the leaf-decoction is used for yaws and ulcers, gravels, and epilepsy or convulsions in infants. In India, it is used for cerebral disorders and as a remedy towards the injury of the eyeball, inflammation of the eyelids, or to kill filaria in the conjunctiva tissue (Burkill, 1995). In Tanganyika, the leaves, flowers and tender shoots are used for leprosy and cancer (Chandramouli, 1995; Singh and Padney, 1997). In Rwanda, the leaves and roots are used for respiratory ailment, leprosy, malaria, scabies and sores, it is also used to alleviate labour pain, stomach disorders and tuberculosis (Hakizanmungu, *et al*., 1994); the plant is also used for the treatment of skin itching, infections, snake bite, epilepsy and jaundice (Burkill, 1995). *Indigofera articulata* has been used for the management of toothache and epilepsy in human (Roig, 1974) and it also has embryotoxic effects (Perez de Alejo *et al.,* 1996).

The leaves of *Indigofera pulchra* is used to treat infected wounds (Hepper, 1976; Burkill, 1995). In Nigeria, decoction of its aerial parts is used in the management of snake-bite (Sule *et al.,* 2003), counteract various poisons (Burkill, 1995), against malaria and dysentery (Adamu *et al*., 2005; Asase *et al*., 2005). The leaves of *Indigofera oblongifolia* is used in the treatment of urinary tract infections, urothliasis and as a remedy for cough (Al-Alwadh *et al*., 2002). Decoction of the aerial parts of *Indigofera tuxellensis* is used for the treatment of gastro-intestinal disorders (Roig, 1988) and diarrhoea (Methabe *et al*., 2006). *Indigofera tinctoria* is widely used in Indian and Chinese traditional medicine for the treatment of epilepsy, nervous disorders, bronchitis, liver ailments, and as an ointment for sores, old ulcers, hemorrhoids, piles, growth of hair and for treating skin diseases, urinary complaints, hepatitis and gastropathy (Satyavati *et al.,* 1987; Liang and Jin, 1997). In Cameroun, stem of *Indigofera*

tinctoria is chewed to cure cough while decoction of the leaves is also used to cure chest pains, epilepsy, nervous disorders, asthma, bronchitis, fever, stomach, liver, kidney and spleen disorders (Takawira-Nyenya and Cardon, 2005);warm decoction of the leaves of this plant is used in the treatment of bruises (Ibe and Martin, 2005).

2.4 Chemistry of Flavonoids

2.4.1 Flavonoids and its distribution

Flavonoids are low molecular weight bioactive polyphenolic compounds which play a tremendous role in photosynthesizing cells (Kumar *et al., 2011*). They are widely distributed throughout the plant kingdom. Flavonoids are found in vegetables, fruits, nuts, seeds, stem, flowers, tea, wine etc. and these are an integral part of our daily diet. The dietary intake of flavonoids is estimated to be 1-2 g/day; the average intake of flavonols and flavones was found to be 23 mg/day, among which, quercetin contributed 16 mg/day (Kumar *et al., 2011*).

2.4.2 Structure of flavonoids

Flavonoids are secondary metabolites. They are a group of structurally related compounds with a chromane-type skeleton having phenyl substituent on C_2 or C_3 position. The basic structural feature of flavonoid can be considered as a derivatives of the 2-phenyl-benzo-γ-pyrane nucleus composed of two benzene rings - A and B joined through a heterocyclic pyran ring C (Fig. 2.1), (Kumar *et al., 2011*).

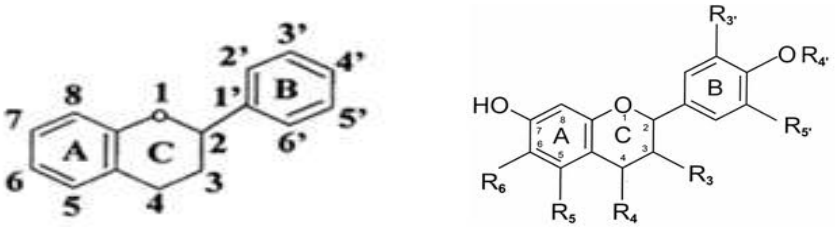


Fig (2.1): Basic structure of Flavonoids

Flavonoids differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings. Naturally occurring flavonoids are generally classified into various classes (Fig. 2.2) according to their molecular structures, C-ring and the functional groups at C-3 and C-4 (Kumar *et al., 2011).*

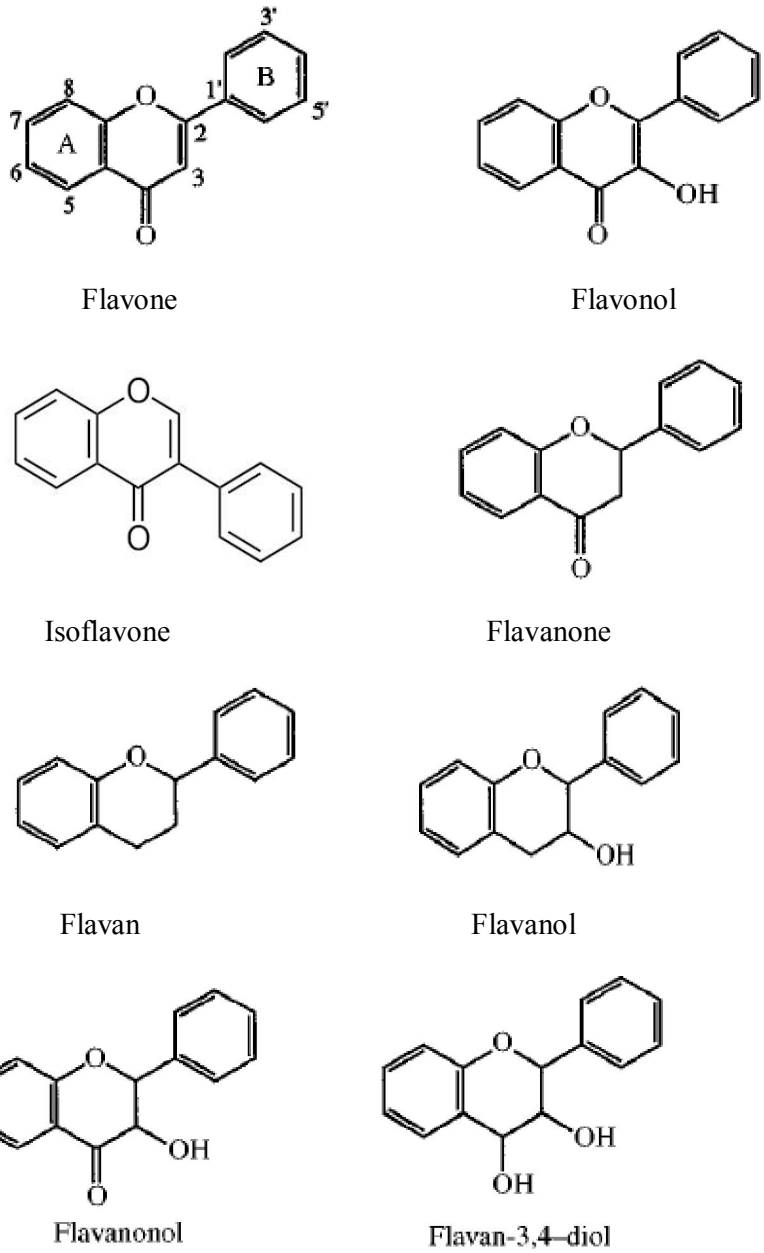
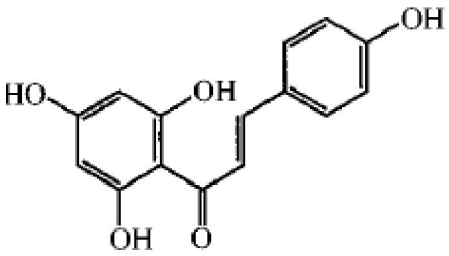
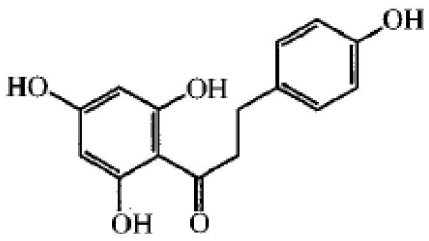


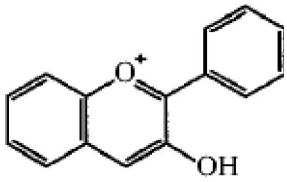
Fig. (2.2): Chemical Structures of the various classes of flavonoids (*Kumar et al.,* 2011; Robards and Antolovich, 1997)



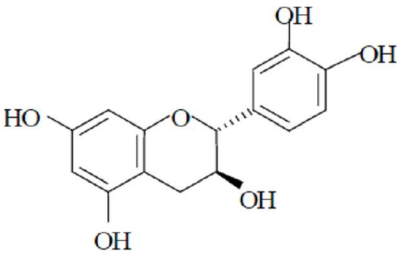
Chalcone



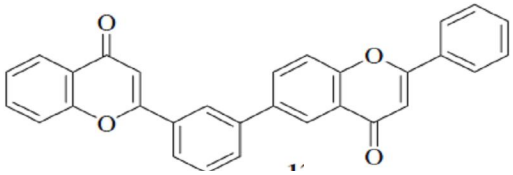
Dihydrochalcone



Anthocyanidin



Catechin



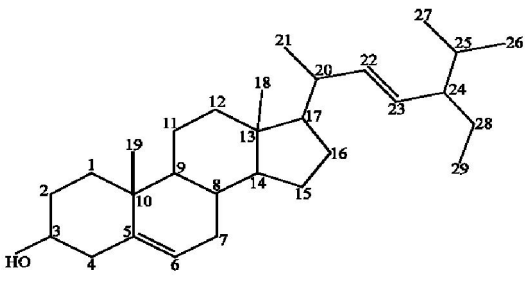
Biflavone (Gautam and Jachak, 2009)

(Fig. 2.2): Continued

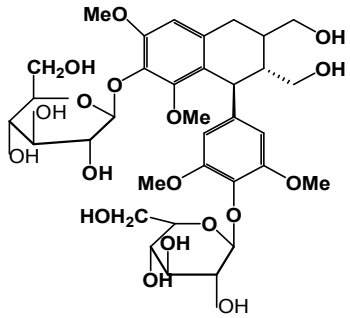
In plants, flavonoids are present as O-glycosides or C-glycosides. The O-glycosides possess sugar substituent attached to –OH of aglycone usually at positions C-3 or C-7, while C-glycosides possess sugar groups attached directly to Carbon of aglycone usually at C-6 or C-8 (Kumar *et al., 2011*).

2.5 Phytochemistry of *Indigofera hirsuta* and other Species in the Genus *Indigofera*

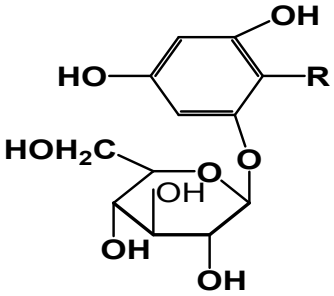
Phytochemical investigations on *Indigofera hirsuta* led to the isolation of stigmasterol (xix) from n-hexane fraction of the aerial parts of methanol extract (Musa *et al.*, 2012). Chemical investigations on other species of *Indigofera include*; isolation of a lignan and two phluroglucinols from the aerial parts of *Indigofera hethtrantha* (Azizur-rahman *et al.*, 2005).The lignans isolated is (+)-lynoresinol-4,4'-bis-O-β-D-glucopyranoside (xx) and the phluroglucinols isolated were 1-[(-methylbutanoyl) phluroglucinyl]-β-D-glucopyranoside (xxi) and 1-[(pentanoyl) phluroglucinyl]-β-D-glucopyranoside (xxii).

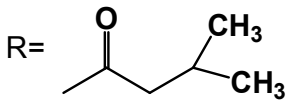


(xix)



(xx)



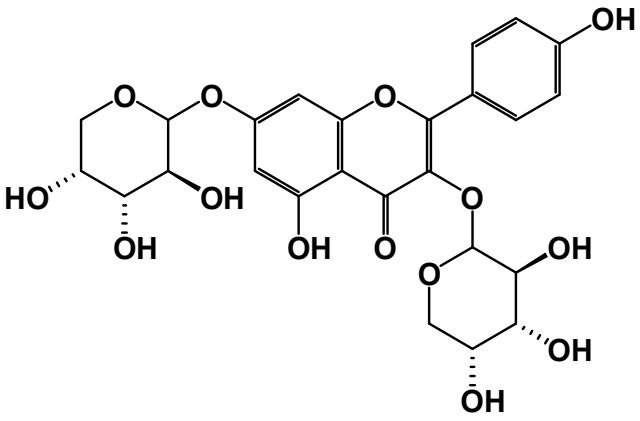


(xxi)

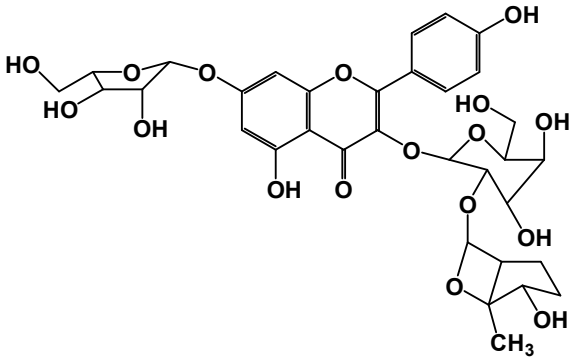


(xxii)

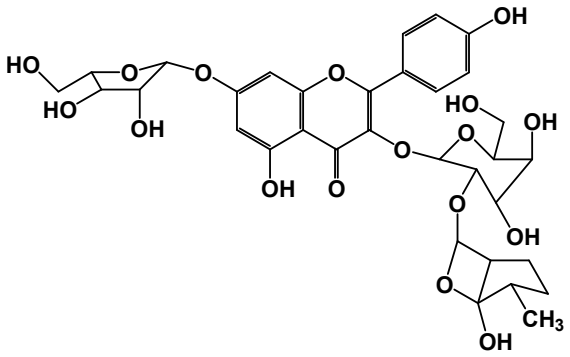
The isolation of kaempferol-3,7-diarabinoside(xxiii) from the leaves of *Indigofera hebepetala* was reported (Hassan *et al*., 1994). Similarly, the isolation of five flavonoid glycosides from the flower of the same plant has been reported (Hassan *et al*., 1996); these glycosides includes: kaempferol-3-O-α-L-rhamnopyranosyl-(1-2)-β-D- galactopyranoside-7-O-α-L-arabinoside (xxiv), kaempferol-3-O-α-L-rhamnopyranosyl-(1-6)-β-D-galactopyranoside-7-O-α-L-arabinofuranoside (xxv), kaempferol-3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside(xxvi), kaempferol-3-O-α-L-arabinopyranoside-7-O-α-L-arabinopyranoside (xxvii) and kaempferol-7-O-rhamnoside (xxviii).



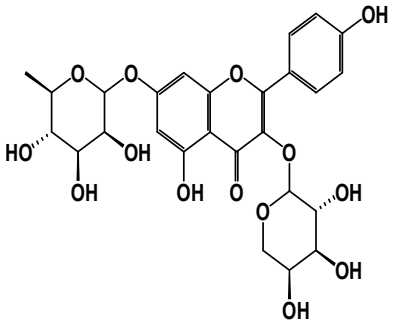
(xxiii)



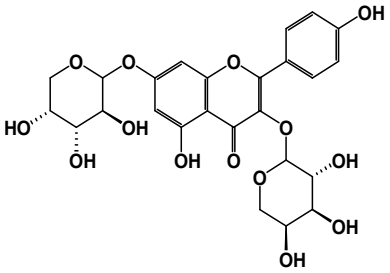
(xxiv)



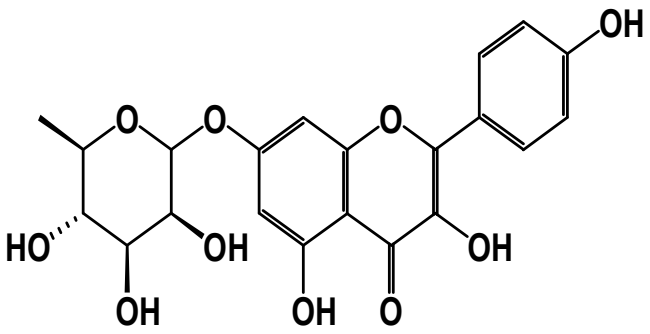
(xxv)



(xxvi)

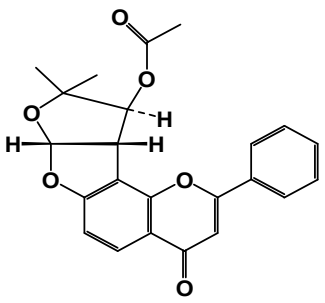


(xxvii)

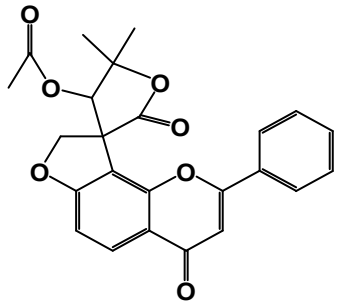


(xxviii)

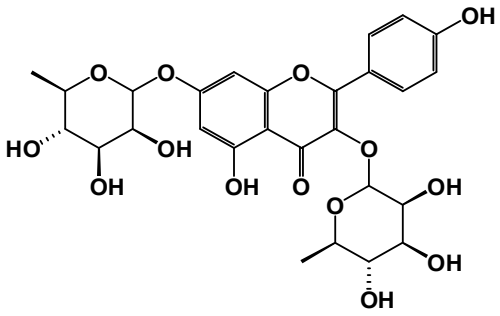
Phytochemical investigation of the aerial parts of *Indigofera tinctoria* led to the isolation of semiglabarin (xxix), glabretephrin (xxx) and kaempferol-3, 7- dirhamnoside (xxxi) (Rao and Raju, 1984).



(xxix)

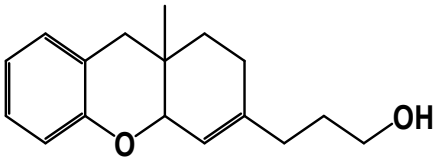


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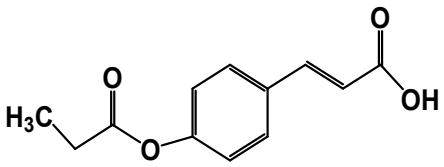


(xxxi)

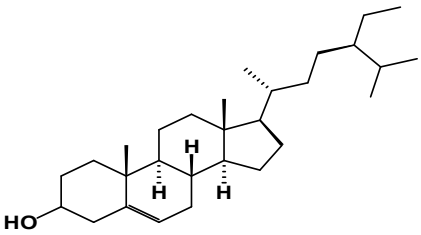
Furthermore, phytochemical investigations of the chloroform fraction of the whole plant of *Indigofera oblongifolia* yielded two new and two known compounds; the new compounds were indigin an alkylated xanthene (xxxii), and indigoferic acid (xxxiii) while the known compounds are β-sitosterol (xxxiv) and 3-hydroxybenzoic acid (xxxv) (Sharif *et al.,* 2005).



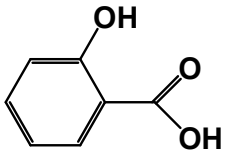
(xxxii)



(xxxiii)

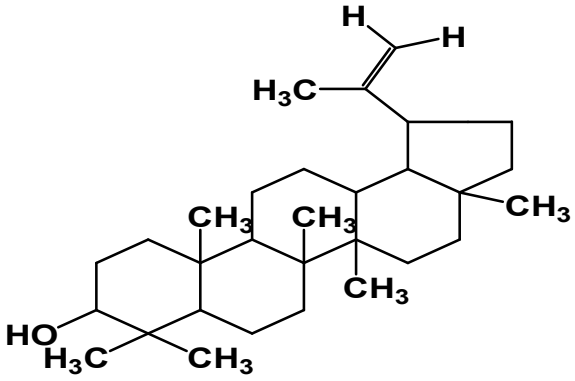


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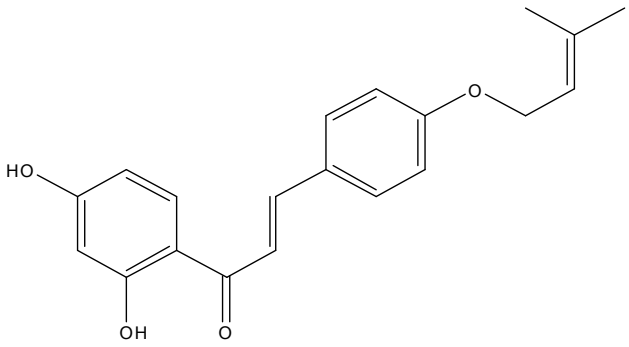


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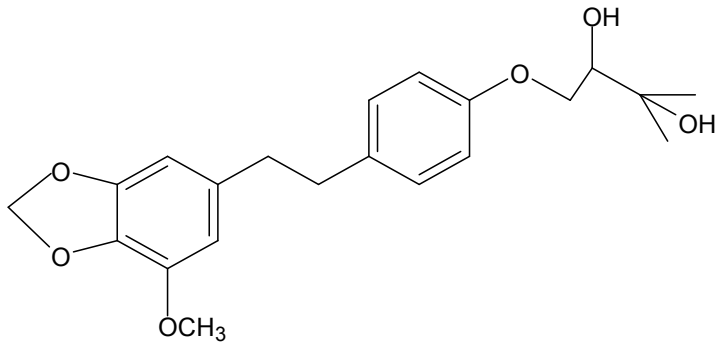
In addition, phytochemical investigations of methanol extract of the aerial parts of *Indigofera pulchra* led to the isolation of β-sitosterol (xxxiv), Lupeol (xxxvi), 2′,4′-dihydroxy-4-prenyloxychalcone (xxxvii) and a mixture of two new dihydrostilbenes; 3-methoxy-4,5-methylenedioxy-4'-*O*-(2,3-dihydroxy-3methylbutyl)-dihydrostilbene (xxxviii) and 3,5-dimethoxy-4'-*O*-(2,3-dihydroxy-3-methylbutyl)-dihydrostilbene (xxxvix) (Musa *et al.,* 2008).



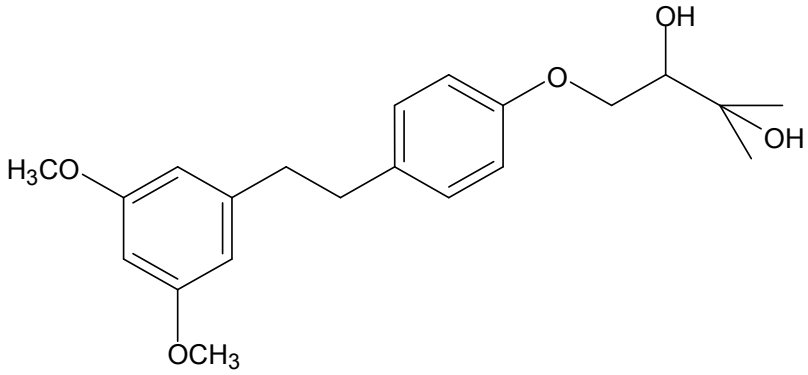
(xxxvi)



(xxxvii)



(xxxviii)



(xxxix)

2.6 Pharmacological Actions of *Indigofera hirsuta* and Other Species of *Indigofera Genus*

Literature search on biological studies of the Indigofera species revealed various activities including: anti-inflammatory, anti-bacterial, cytotoxicity, anti-ulcerogenic, anti-diabetic, anti-cancer, anti-oxidant, antispasmodic, anti-depressant, antimicrobial and anti-allergic. *Indigofera hirsuta* was reportedly shown to possess anti-bacterial activity (Yasodamma *et al.,* 2009), analgesic and anti-inflammatory activity (Musa *et al.,* 2012).

Other species with pharmacological actions are; *Indigofera dendroides* reported to have antibacterial activities (Mathabe *et al*., 2006; Esimone *et al.*, 1999) and smooth muscle contraction effect (Amos *et al*., 2003). *Indigofera pulchra* has been reported to have

antimicrobial activity (Musa *et al*., 2007), analgesic and anti-inflammatory activities (Abubakar *et al*., 2007; Musa *et al*., 2010), venom detoxifying effect (Abubakar *et al*., 2006), hypoglycemic (Tanko *et al.,* 2008) and anti-oxidant activity (Bakasso *et al.,* 2008). *Indigofera longercemosa* has been reported to possess antimicrobial activity (Thangadurai *et al*., 2001). *Indigofera asphalatoides* has been reported to posses anti-inflammatory (Bhaskr *et al*., 1982; Amala *et al*., 1982), it has been reported to have the following activities; anticancer (Rajkapoor *et al*., 2004), anti-bacterial (Bhuvaneswari and Balasundaram, 2006) and hepatoprotective activities (Rajkapoor *et al*., 2005). *Indigofera tuxellensis* has been reported to have anti-ulcerogenic activity (Cola-miranda *et al*., 2006) and mutagenic activity (Calvo *et al.,* 2009).

Indigofera tinctoria has been reported to posses hepatoprotective (Singh *et al*., 2006), anti-diabetic (Verma *et al.,* 2010), depressant action (Rahamathullah *et al.,* 1991) anti-oxidant action (Sreepriya *et al*., 2001; Bakasso *et al.,* 2008), and hypolipidemic activity was also reported (Puri *et al.,* 2007). *Indigofera oblongifolia* has been reported to have anti-microbial (Ali *et al.,* 1999; Al-Awadh *et al*., 2001; Dahot, 2001), hepatoprotective (Shajahan *et al*., 2005) and lipoxygenase inhibitory effects (Sharif *et al*., 2005). *Indigofera suffruticosa* possess mutagenic activity (Calvo *et al.,* 2009).

Other species are also associated with analgesic and anti-inflammatory activity (Anonymous, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents used were of analytical grade obtained from Sigma Aldrich, Germany. Sephadex LH-20 (GE Health care Bio-Sciences AB SE-75184 Uppsala) and thin-layer chromatography (TLC) 60 GF_{254} precoated glass plate all were also obtained from Sigma Aldrich, Germany.

3.1.2 Equipment

- Ultraviolet (UV): Pye Unicam 5000 spectrophotometer available in the Pharmaceutical Chemistry department and was used for visualizing spots under UV lamp (254 and 365 nm).
- UV-Visible spectrophotometer, UV – 2500pc series used for running spectra of the isolated Compounds at National Research Institute for Chemical Technology, Zaria.
- Shimadzu FTIR-fourier transform infrared spectrophotometer, 8400S model was used for running the spectra of the isolated compounds at NARICT, Zaria.
- Bruker AVANCE III NMR spectrometers, Japan operating at 400 MHz (^{1}H) and 100 MHz (^{13}C) were used for the samples, using TMS as the internal standard with CD_3OD as solvents. Chemical shifts are reported in δ units and coupling constants (J) in Hz, carried out at School of Chemistry, University of Kwa-Zulu Natal-Durban, South Africa.

3.2 Methods

3.2.1 Collection and identification of plant material

Indigofera hirsuta plant sample was collected from bush near Samaru, Zaria in September, 2010. It was identified by U.S. Gallah of the herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria, by comparing with herbarium reference voucher specimen (No.390). The aerial parts were cut into pieces, air-dried and powdered.

3.2.2 Extraction procedures

The aerial parts powdered of the plant were exhaustively extracted with methanol (2.5L x 2) using Soxhlet apparatus for 48hrs. The solvent was removed *in vacuo* using Rotary evaporator which yielded a solid residue coded as the methanol extract of *I. hirsuta* (MEIH), its colour and weight were recorded. MEIH was suspended in distilled water and filtered. The filtrate was successively partitioned with ethyl acetate and n-butanol to afford ethyl acetate, n-butanol and aqueous fractions coded EAF, NBF and AF respectively.

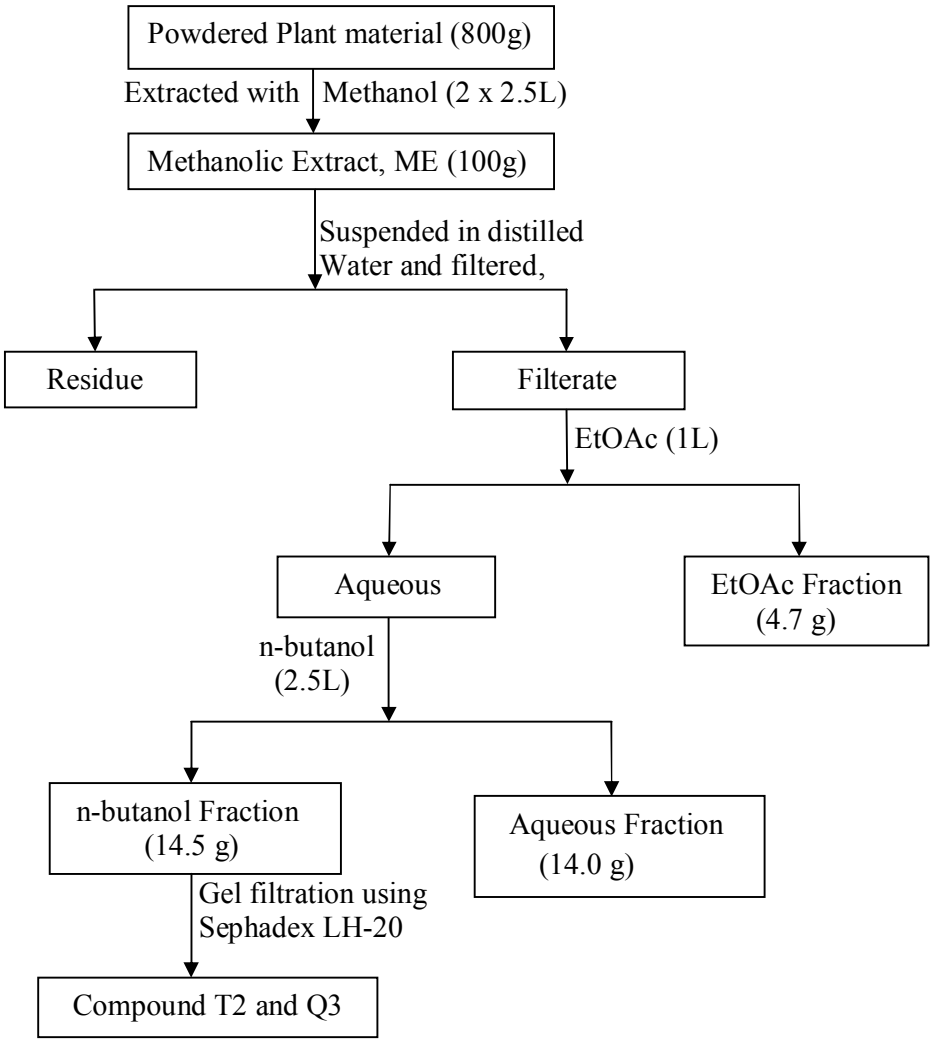


Fig 3.1: The chart of extraction protocol.

Having conducted preliminary TLC on all the fractions mentioned, the TLC profile of NBF revealed the most prominent spots (plate: 4.1) and therefore was chosen for further study.

3.2.3 Preliminary phytochemical screening

The n-butanol soluble fraction was subjected to preliminary phytochemical screening using standard methods as summarized below:

3.2.2.1 Test for flavonoids

(i) Shinoda Test

0.5g Of the fraction was dissolved in 1-2ml of methanol and pieces of metallic magnesium chips were added followed by a few drops of concentrated hydrochloric acid. The colour change was observed (Silva *et al*., 1998)

(ii) Sodium Hydroxide Test

2-3 Drops of 10% sodium hydroxide were added to the solution of the fraction. The colour change was observed (Silva *et al*., 1998).

(iii) Ferric Chloride Test

2-3 Drops of ferric chloride solution were added to the solution of the fraction. The colour change was observed (Silva *et al*., 1998).

3.2.2.2 Test for saponins

Frothing Test

About 10ml of distilled water was added to a portion of n-butanol fraction in a test tube and shaken vigorously for 30seconds.The tube was allowed to stand in a vertical position and observed for 30mins. Presence of a honeycomb froth lasting for 10-15mins was observed (Silva *et al*., 1998).

3.2.2.3 Test for tannins

(i) Lead Sub-acetate Test:

To a small portion of the fraction, 3-5 drops of lead acetate solution was added. Presence of precipitate was observed (Silva *et al*., 1998).

3.2.2.4 Test for alkaloids

(i) Dragendoff's Test

2-3 Drops of Dragendoff's reagent was added to the solution of the fraction. The colour change was observed (Silva *et al.*, 1998).

(ii) Wagner's Test

2-3 Drops of Wagner's reagent was added to a small amount of the diluted fraction. Presence of a whitish precipitate was observed (Silva *et al.*, 1998).

(iii) Meyer's test

2-3 Drops of Meyer's reagent was added to the solution of the fraction. Presence of a white to yellowish precipitate was observed (Silva *et al.,* 1998).

3.2.4 Chromatographic methods

3.2.3.1 Thin-layer chromatography (TLC)

Thin-layer chromatography was performed on precoated TLC plate (silica gel 60 PF_{254}) by one way ascending technique. Spots were applied manually using capillary tube and developed at room temperature in an air tight chromatographic tanks using either of the following: EtOAc-$CHCl_3$-MeOH-H_2O (15:8:4:1) and EtOAc-MeOH-H_2O (100:16.5:13.5) solvent systems. Developed chromatograms were air-dried and visualized;

- under ultraviolet light (254 and 366nm)
- by spraying with Gibb's reagent and exposure to ammonia fumes.
- spraying with 10% sulphuric acid followed by heating at 100^0c for 3-5 minutes in an oven.

3.2.5 Chromatographic separation

3.2.5.1 Gel filtration column chromatography using sephadex LH-20

4 g Of n-butanol soluble fraction (NBF) was subjected to gel filtration over sephadex LH-20 column and eluted with methanol. A total of 52 fractions (5ml each) were collected and were combined based on their TLC profiles to give eleven (11) major fractions coded A-K (Table 4.3). Fraction H was further purified through repeated gel filtration to give fraction MP consisting of 2 spots. MP was subjected to preparative TLC.

3.2.5.2 Preparative thin-layer chromatography (PTLC)

Preparative TLC was performed using (pre-coated glass plate 20 x 20 cm and 0.25mm thickness). Fraction MP was applied uniformly on a thin line marked with pencil about 1.5cm from the bottom of the plate using capillary tube and allowed to dry. Dried plates were developed using EtOAc-$CHCl_3$-MeOH-H_2O (15: 8: 4: 1) solvent system. The regions containing band of interest were marked and scrapped off. The scrapped sorbent was size reduced using pestle and mortar, transferred to a sintered glass funnel and extracted repeatedly with methanol; the solution obtained was evaporated to afford a yellow solid compounds coded T2 and Q3 (Gibbons and Gray, 1998).

3.2.6 Characterization of T2 and Q3

a. Solubility Test:

The solubility of T2 and Q3 were observed in $CHCl_3$, Acetone, ethyl acetate and Methanol.

b. Chemical Tests:

Both T2 and Q3 were subjected to shinoda, Sodium hydroxide and Ferric Chloride tests.

3.2.7 Spectral analysis for T2 and Q3

The isolated compounds were subjected to UV, IR and NMR analysis

3.3 Pharmacological Studies

3.3.1 Animals

Wister rats (109-198 g) of both sexes were used. The animals were purchased from the animal house facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria-Nigeria. They were kept in standard animal cages at room temperature and provided with standard laboratory diet and water *ad libitum*. The studies were conducted in accordance with the "Principles of laboratory animal care" (NRC, 1985).

3.3.2 Drugs and dosage

Ketoprofen injection (Lek, Slovenia; 10mg/kg) was used as standard drug, 1% solution of Carrageenan (sigma; 0.1cm^3/animal), n-butanol soluble fraction of the methanol extract of *I. hirsuta* aerial parts (75, 150, and 300 mg/kg) and vehicle (Normal saline; DANA, Nigeria; equivalent volume administered with extract). All test solutions were administered intraperitoneally.

3.3.3 Acute toxicity study

The median Lethal dose (LD_{50}) of n-butanol fraction of the methanol extract of *I. hirsuta* was determined using rats, as described by Lorke's (1983).The determination was carried out in two phases. In the first phase, three groups (1, 2 and 3) of three rats each were administered with extract at doses of (10, 100 and 1000 mg/kg) body weight intraperitoneally. They were observed for signs and symptoms of toxicity and death for 24h. In the second phase, four groups each consisting one rat were treated based on the result of the first phase. The 1st, 2^{nd,} 3rd and 4th groups were treated with the extract at doses of 200, 400, 800 and 1600 mg/kg, respectively. They were observed for signs and symptoms of toxicity and death for 24hr. The LD_{50} was calculated as the geometric mean of the lowest lethal dose and the highest non-lethal dose.

3.3.4 Screening for anti-inflammatory activity.

The test was conducted according to the method of (Winter *et al*., 1962). Thirty rats were divided into 5 groups, each group of six animals. Group 1 (negative control) received 1ml/kg saline. Group 2, 3 and 4 received extract at doses of (75, 150 and 300 mg/kg respectively), while group 5 received positive control (ketoprofen; 10 mg/kg body weight. One hour later, 0.1ml of freshly prepared Carrageenan suspension (1% w/v in 0.9% normal saline) was injected into the sub planter region of the left hind paw of each rat. The paw diameter was measured immediately before carrageenan injection and 1, 2, 3, 4 h after carrageenan injection using Vernier calliper.

3.3.5 Statistical analysis

It was conducted using SPSS statistical package 17.0. Data were expressed as mean ± SEM and the mean values of the control groups were compared with the mean values of the treated groups using one way ANOVA followed by Posthoc Dunnet's t-test for multiple comparison. Results were considered statistically significant at ($P< 0.05$). The lowest confidence level is usually indicated $P < 0.05$ (95% C.I).

CHAPTER FOUR

4.0 RESULTS

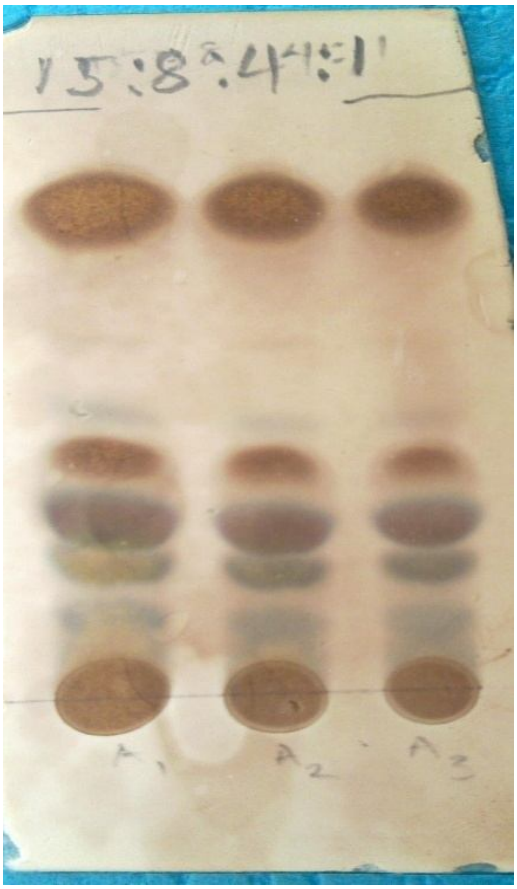
4.1 Extraction and Fractionation Yield

Extraction of the powdered aerial parts of *I. hirsuta* gave a dark greenish residue weighed 100g. The ethyl acetate fraction (EAF) gave 4.7g, n-butanol fraction (NBF) yielded 14.5g and that of aqueous fraction (AF) weighed 14.0 g. The n-butanol fraction was mainly used in this work because of its most prominent spots shown on the preliminary TLC profile (Chromatogram 4.1).

4.2 Result of Phytochemical Studies

4.2.1 Thin-layer chromatography of n-butanol fraction

TLC profile of n-butanol fraction (NBF) using EtOAc-$CHCl_3$-MeOH-H_2O (15: 8: 4:1) solvent system revealed 7 major spots when sprayed with Gibb's reagent and then exposed to ammonia fumes (Chromatogram 4.1).



Chromatogram 4.1: TLC profile of n-butanol fraction

4.2.2 Preliminary phytochemical screening

Preliminary phytochemical screening of n-butanol fraction revealed the presence of flavonoids, phenolic compounds, tannins and saponins (Table 4.1).

Table 4.1: showing result of preliminary phytochemical screening of NBF

Constituents	Test	Observation	Inference
			NBF
Flavonoids	Shinoda	Pink-Red Colour	+
	Sodium hydroxide	Yellow colour	+
Phenolic	Ferric Chloride	Green Precipitate colour	+
Saponins	Frothing	Frothing persisted for 10-15 minutes	+
Tannins	Lead Sub-acetate	Cream precipitate formed	+
Alkaloids	Dragendoff's	Red precipitate formed	−
	Meyer's reagent	White precipitate	−
	Wagner's reagent	White precipitate	−

Key: + = present of Constituents, − = Absent of Constituents

4.3 Result of Sephadex Column Chromatographic Separation

A total of 52 fractions (5ml each) were collected and were pooled based on their TLC profiles to give eleven major fractions coded A-K (Table 4.3). Repeated gel filtration of fraction H gave fraction MP containing two (2) major spots.

Table 4.2: Result of Sephadex column chromatographic separation of n-butanol (NBF)

Eluents No.	Fraction No.	No. of spots	Code	Yield
1	1 - 7	mixture	A	major
2	8 - 10	8	B	major
3	11-14	7	C	minor
4	15-18	6	D	minor
5	19-21	4	E	minor
6	22-26	7	F	major
7	27-32	5	G	minor
8	**33-36**	**3**	**H**	**major**
9	37-41	4	I	minor
10	42-47	3	J	major
11	48-52	4	K	major

4.3.1 Isolation of T2 and Q3

4.3.1.1 Result of preparative TLC

Fraction MP, when subjected to PTLC gave three bands with R_f values of 0.28, 0.31 and 0.35.

4.3.1.2 TLC profile of T2

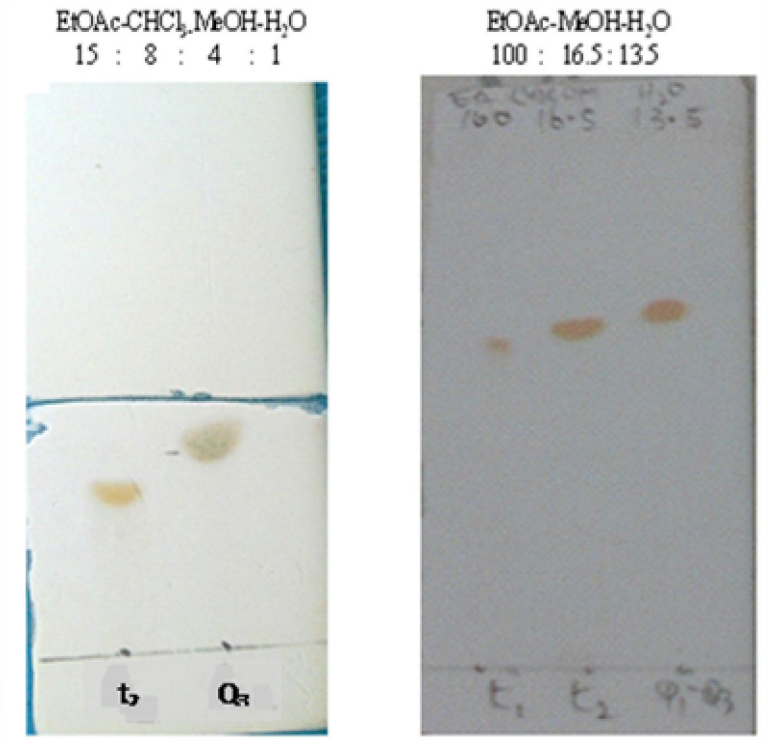
The second band with R_f value 0.31 was scrapped off from the plate and washed with methanol to obtained light yellow solid weighed 9.7mg and coded as T2. It gave a single homogenous spot with two solvent systems (Chromatogram 4.2 and Table 4.3).

4.3.1.3 Solubility profile of T2

T2 was found to be Soluble in methanol, insoluble in $CHCl_3$, acetone and ethyl acetate.

4.3.1.4 Chemical tests of T2 result

It produced a positive test with Shinoda reagent and ferric chloride solution.



Chromatogram 4.2: TLC Profile of T2 and Q3

Table 4.3: showing TLC profile of T2

Solvent system	spraying agent	colour of spot after heating	No. of spot	R_f value
EtOAc-$CHCl_3$-MeOH-H_2O **(**15**:** 8: 4: 1**)**	Gibb's	light-brown	1	0.31
EtOAc-MeOH-H_2O (100: 16.5:13.5)	10% H_2SO_4	dark-yellow	1	0.62

4.3.2 Spectral analysis of T2

4.3.2.1: Ultraviolet (UV) spectral analysis of T2

The UV λmax (MeOH) spectrum showed absorption bands at 267nm and 346nm (Figure4.1).

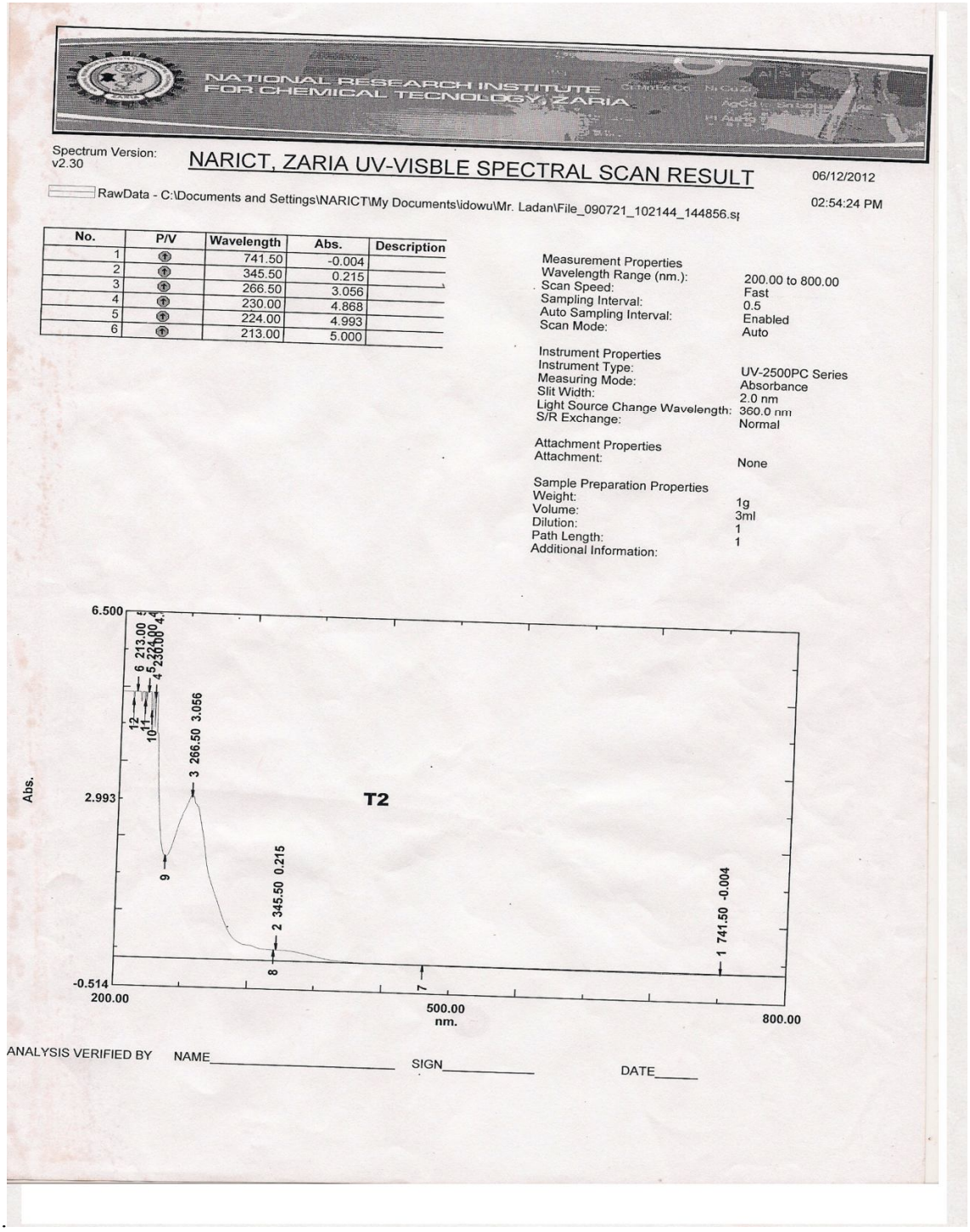
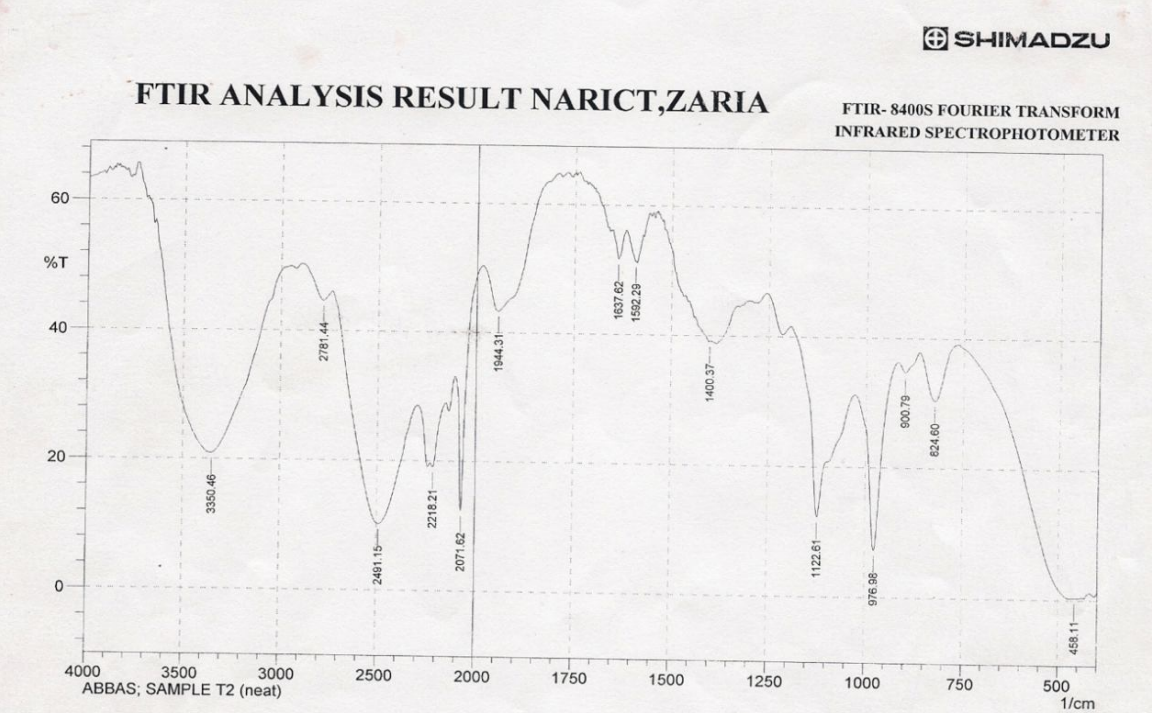


Figure 4.1: UV spectrum of T2

4.3.2.2 Infrared (IR) spectroscopy of T2

FT-IR spectrum Vmax (MeOH) values are 3350, 2781 1637, 1592, 1400, and 1122cm[1] (Figure 4.2 and Table 4.4).



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	458.11	0.352	0.002	459.07	457.14	4.731	0.002
2	824.6	30.372	8.043	865.1	768.66	44.436	4.465
3	900.79	34.723	2.142	919.11	865.1	24.02	0.768
4	976.98	7.284	26.346	1029.06	919.11	70.488	18.506
5	1122.61	12.339	24.73	1194.94	1029.06	96.758	23.212
6	1400.37	39.151	0.065	1402.3	1398.44	1.57	0.002
7	1592.29	51.351	0.201	1617.37	1591.33	7.05	0.022
8	1637.62	51.701	4.273	1652.09	1621.22	8.309	0.527
9	1944.31	43.31	2.175	1985.78	1932.74	17.835	0.508
10	2071.62	12.463	25.825	2108.27	1985.78	58.761	11.134
11	2218.21	19.1	2.106	2230.75	2154.56	47.497	0.124
12	2491.15	10.237	26.033	2737.08	2299.22	301.983	109.317
13	2781.44	44.676	2.655	2893.32	2737.08	51.192	1.659
14	3350.46	20.88	0.073	3351.43	2949.26	189.022	0.948

Comment;
ABBAS; SAMPLE T2 (neat)

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Figure 4.2: IR spectrum of T2

Table 4.4: FT-IR data of Compound T2

Absorption bands (cm^{-1})	Intensity	Vibrations
3350	broad	O-H Stretch
2781	m	C-H Stretch
1637	s	C=O Stretch
1592	m	C=C Stretch
1400	s	C=C Stretch
1122	m	C-O Stretch

S = strong, m = medium

The IR spectrum showed strong absorption at 1637 cm^{-1} (C=O) and a broad band at 3350 cm^{-1} (hydrogen-bonded OH).

4.3.2.3 Proton Nuclear magnetic resonance spectral analysis of T2

The ^{1}H-NMR spectrum of T2 revealed resonances at δ 7.82 (2H, d, J=8.8Hz, H-2′, 6′), 6.66 (2H, d, J=8.8Hz, H-3′, 5′), 6.11 (1H, d, 1.9Hz, H-8), 5.93 (1H, d, J=1.9Hz, H-6), 5.00 (1H, d, J=7.2Hz, H-1″), 3.24 (1H, s, H-2″), 3.20 (1H, t, J=6.68, 7.72Hz, H-3″), 3.16 (1H, s, H-4″), 2.97 (m, H-5″), 3.46 (1H, dd, J=2.2, 2.2Hz, H-6a″), 3.31 (1H, dd, J=5.4, 5.4Hz, H-6b″) for aromatic and sugar regions respectively, (Figure. 4.3a-c) and (Table 4.5 summary of spectral data).

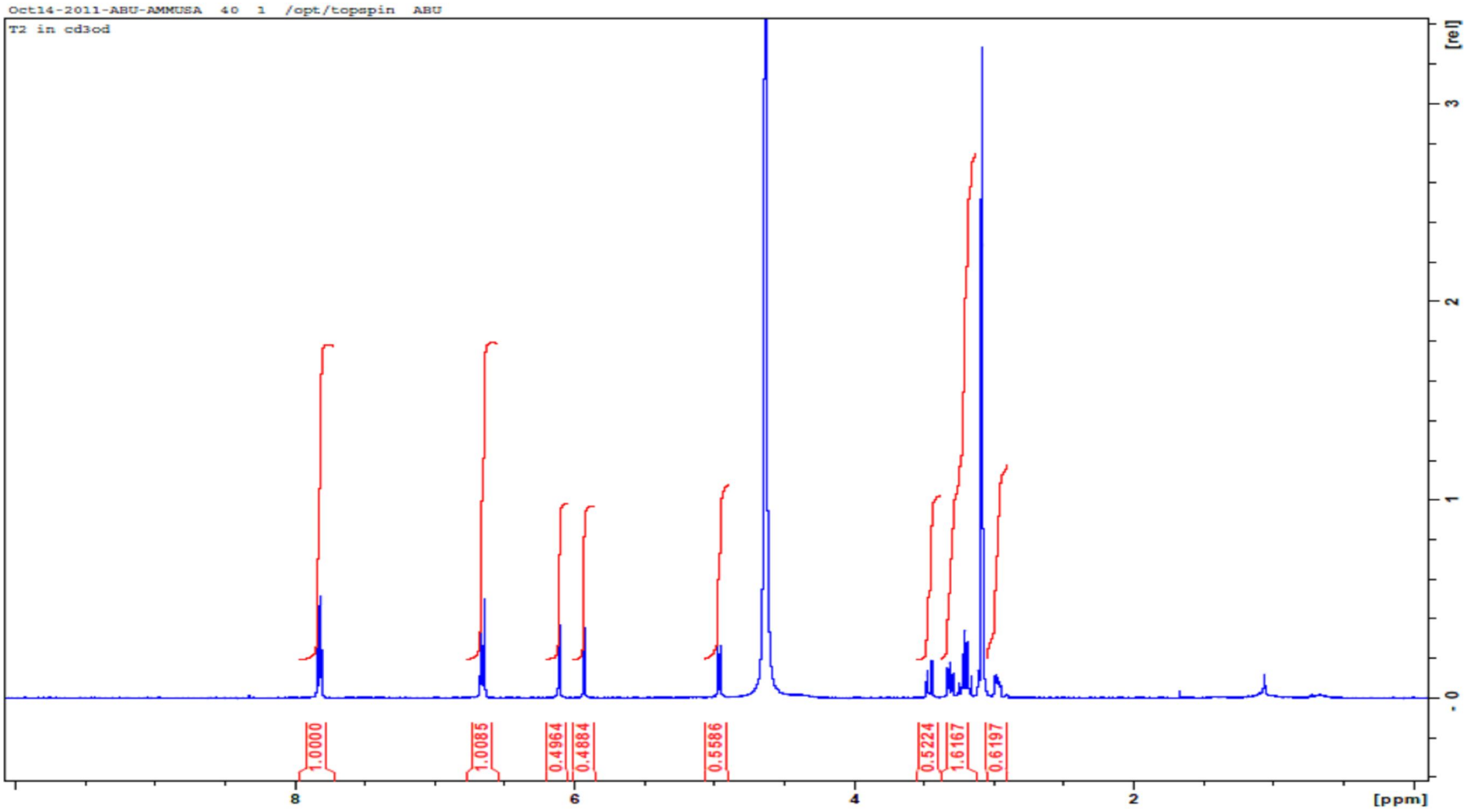


Figure 4.3a: ^{1}H-NMR spectrum of compound T2

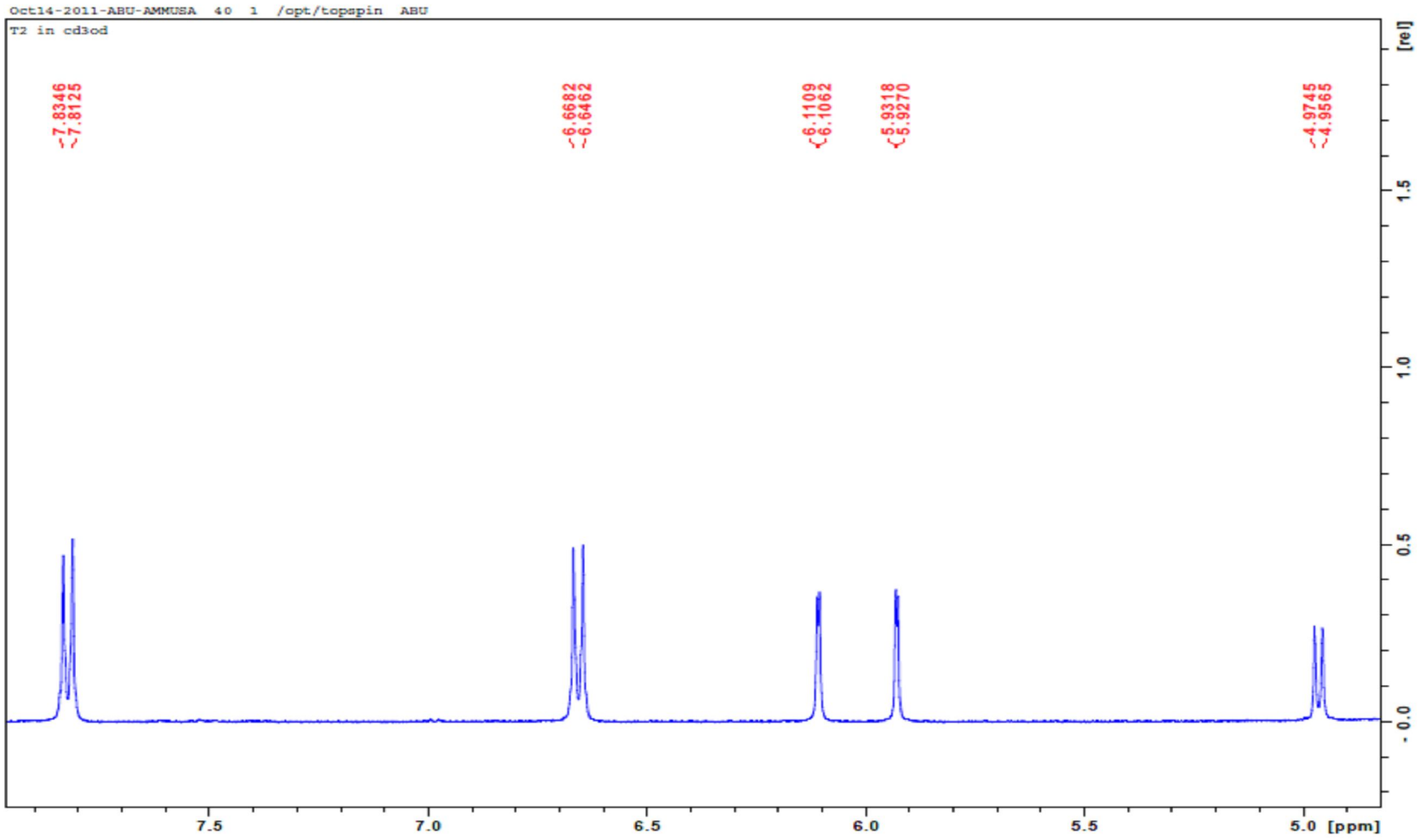


Figure 4.3b: ^{1}H-NMR spectrum of T2 (expanded aromatic region

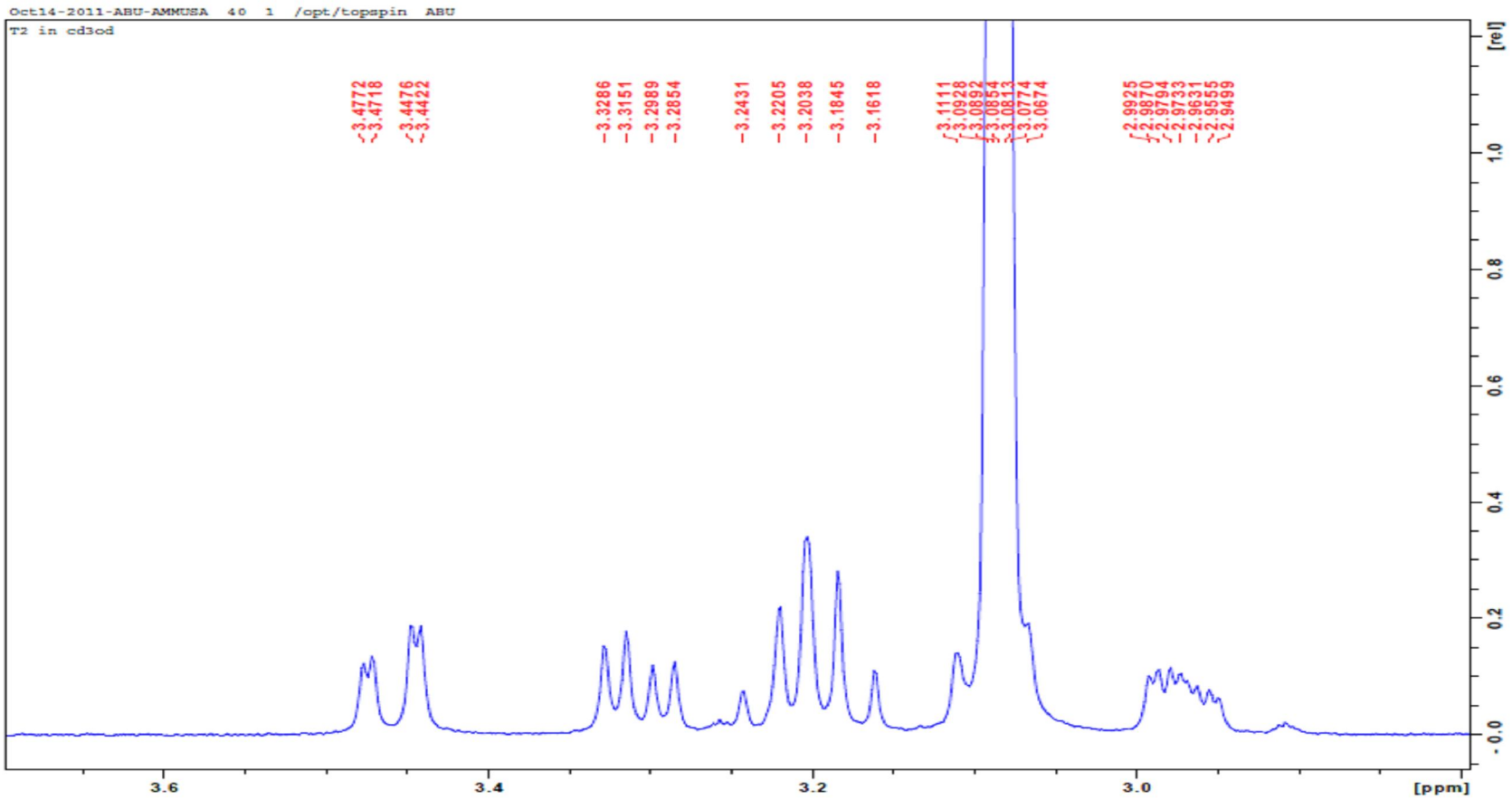


Figure 4.3c: ^{1}H-NMR spectrum of T2 (expanded sugar region)

4.3.2.4 13C-NMR and ^{13}C- DEPT NMR spectral analysis of T2

The ^{13}C-NMR and ^{13}C-DEPT spectra of T2 revealed signals at δ 159 (C-2), 135.4 (C-3), 179.5 (C-4), 163.0 (C-5), 99.8 (C-6), 165.7 (C-7), 95.5 (C-8), 159.0 (C-9), 105.0 (C-10), 122.0 (C-1′), 132.0 (C-2′, 6′), 116.0 (C-3′, 5′), 162.0 (C-4′), 104.8 (C-1″), 76.0 (C-2″), 79.0 (C-3″), 72.0 (C-4″), 78.0 (C-5″) and 63.0 (C-6″).

^{13}C-NMR spectra and the DEPT experiments of T2 indicated presence of 21 carbon atoms including the double signals observed at δ 132.0 and 116.0. The spectra showed 9 quaternary carbon atoms 7 of which are oxygenated including the downfield signal at 179.5 due to carbonyl group; the resonances around 79-63 is characteristic of sugar nucleus and the one methylene carbon absorption shown in the DEPT experiment suggests a glucose moiety (Figures 4.4-4.5) and (Tables 4.5-4.6, summary of spectral data).

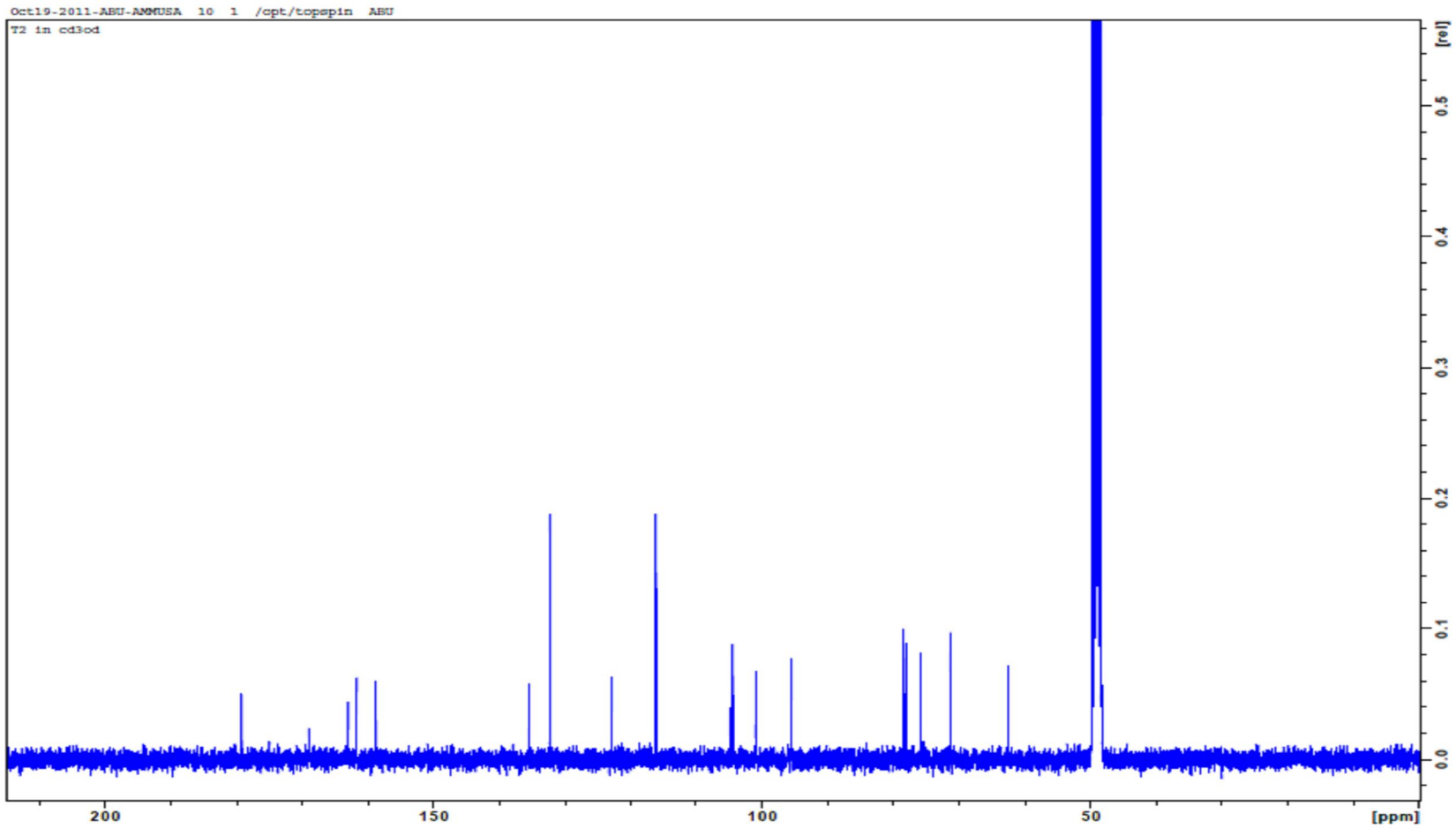


Figure 4.4: ^{13}C-NMR spectrum of T2.

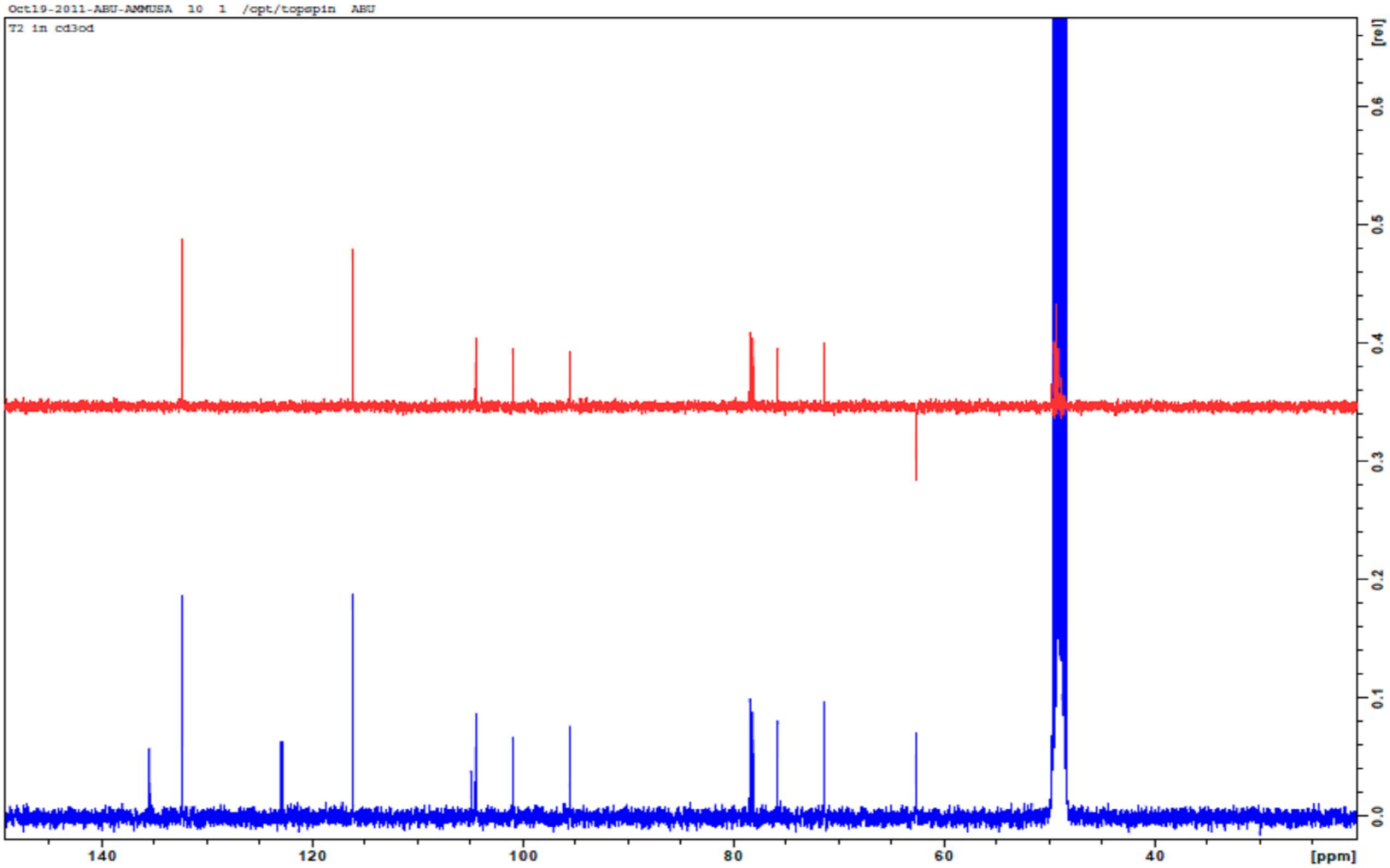


Figure 4.5: ^{13}C-DEPT NMR spectrum of T2

4.3.2.5 HSQC analysis of T2

The HSQC spectrum of T2 shown in Figure 4.6 was used in the assignment of protons to their respective carbon atoms at: (Table 4.7).

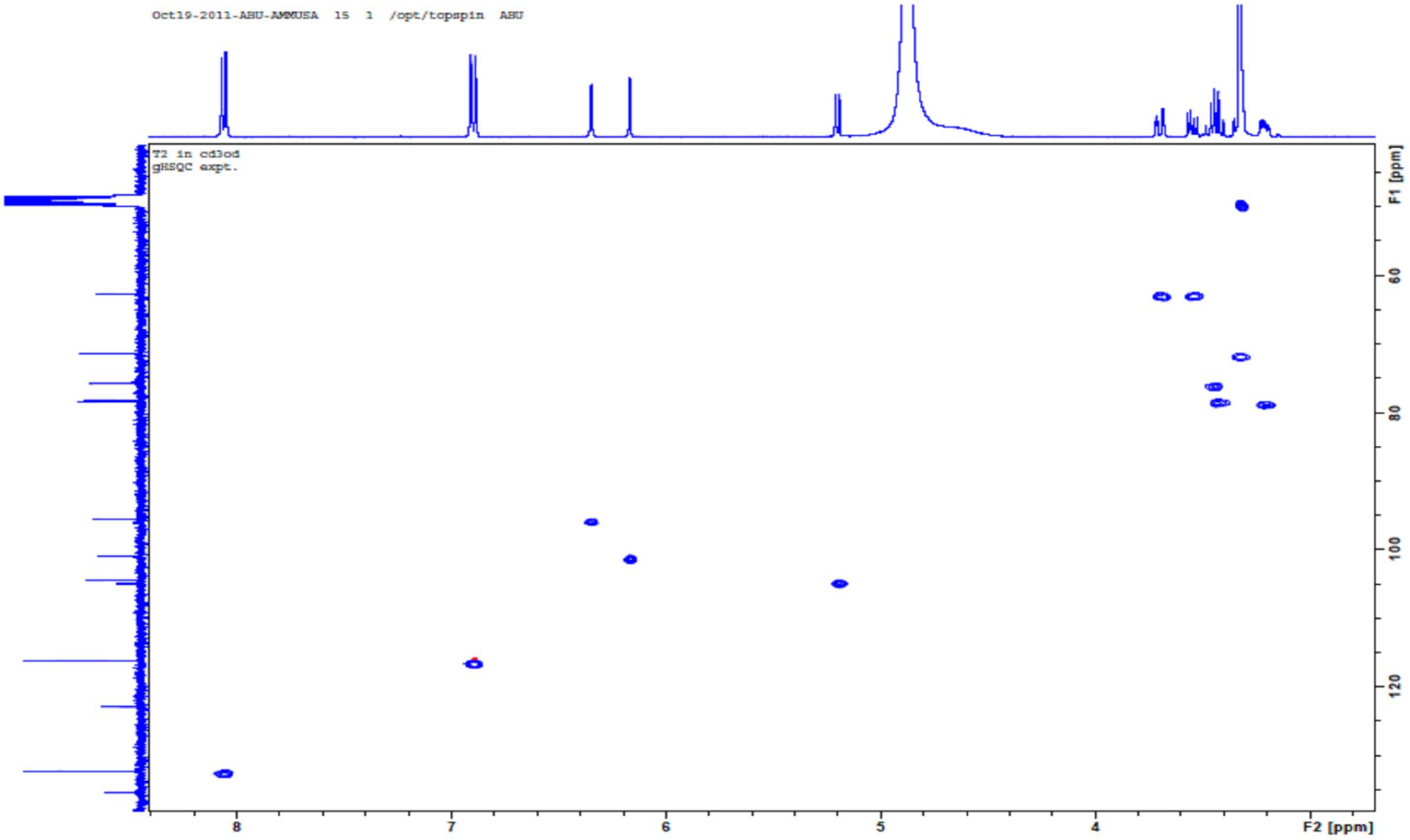


Figure 4.6: HSQC spectrum of T2

4.3.2.6 ^{1}H-^{1}H COSY spectral analysis of T2

The 2D ^{1}H-^{1}H COSY correlation spectrum of T2 indicates the protons that are situated in the same environment and showed the nature of the aromatic substitution. Protons at δ (7.82 and 6.66), (6.11 and 5.93) and the ^{1}H δ 5.0 and δ 3.24 are in the same environment. The 2D ^{1}H-^{1}H COSY results and the J-values from the ^{1}H-NMR indicates presence of one 1, 2, 4, 6-tetra-substituted benzene A ring (meta-coupled) and a di-substituted (ortho-coupled) benzene B ring (Figure 4.7) and (Table 4.6 summary of spectral data).

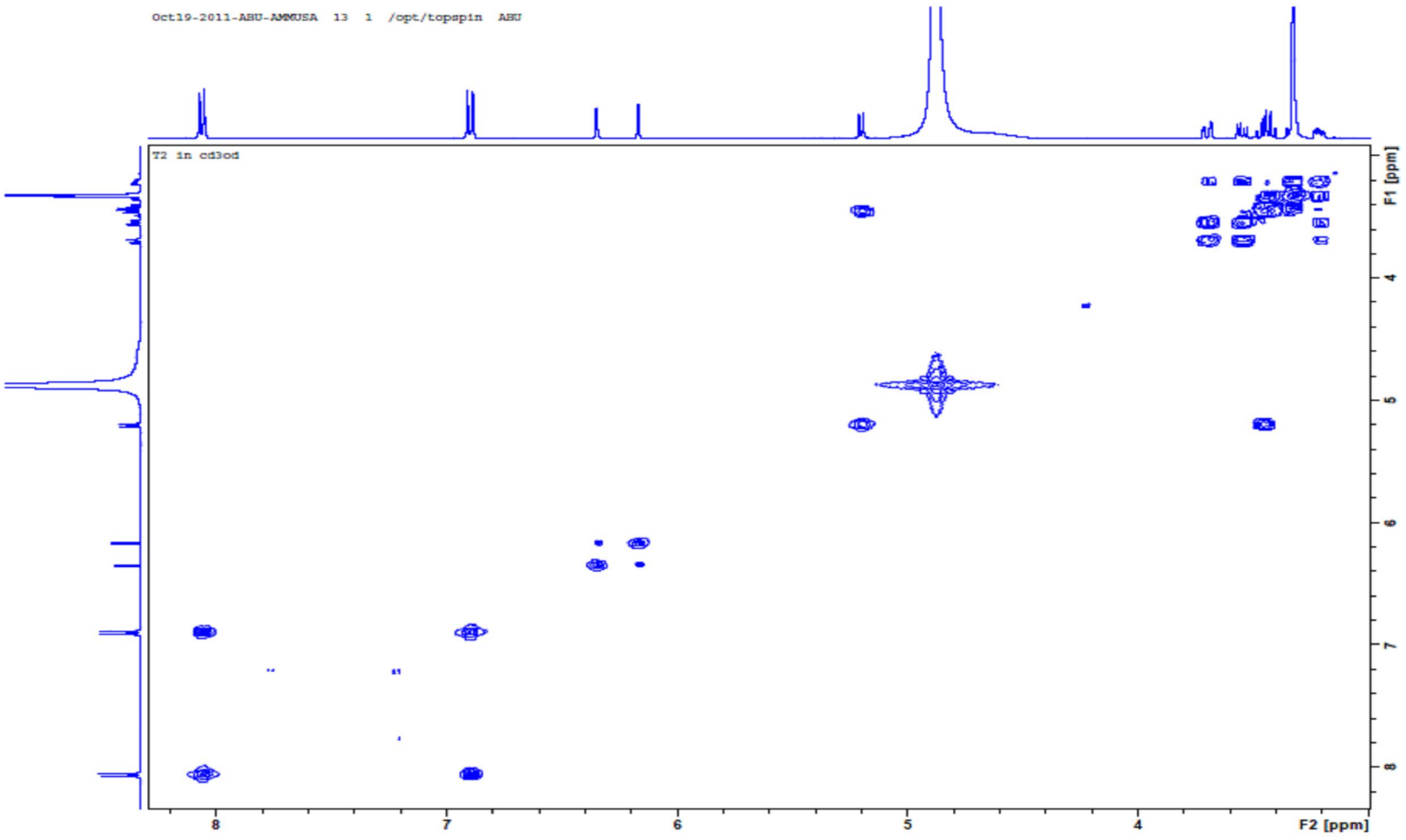


Figure 4.7: COSY spectrum of T2

4.3.2.7 HMBC analysis of T2

The HMBC spectrum allowed establishing the connectivity between the various units of the molecule.

Correlations were established between the 2 protons on ring A: Proton H-8 correlated with C-6, C-7, C-8, C-9 and C-10 while Proton H-6 correlated with C-5, C-6, C-7, C-8 and C-10.

Correlations were established between the 2 ortho-coupled protons on ring B: Proton H-2′, H-6′ correlated with C-2, C-2′, C-4′ and C-6′ while proton H-3′, 5′ correlated with C-1′, C-3′, C-4′ and C-5′

Correlation was established between the sugar unit and the flavonoid moiety; anomeric Proton H-1″ correlated with C-1″, C-3″, C-5″ and C-3 of the flavonoid (Figure 4.8 and Table 4.6 summary of spectral data).

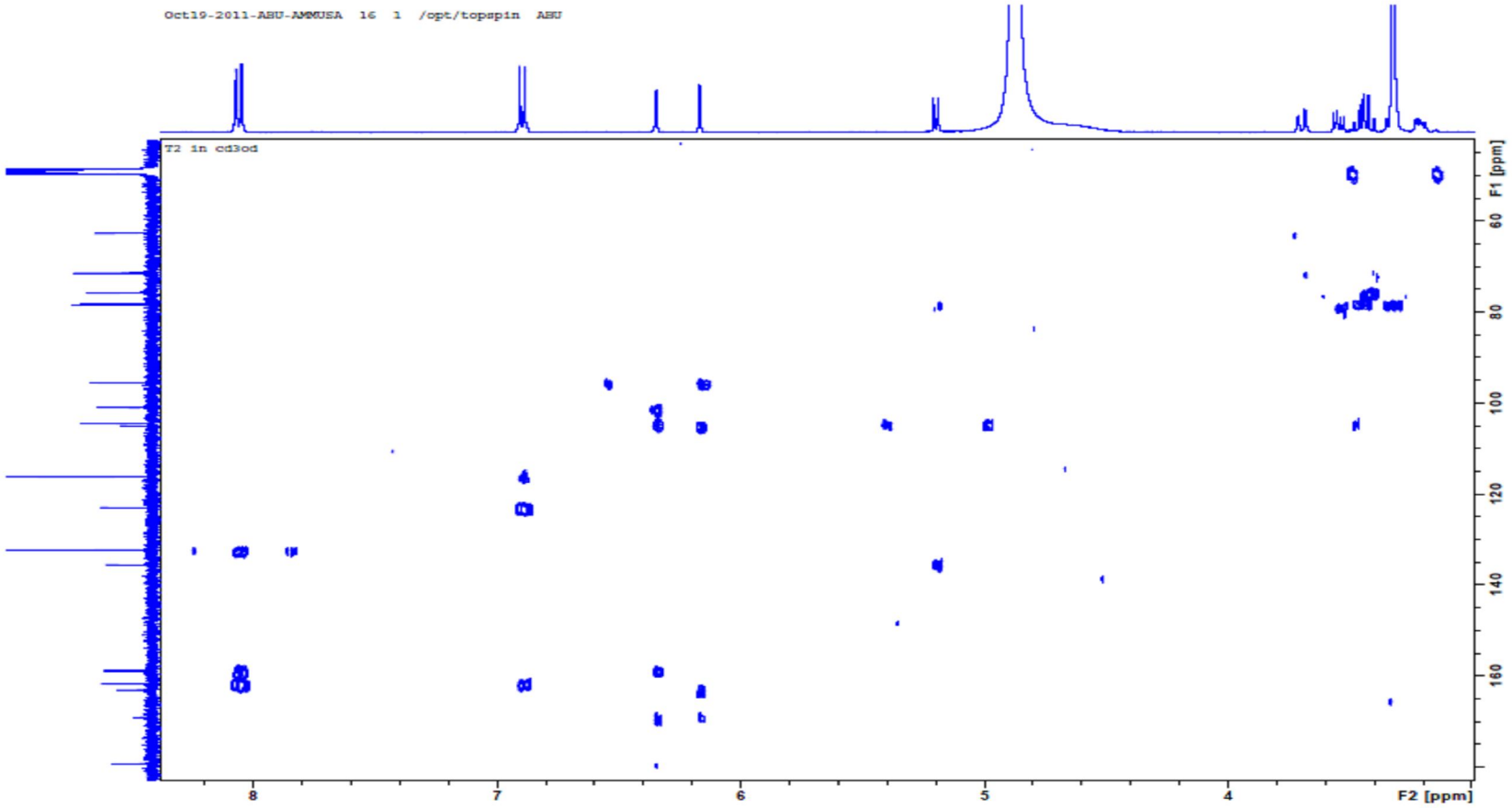


Figure 4.8: HMBC spectrum of T2

Table 4.5: ^{1}H and ^{13}C-NMR spectral summary data of Compound **T2** (CD_3OD), ^{1}H (400MHz) and ^{13}C (100 MHz)

Position	δ ^{13}C	δ ^{1}H (J in Hz)
2	159.0	-
3	135.4	-
4	179.5	-
5	163.0	-
6	99.8	5.93 (d, 1.9)
7	165.7	-
8	95.5	6.11 (d, 1.9)
9	159.0	-
10	105.0	-
1′	122.0	-
2′, 6′	132.0	7.82 (d, 8.8)
3′, 5′	116.0	6.66 (d, 8.8)
4′	162.0	-
Glc-1″	104.8	5.00 (d, 7.2)
2″	76.0	3.24 (s)
3″	79.0	3.20 (t, 6.68, 7.72)
4″	72.0	3.16 (s)
5″	78.0	2.97 (m)
6″	63.0	3.46 (dd, 2.2, 2.2)
		3.31 (dd, 5.4, 5.4)

Table 4.6: 1D and 2D NMR spectral summary data for compound **T2** in CD_3OD

Position	δ ^{1}H (J in Hz)	δ ^{13}C	DEPT	COSY	HMBC
2	-	159.0	C	-	-
3	-	135.4	C	-	-
4	-	179.5	C=O	-	-
5	-	163.0	C	-	-
6	5.93 (d, 1.9)	99.8	CH	H-8	C-5, C-6, C-7, C-8, C-10
7	-	165.7	C	-	-
8	6.11 (d, 1.9)	95.5	CH	H-6	C-6, C-7, C-8, C-9, C-10
9	-	159.0	C	-	-
10	-	105.0	C	-	-
1′	-	122.0	C	-	-
2′, 6′	7.82 (d, 8.8)	132.0	CH	H-3′, H-5′	C-2, C-2′ C-4′, C-6′
3′, 5′	6.66 (d, 8.8)	116.0	CH	H-2′, H-6′	C-1′, C-3′, C-4′, C-5′
4′	-	162.0	C	-	-
1″	5.00 (d, 7.2)	104.8	CH	H-2″	C-1″, C-3, C-3″, C-5″
2″	3.24 (s)	76.0	CH	H-1″, H-3″	-
3″	3.20 (t, 6.68, 7.72)	79.0	CH	H-2″, H-4″	-
4″	3.16 (s)	72.0	CH	H-3″, H-5″	-
5″	2.97(m)	78.0	CH	H-4″, H-6a″, H-6b″	-
6″	3.46 (dd, 2.2, 2.2)	63.0	CH_2	H-5″, H-6b″	C-6″
	3.31 (dd, 5.4, 5.4)			H-5″, H-6a″	C-3″, C-5″

Table 4.7: C-H Correlation spectral summary data (HSQC) for Compound **T2**

Position	δ ^{13}C	δ ^{1}H (J in Hz)
2	-	-
3	-	-
4	-	-
5	-	-
6	99.8	5.93 (d, 1.9)
7	-	-
8	95.5	6.11 (d, 1.9)
9	-	-
10	-	-
1′	-	-
2′, 6′	132.0	7.82 (d, 8.8)
3′, 5′	116.0	6.66 (d, 8.8)
4′	-	-
Glc 1″	104.8	5.00 (d, 7.2)
2″	76.0	3.24 (s)
3″	79.0	3.20 (t, 6.68, 7.72)
4″	72.0	3.16 (s)
5″	78.0	3.97 (m)
6″	63.0	3.46 (dd, 2.2, 2.2)
		3.31 (dd, 5.4, 5.4)

Table 4.8: A Comparison of the ^{1}H and ^{13}C-NMR spectral data for Compound T2* and Reference compound (Kaempferol-3-*O*-*β*-D-glucoside)*

Carbon	Chemical shifts in ppm (δ, J (Hz)			
	Compound T2		Kaempferol-3-O-β-D-glucoside	
Position	**δ ^{13}C**	**δ ^{1}H**	**δ ^{13}C**	**δ ^{1}H**
2	159.0	-	158.2	-
3	135.4	-	135.3	-
4	179.5	-	179.2	-
5	163.0	-	162.7	-
6	99.8	5.93 (d, 1.9)	99.8	6.16 (d, 1.4)
7	165.7	-	165.7	-
8	95.5	6.11 (d, 1.9)	94.7	6.35 (d, 1.4)
9	159.0	-	158.8	-
10	105.0	-	105.6	-
1′	122.0	-	122.6	-
2′, 6′	132.0	7.82 (d, 8.8)	132.2	8.03 (d, 8.8)
3′, 5′	116.0	6.66 (d, 8.8)	115.9	6.86 (d, 8.8)
4′	162.0	-	161.3	-
1″	104.8	5.00 (d, 7.2)	104.0	5.22 (d, 7.6)
2″	76.0	3.24 (s)	75.6	3.21~3.48 (m, H-2″,
3″	79.0	3.20 (t, 6.68, 7.72)	78.2	H-3″, H-4″, H-5″)
4″	72.0	3.16 (s)	71.2	
5″	78.0	2.97 (m)	77.9	
6″	63.0	3.46 (dd, 2.2, 2.2)	62.5	3.69 (dd, 12.0, 2.2)
		3.31 (dd, 5.4, 5.4)		3.53 (dd, 12.0, 5.4)

*400 MHz ^{1}H, *100 MHz ^{13}C, CD_3OD {(T2) and Reference Compound (Han *et al.*, 2004)}.

4.4 Thin-Layer Chromatographic Analysis of Q3

The third band from Prep-TLC with R_f value 0.35 was scrapped off from the plate and washed with methanol to obtained light yellow solid (7.2mg) and was coded as Q3. It gave a single homogenous spot with two solvent systems (Chromatogram 4.2 and Table 4.9).

4.5 Solubility Profile of Q3

Q3 was found to be soluble in methanol, insoluble in $CHCl_3$, acetone and ethyl acetate

4.6 Chemical Tests for Q3

It produced a positive test with Shinoda reagent and ferric chloride solution.

Table 4.9: showing TLC profile of Q3

Solvent system	spraying agent	colour of spot after heating	No. of spot	R_f value
EtOAc-$CHCl_3$-MeOH-H_2O **(**15**:** 8: 4: 1**)**	Gibb's	darkt-brown	1	0.38
EtOAc-MeOH-H_2O (100: 16.5:13.5)	10% H_2SO_4	dark-yellow	1	0.71

4.7 Spectral Analysis of Q3

4.7.1 ^{1}H-NMR spectral analysis of Q3

The ^{1}H-NMR of Q3 revealed signals at δ 6.22 (1H, d, J=2.0Hz, H-6), 6.50 (1H, d, J=2.0Hz, H-8), 8.07 (2H, dd, 2.0, 8.9Hz, H-2′, 6′), 6.91 (2H, dd, J=2.0, 8.9Hz, H-3′, 5′), 5.30 (1H, d, J=7.4Hz, H-1″), 3.44 (1H, t, 5.9, 7.0Hz, H-2″), 3.21 (m, H-3″), 3.48 (1H, s, H-4″), 3.40 (H-5″), 3.72 (1H, dd, J=2.2, 2.3Hz, H-6a″), 3.56 (1H, dd, J=5.5, 5.5Hz, H-6b″) for aromatic and sugar regions respectively (Figure 4.9a-c) and (Table 4.10 summary of spectral data).

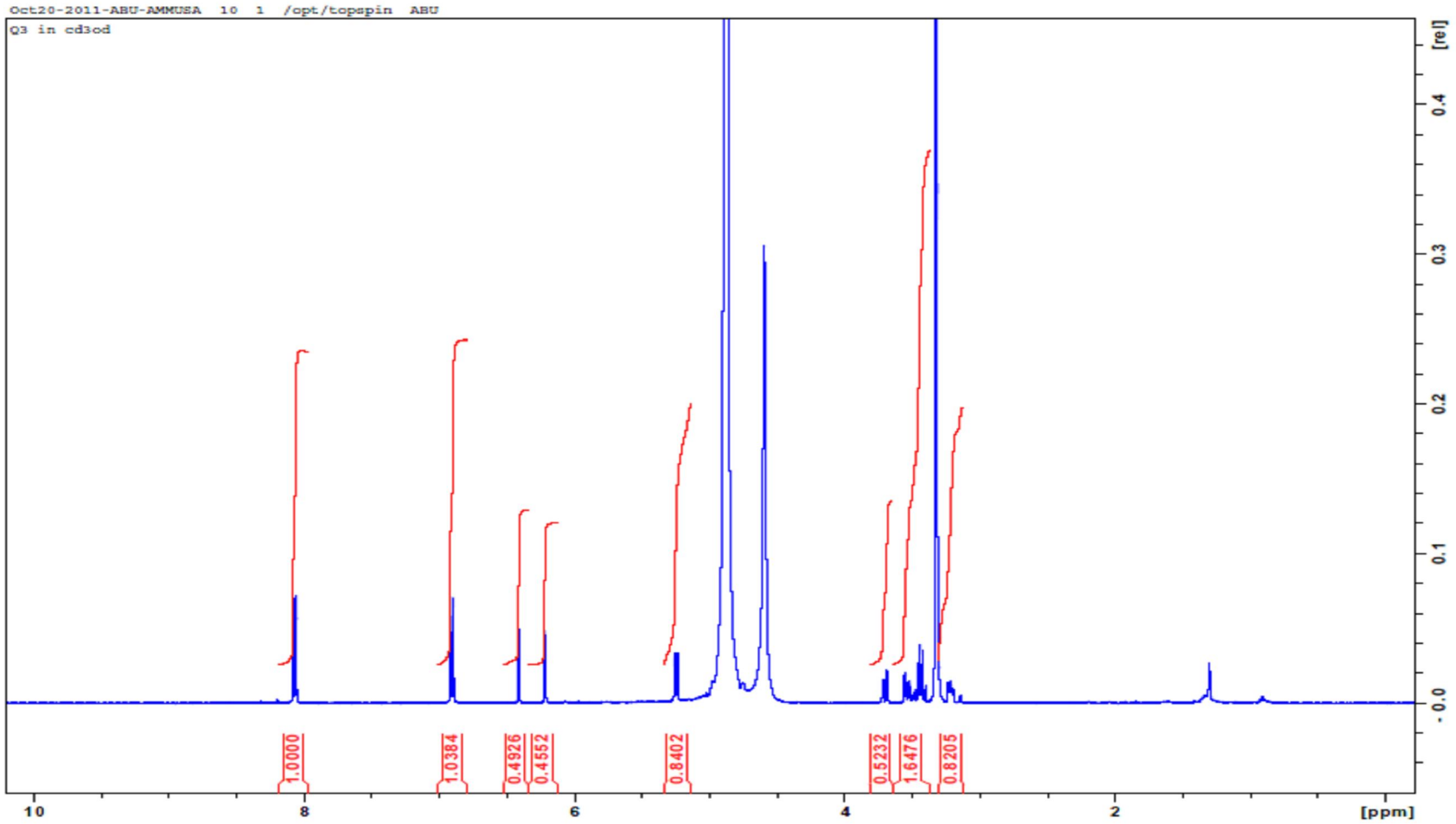


Figure 4.9a: ^{1}H-NMR spectrum of compound Q3

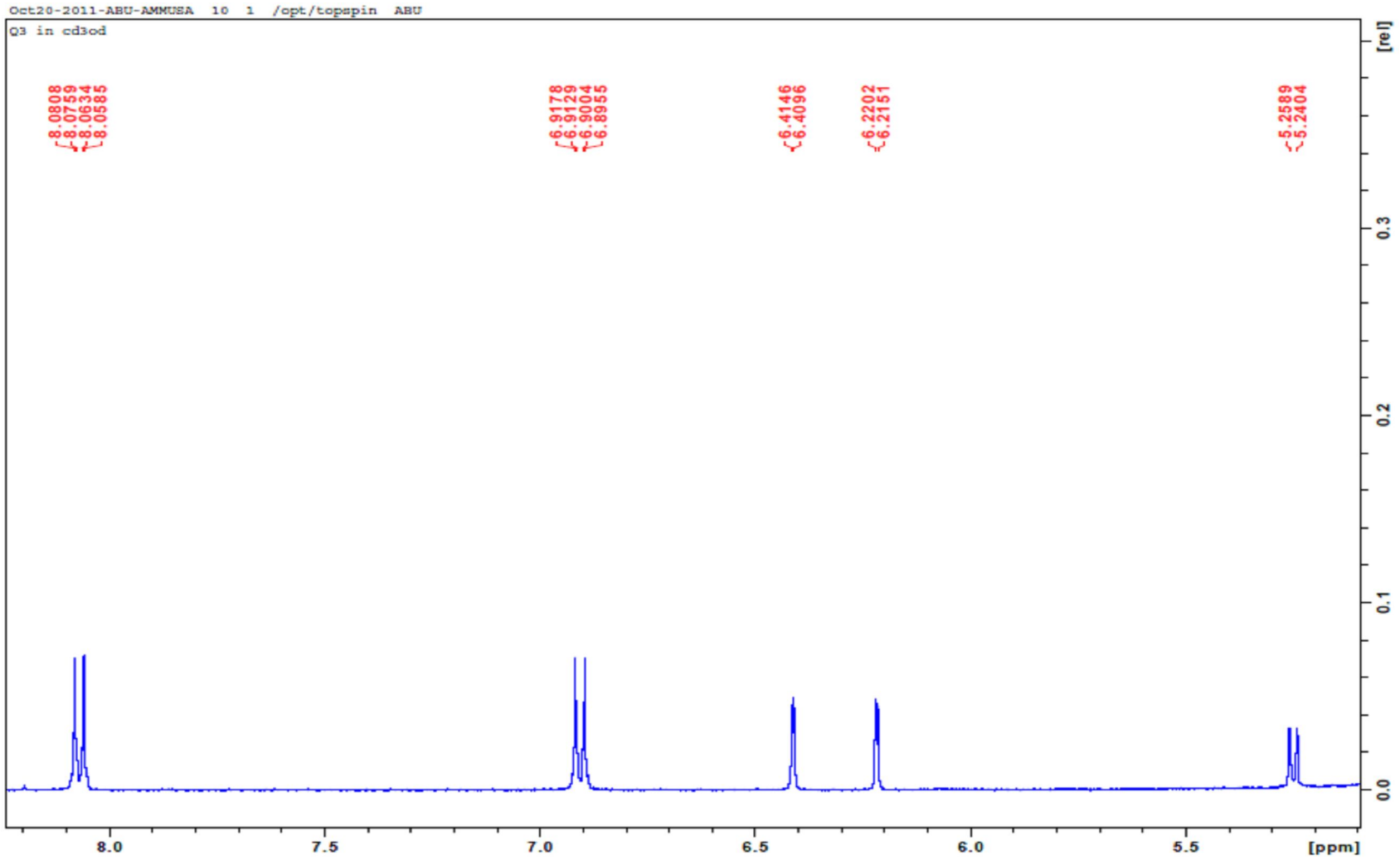


Figure 4.9b: ^{1}H-NMR spectrum of Q3 (expanded aromatic region

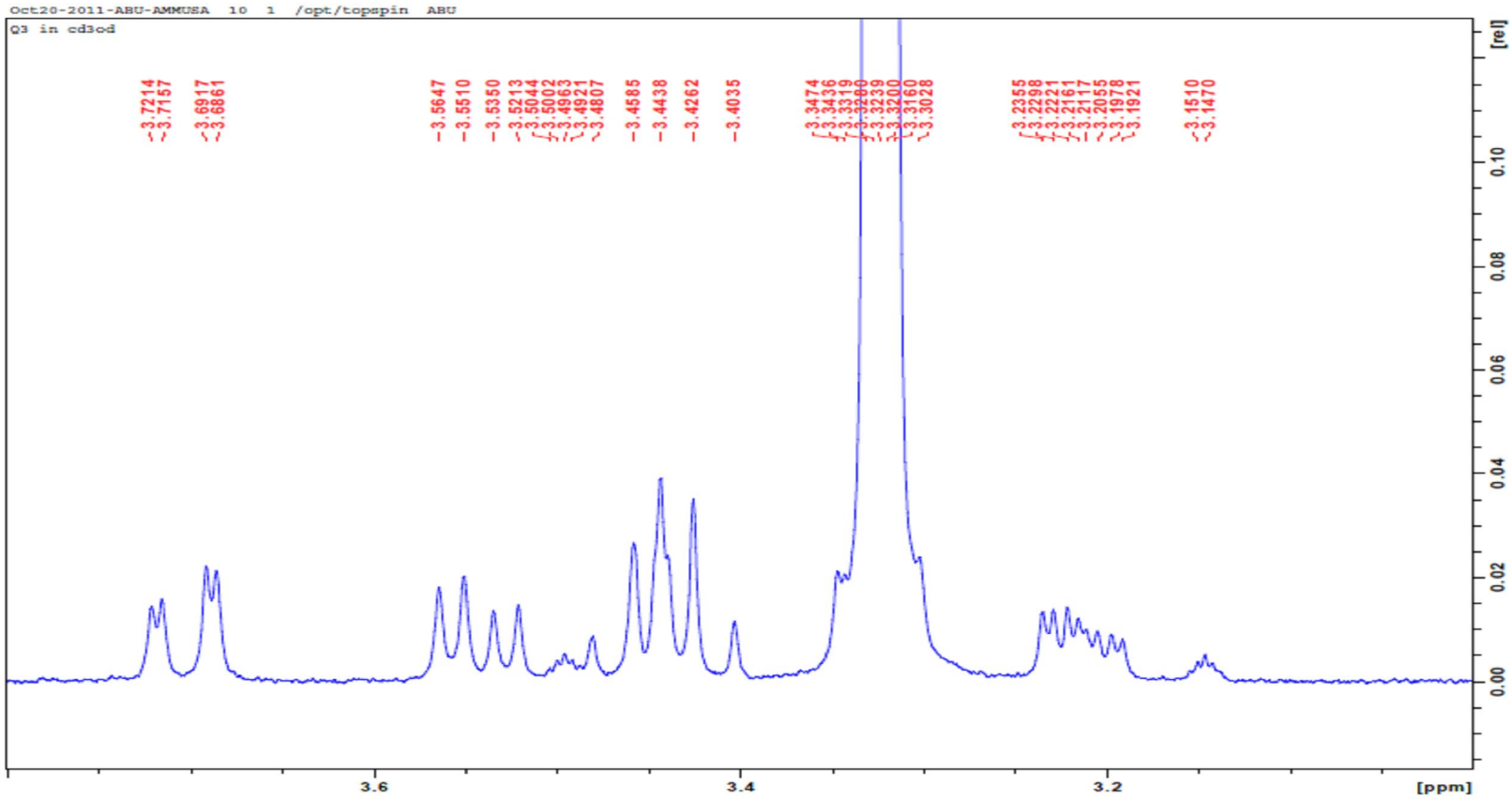


Figure 4.9c: H-NMR spectrum of Q3 (expanded sugar region)

4.7.2 ^{13}C-NMR and ^{13}C- DEPT NMR spectral analysis of Q3

The ^{13}C-NMR and ^{13}C-DEPT spectra, δ (ppm) in CD_3OD: 159.0 (C-2), 136.1 (C-3), 179.5 (C-4), 163.1 (C-5), 102.1 (C-6), 169.0 (C-7), 97.0 (C-8), 159 (C-9), 105.3 (C-10), 122.8 (C-1′), 132.3 (C-2′, 6′), 116.1 (C-3′, 5′), 162 (C-4′), 104.1 (C-1″), 75.7 (C-2″), 78.4 (C-3″), 72 (C-4″), 78.1 (C-5″) and 62.6 (C-6″)

^{13}C-NMR spectra and the DEPT experiments of Q3 indicated presence of 21 carbon atoms including the double signals observed at δ 132.3 and 116.1. The spectra showed 9 quaternary carbon atoms 7 of which are oxygenated including the downfield signal at 179.5 due to carbonyl group; the resonances around 78.4-62.6 is characteristic of sugar nucleus and the one methylene carbon absorption shown in the Dept experiment suggests a glucose moiety (Figures 4.10-4.11) and (Tables 4.10 - 4.11 summary of spectral data)

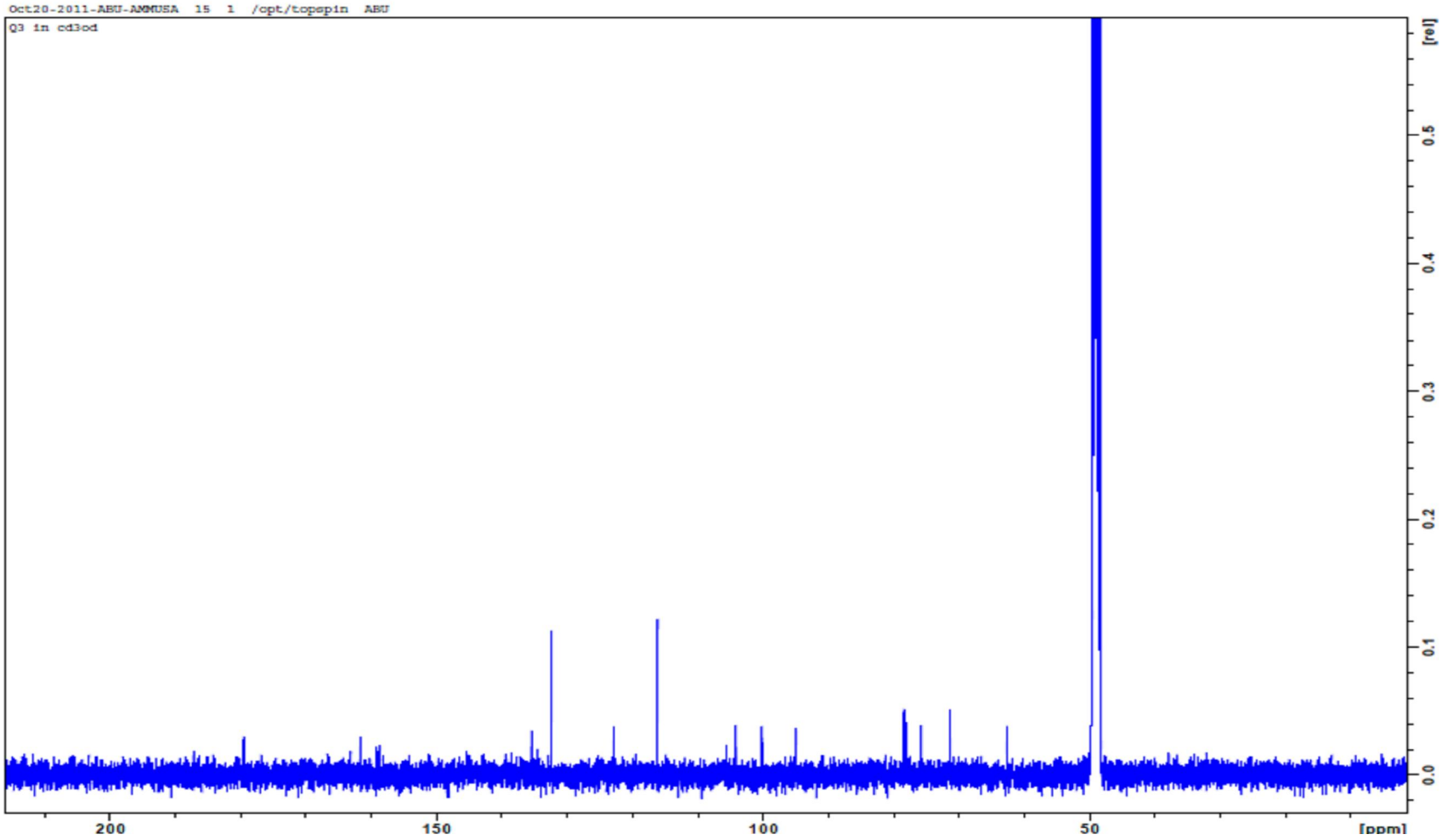


Figure 4.10: ^{13}C-NMR spectrum of Q3

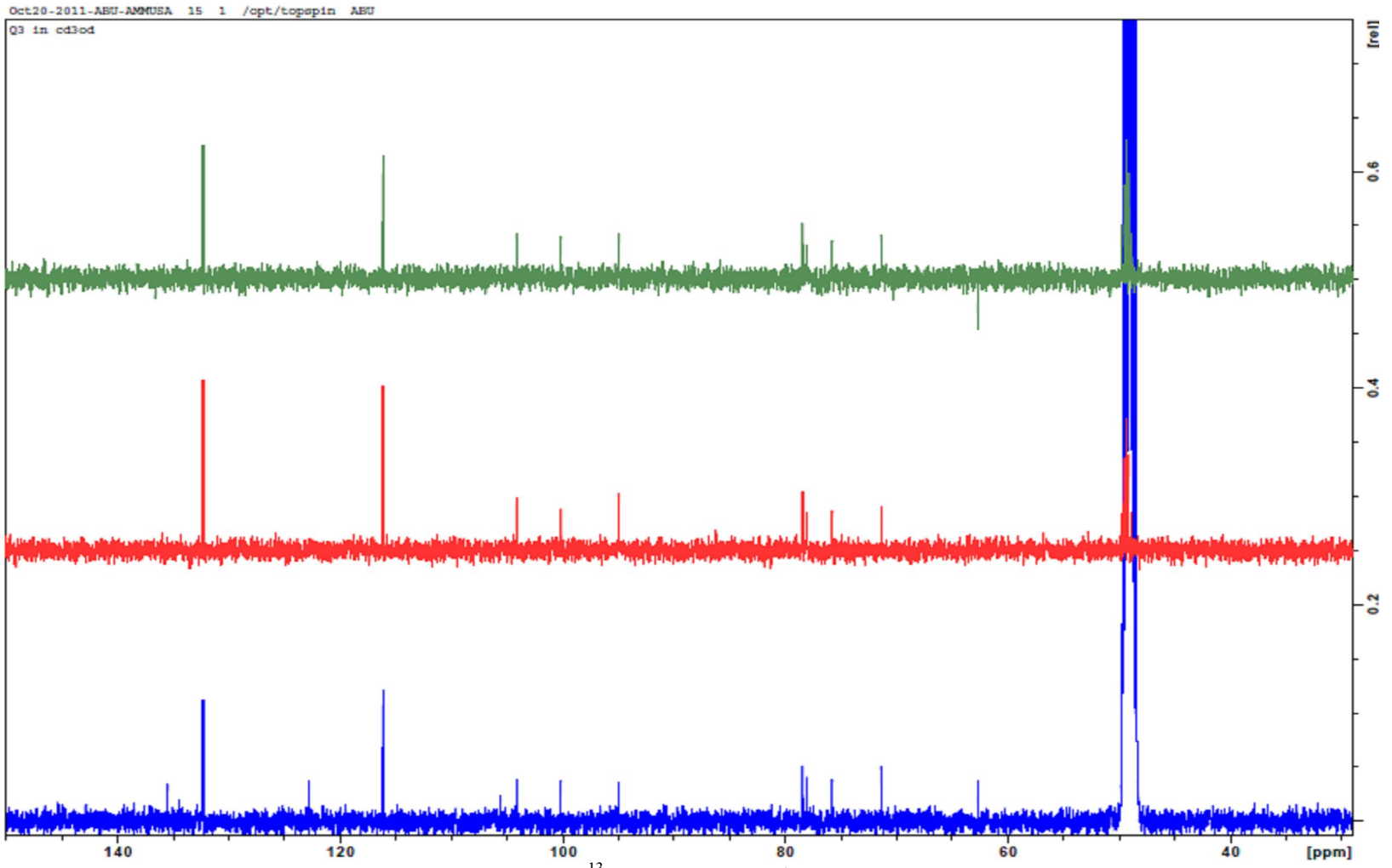


Figure 4.11: ^{13}C-DEPT NMR of Q3

4.7.3 HSQC spectral analysis of Q3

The HSQC spectrum of Q3 shown in Figure 4.12 was used in assigning protons to their respective carbons (Table 4.11 summary of spectral data).

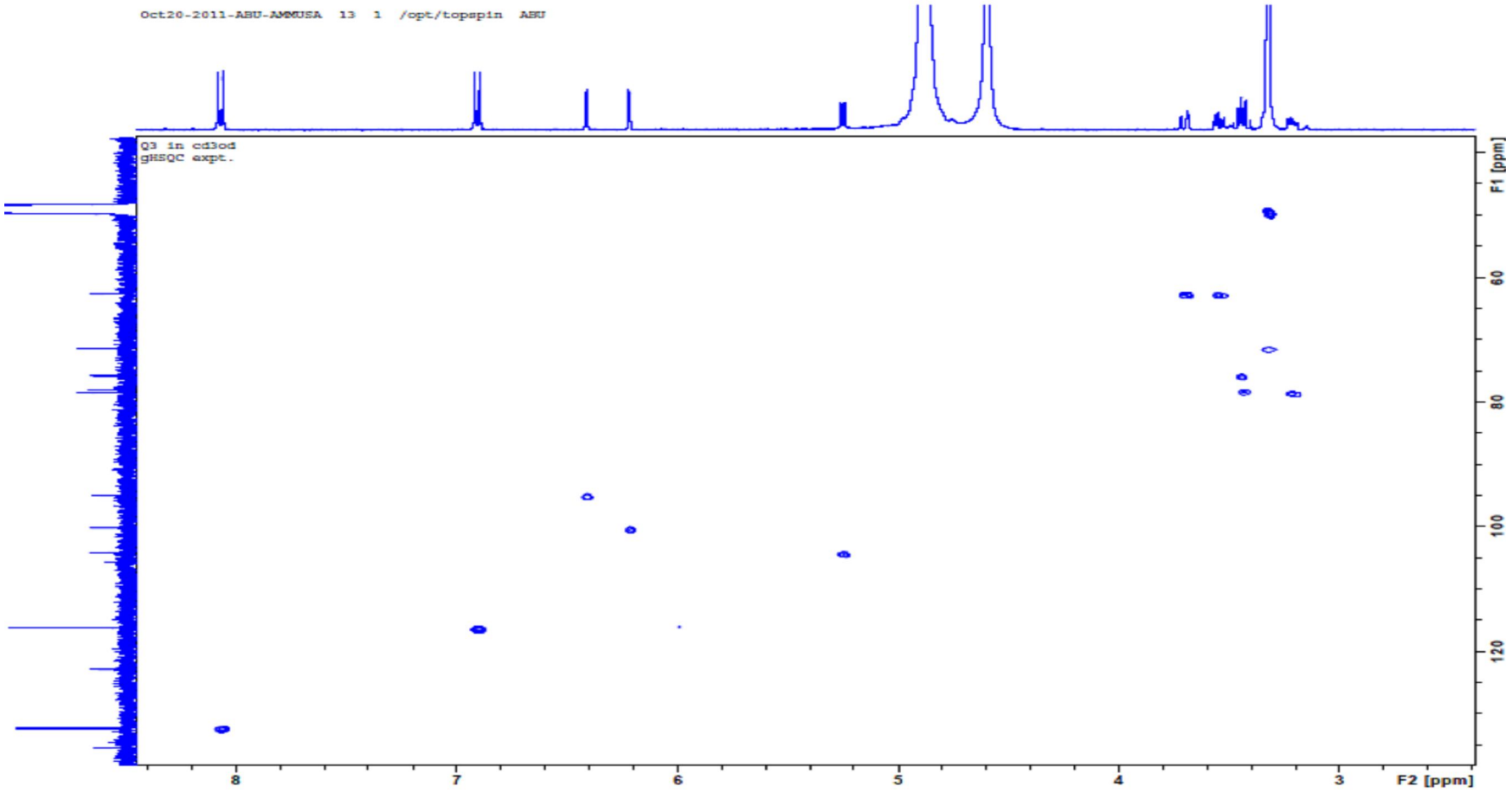


Figure 4.12: HSQC spectrum of Q

4.7.4 HMBC spectral analysis of Q3

The HMBC spectrum allowed establishing the connectivity between the various units of the molecule.

Correlations were established between the 2 protons on ring A: proton H-8 correlated with C-6, 7, 9 and 10 while proton H-6 correlated with C-8 and 10

Correlations were established between the 2 ortho-coupled protons on ring B: proton H-2′, H-6′ correlated with C-2′, C-4′, 6′ and 9 while proton H-3′, 5′ correlated with C-1′, C-3′, C-4′ and C-5′ (Figure 4.13) and (Table 4.10 summary of spectral data)

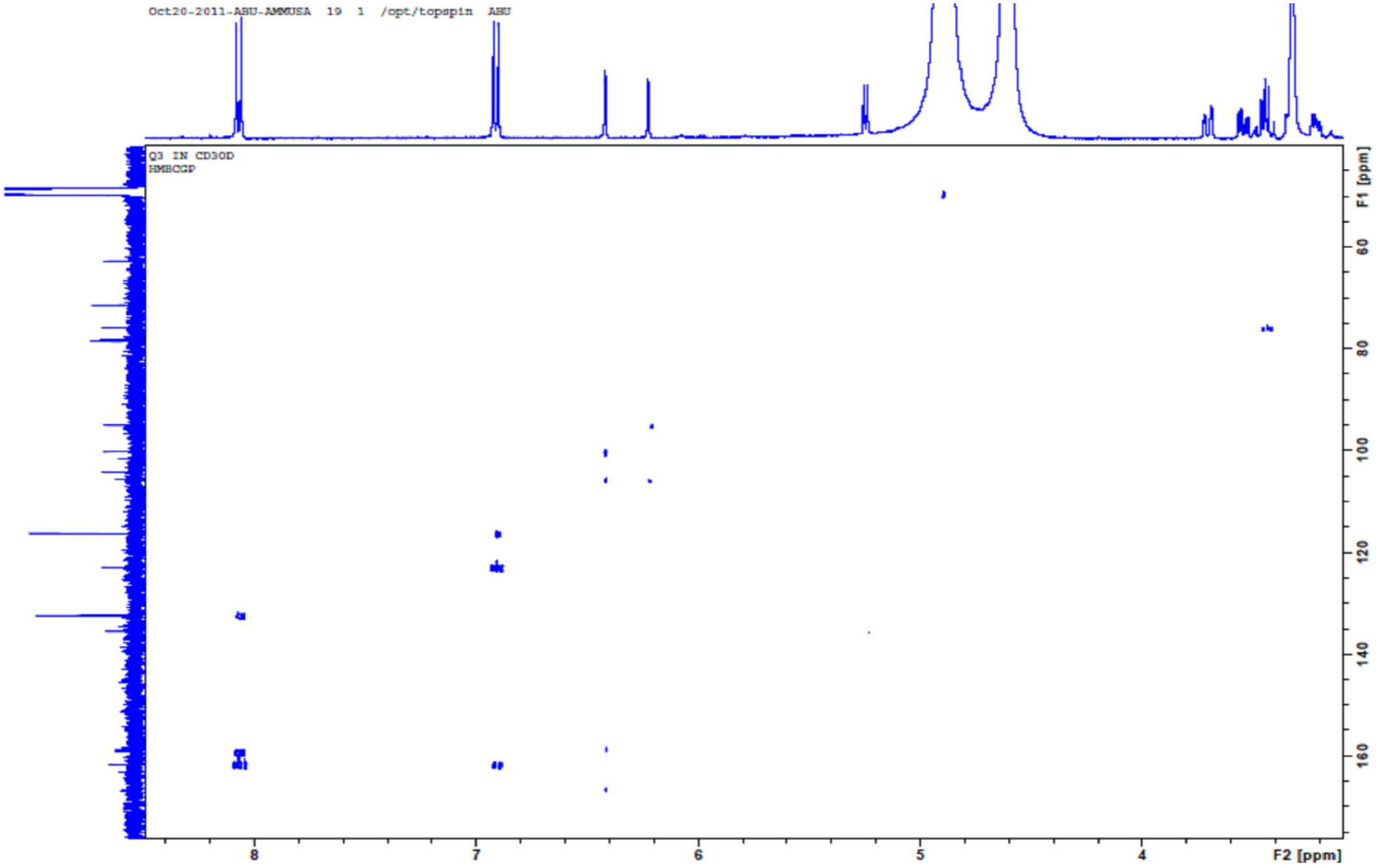


Figure 4.13: HMBC spectrum of Q3

Table 4.10: ^{1}H and ^{13}C-NMR spectral summary data of Compound **Q3** (CD_3OD), ^{1}H (400MHz) and ^{13}C (100 MHz)

Position	δ ^{13}C	δ ^{1}H (J in Hz)
2	159.0	-
3	136.1	-
4	179.5	-
5	163.1	-
6	102.1	6.22 (d, 2.0)
7	169.0	-
8	97.0	6.50 (d, 2.0)
9	159.0	-
10	105.3	-
1′	122.8	-
2′, 6′	132.3	8.07 (dd, 2.0, 8.9)
3′, 5′	116.1	6.91 (dd, 2.0, 8.9)
4′	162.0	-
glc1″	104.1	5.30 (d, 7.4)
2″	75.7	3.44(t, 5.9, 7.0)
3″	78.4	3.21 (m)
4″	72.0	3.48 (s)
5″	78.1	3.40 (s)
6″	62.6	3.72 (dd, 2.2, 2.3)
		3.56 (dd, 5.5, 5.5)

Table 4.11: 1D and 2D NMR spectral summary data for compound **Q3** in CD_3OD

Position	δ ^{1}H (J in Hz)	δ ^{13}C	DEPT	COSY	HMBC
2	-	159.0	C	-	
3	-	136.1	C	-	-
4	-	179.5	C=O	-	-
5	-	163.1	C	-	-
6	6.22 (d, 2.0)	102.1	CH	H-8	C-8, C-10
7	-	169.0	C	-	-
8	6.50 (d, 2.0)	97.0	CH	H-6	C-6, C-7, C-9, C-10
9	-	159.0	C	-	-
10	-	105.3	C	-	-
1′	-	122.8	C	-	-
2′, 6′	8.07 (dd, 2.0 8.9)	132.3	CH	H-3′, H-5′	C-2, C-9, C-2′ C-4′,C-6′
3′, 5′	6.91 (dd, 2.0 8.9)	116.1	CH	H-2′, H-6′	C-1′, C-3′, C-4′, C-5′
4′	-	162.0	C	-	-
1″	5.30 (d, 7.4)	104.1	CH	H-2″,H-5″	-
2″	3.44 (t, 5.9, 7.0)	75.7	CH	H-6a″, H-6b″	C-2″, C-5″
3”	3.21 (m)	78.4	CH	H-2″, H-5″, H-6b″	-
4″	3.48 (s)	72.0	CH	H-6a″	-
5″	3.40(m)	78.1	CH	H-6a″, H-6b″	-
6″	3.72 (dd, 2.2, 2.3)	62.6	CH_2	H-6b″	C-6″
	3.56 (dd, 5.5, 5.5)			H-6a″	C-3″, C-5″

Table 4.12: C-H Correlation spectral summary data (**HSQC**) for Compound **Q3**

Position	δ_C	δ_H **(J in Hz)**
2	-	-
3	-	-
4	-	-
5	-	-
6	102.1	6.22 (d, 2.0)
7	-	-
8	97.0	6.50 (d, 2.0)
9	-	-
10	-	-
1′	-	-
2′, 6′	132.3	8.07 (dd, 2.0, 8.9)
3′, 5′	116.1	6.91 (dd, 2.0, 8.9)
4′	-	-
glc 1″	104.1	5.30 (d, 7.4)
2″	75.7	3.44 (t, 5.9, 7.0)
3″	78.4	3.21 (m)
4″	72.0	3.48 (s)
5″	78.0	3.40 (s)
6″	62.6	3.72 (dd, 2.2, 2.3)
		3.56 (dd, 5.5, 5.5)

4.8 Pharmacological Studies

4.8.1 Result of acute toxicity studies

The intraperitoneal LD_{50} value of n-butanol fraction in rats was found to be 1264.91mg/kg body weight. The rats experienced respiratory distress, weakness, immobility and death.

4.8.2 Anti-inflammatory study

4.8.3 Carrageenan-induced paw oedema

Effects of n-butanol fraction and Ketoprofen on carrageenan-induced paw oedema were studied and the results were recorded in (Tables 4.13 - 4.14).

Table 4.13: Effect of n-butanol soluble fraction of the methanol extract of *Indigofera hirsuta* and Ketoprofen on Carrageenan-induced Paw Oedema in Rats.

		Mean Paw diameter (cm) ± SEM, t(hr)			
Treatment	Dose(mg/kg)	1h	2h	3h	4h
Normal saline	1ml/kg	1.58 ± 0.42	2.30 ± 0.26	2.91 ± 0.24	2.10 ± 0.25
Extract	75	0.93 ± 0.18	1.84 ± 0.25	2.47 ± 0.33	1.71 ± 0.23
Extract	150	0.26 ± 0.06**	0.75 ± 0.18***	1.07 ± 0.09**	0.54 ± 0.12***
Extract	300	0.42 ± 0.11**	0.77 ± 0.08***	1.00 ± 0.12***	0.52 ± 0.09***
Ketoprofen	10	1.08 ± 0.13	1.37 ± 0.22	2.03 ± 0.16	1.46 ± 0.18

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to control, Dunnet's t-test, ANOVA (n=6)

In the normal saline treated animals, sub plantar injection of 1% Carrageenan suspension produced a local oedema reaching its maximum at 3h. The n-butanol fraction of *I. hirsuta* at doses of 150 and 300 mg/kg significantly ($p < 0.05$) inhibited the paw oedema over a period of 4 hrs (Tables 4.10-4.11). The effects observed were dose independent. The percentage anti-inflammatory effect of the highest dose tested (300 mg/kg) at the peak hour was higher than that of ketoprofen (10 mg/kg), the standard anti-inflammatory agent.

Table 4.14: Percentage inhibition expressed by n-butanol fraction of the methanolic extract of *Indigofera hirsuta* on carrageenan induced paw oedema in Rat.

		% Inhibition of oedema (time/h)			
Treatment	[(Dose(mg/kg)]	1h	2h	3h	4h
Extract	75	41.1	20	15.1	18.6
Extract	150	83.5	67.4	63.2	74.3
Extract	300	73.4	66.5	65.6	75.2
Ketoprofen	10	31.6	40.4	30.2	30.5

(n=6 for each treatment, experimental groups compared with control group.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical Studies

Preliminary phytochemical screening of n-butanol soluble fraction of the methanolic extract of the aerial parts of *Indigofera hirsuta* revealed the presence of flavonoids, phenolic compounds, saponins and tannins. The anti-inflammatory effect of flavonoids, tannins and saponins has been reported (Reanmongkol *et al*., 2005).

Repeated gel filtration of n-butanol soluble fraction over a Sephadex LH-20 column eluted with methanol followed by preparative thin-layer chromatography resulted in the isolation of yellow solid compound coded T2 (9.7mg). This compound revealed single homogenous spot using two different TLC solvent systems (Chromatogram 4.2 and table 4.3).

The positive colour reaction with ferric chloride suggested the presence of phenolic hydroxyl compound. The pink colour observed when subjected to shinoda test and a yellow colour when treated with sodium hydroxide suggested the likely presence of flavones, flavononol, flavonol or isoflavone (Finar, 2007).

The UV spectrum of T2 (Fig.4.1) showed characteristic flavonol absorption bands at 267 nm and 346 nm typical of a kaempferol derivative (Mabry *et al.,* 1970; Andersen and Markham, 2006); the 267 nm absorption is due to benzoyl system (band II) while 346 nm is due to cinnamoyl system (band I) representing A and B ring, respectively.

The IR spectrum of T2 exhibited OH stretching frequency at (3350 cm^{-1}), conjugated carbonyl group (1637 cm^{-1}) and conjugated C=C aromatic absorptions at (1592 and 1400 cm^{-1}); other prominent peaks were 2781 cm^{-1} assignable to an sp^3 C-H and 1122 cm^{-1} due to C-O. This also suggests a flavonol nucleus (Han *et al.*, 2004; Lee *et al*., 2007).

The ^{1}H-NMR of T2 (Figure 4.3a-c and Table 4.5) displayed signals for two meta-coupled protons at δ 5.93 (1H, d, J=1.9 Hz) and δ 6.11 (1H, d, J=1.9 Hz) corresponding to H-6 and H-8 respectively; this tetra-substituted benzene ring A has a 5, 7-dihydroxylation of an AX system (Yekta *et al.,* 2008). The ^{1}H-NMR also revealed the presence of 1′,4′-di-substituted ring comprising of orthor-coupled AB system signals at δ 7.82 (2H, d, J=8.8 Hz) and δ 6.66 (2H, d, J=8.8 Hz) corresponding to H-2′/ H-6′ and H-3′/ H-5′ on ring B, characteristic of kaempferol moiety (Han *et al.,* 2004). The ^{1}H-NMR further revealed the presence of one sugar unit –with the anomeric proton at δ 5.0 (1H, d, J=7.2 Hz, H-1″); the other sugar proton resonances observed around δ 3.46-2.97 (Fig. 4.3c and Table 4.5) and the large coupling constant on the anomeric proton is an indication of a β-glucosyl moiety (Mabry *et al.,* 1970). The above observations were further corroborated through the 2D experiments (^{1}H-^{1}H COSY (Figures 4.7 and Table 4.6) and HSQC (Figure 4.6 and Table 4.7).

Further elucidation of the T2 structure was accomplished with the aid of ^{13}C-NMR spectrum and the DEPT experiments. The ^{13}C-NMR spectra indicated the presence of 21 carbon atoms including the double signals observed at *δ* 132.0 and 116.0. The DEPT experiments showed 9 quaternary carbon atoms 7 of which were oxygenated, including the downfield signal at 179.5 due to a carbonyl group; the HSQC further facilitated the assignment of the ^{13}C signals at δ 99.8(C-6), δ 95.5(C-8), δ 132.0 (C-2′/6′) and δ 116.0 (C-3′/5′) with their corresponding ^{1}H signals at δ 5.93(H-6), δ 6.11(H-8), δ 7.82(H-2′/6′) and δ 6.66 (H-3′/5′). This further supports the presence of a kaempferol aglycone (Han *et al*., 2004; Lee *et al.,* 2007). The resonances around δ 79-63 is characteristic of sugar nucleus; the anomeric carbon δ 104.8 (C-1″) and other signals due to 5 carbons were observed at 79.0 (C-3″), 78.0 (C-5″), 76.0 (C-2″), 72.0 (C-4″) and 63.0 (C-6″) - the DEPT spectrum indicated a secondary (methylene C-6″) suggesting the presence of a glucopyranosyl moiety (Roberta *et al*., 2003; Andersen and Markham, 2006). These assignments were further confirmed by the HSQC data (Figure 4.6 and Table 4.7).

The HMBC spectrum revealed the correlations between protons and the neighboring carbons, up to 3 bonds. The connectivity between the various atoms and units in the molecule was facilitated (Figure 4.8 and Table 4.6). Correlations were established between the 2 protons on ring A through Proton H-8 with C-6, C-7, C-8, C-9 and C-10, and Proton H-6 with C-5, C-6, C-7, C-8 and C-10. Correlations were also established between the 2 ortho-coupled protons on ring B: Proton H-2′, 6′ correlated with C-2, C-2′, C-4′ and C-6′ and Proton H-3′, 5′ correlated with C-1′, C-3′, C-4′ and C-5′. The flavonoid molecule was thus confirmed to be kaempferol. The connectivity between the sugar unit and the kaempferol moiety was established through the correlation observed between the anomeric Proton H-1″ and C-3 of the kaempferol aglycon. Further correlations were observed between the anomeric proton and sugar carbons at C-1″, C-3″ and C-5″ (Figure 4.8). This firmly confirmed the structure of T2 as Kaempferol-3-O-β-D-glucopyranoside.

Further comparison with reference data (Han *et al.*, 2004) was also undertaken (Table 4.8).

Based on the physical and chemical tests, spectral analysis (UV, FIRT, 1D and 2D NMR) and by comparison with ^{1}H-NMR and ^{13}C-NMR reference data, compound T2 was unambiguously elucidated as Kaempferol-3-O-β-D-glucopyranoside (Figure 4.14).

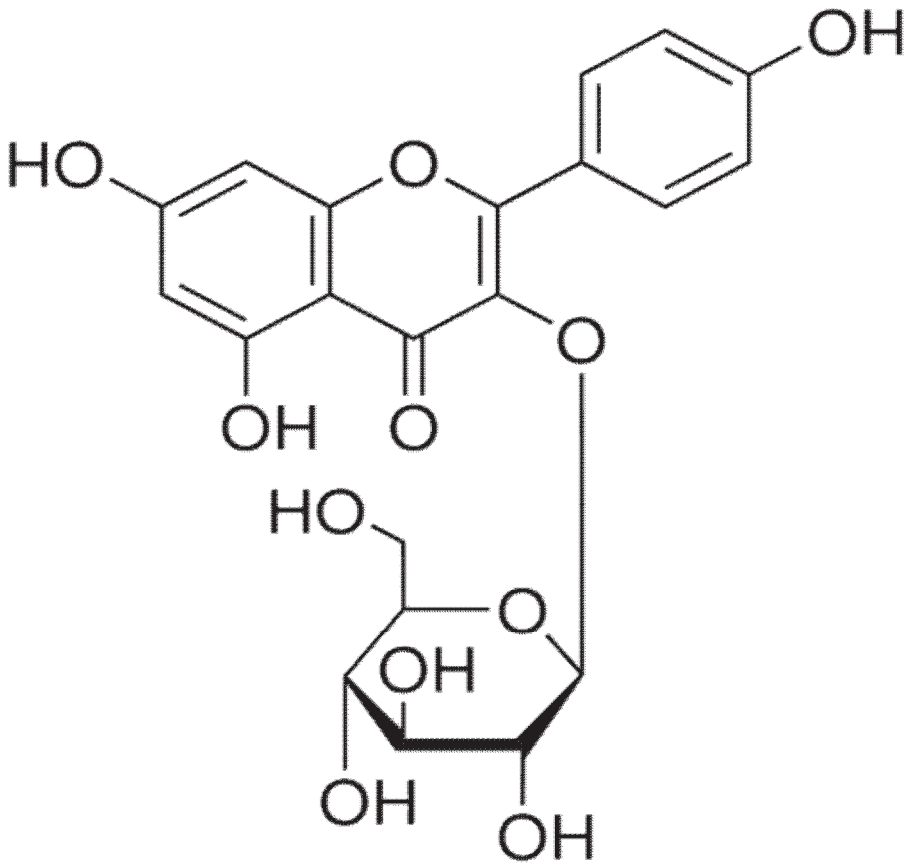


Figure 4.14: structure of T2 (Kaempferol-3-O-β-D-glucopyranoside)

Q3 Repeated gel filtration of n-butanol soluble fraction on a sephadex LH-20 column using methanol as the eluting solvent followed by preparative thin-layer chromatography led to the isolation of compound coded Q3. It was obtained as a pale yellow solid weighed (7.2 mg) and revealed single homogenous spot using two (2) different TLC solvent systems (Chromatogram 4.2 and Table 4.9).

This reacted positively for flavonoids using ferric chloride suggesting the presence of phenolic hydroxyl compound. The pink colour observed when subjected to shinoda test and a yellow colour when treated with sodium hydroxide suggested the likely presence of flavones, flavononol, flavonol or isoflavone (Finar, 2007).

The ^{1}H and ^{13}C-NMR spectral data indicated that Q3 is fundamentally similar structure to that of T2 except for the position of attachment of sugar moiety to the aglycone. The down field resonance of C-7 in ring A suggested that the glucose unit in Q3 is attached to C-7. Moreover, the chemical shifts values at δ 5.30 for anomeric (H-1″, J=7.4 Hz), δ 6.22 for H–6 and δ 6.50 for H–8 and their corresponding carbons shifts at δ 104.1 (C1″), 102.1 (C-6) and δ97.0 (C-8) further suggested the attachment of sugar unit at 7 position (Mabry *et al.*, 1970; Pereira *et al.*, 2012). This firmly confirmed the structure of Q3 as Kaempferol-7-O-β-D-glucopyranoside.

Based on the physical and chemical tests, NMR Spectral data and by comparison with ^{1}H-NMR and ^{13}C-NMR published data, compound Q3 was structurally elucidated as Kaempferol-7-O-β-D-glucopyranoside (Figure 4.15).

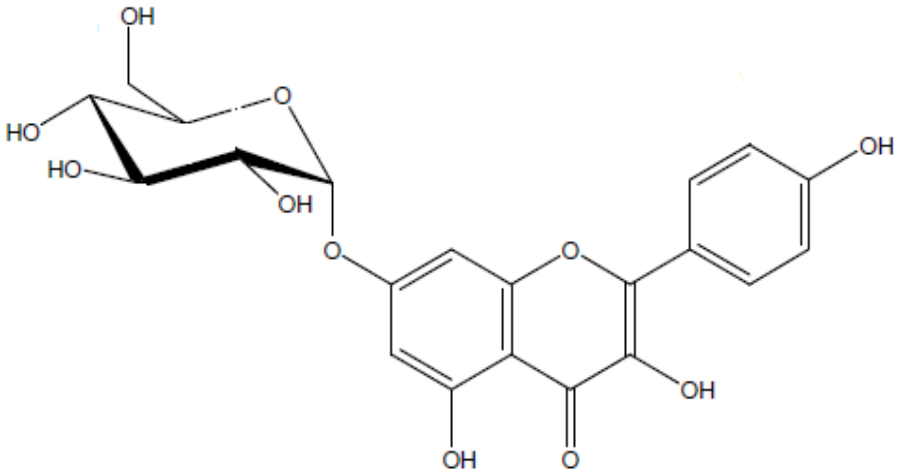


Fig. 4.15: Structure of Q3 (Kaempferol-7-O-β-D-glucopyranoside)

5.2 Pharmacological Studies

The intraperitoneal median lethal dose (LD_{50}) of n-butanol fraction was estimated to be 1264.91mg/kg in rats. This suggests that it is relatively toxic (Lork's 1983), but it is relatively safe at the doses employed in the studies.

The results of the present investigation suggest that n-butanol soluble fraction of the methanolic extract of the aerial parts of *I. hirsuta* possesses significant anti-inflammatory activity. Carrageenan-induced inflammatory process has proven to be bi-phasic (Banerjee *et al.*, 2003). The initial phase observed at the first hour is associated with the release of histamine and probably increased synthesis of prostaglandins in the damaged tissue (Crunkhon and Meacock, 1971). The second phase which occurs in the second and third hours is attributable to the release of prostaglandins, bradykinin, leukotrienes and polymorphonuclear cells (Brito and Antonio, 1988).

The ability of the n-butanol soluble fraction to inhibit inflammation in both first and second phases of carrageenan-induced inflammation suggest that its action may include inhibition of release of histamine, synthesis of prostaglandin, or release of the mediators of inflammation. Several flavonoids isolated from medicinal plants have been discovered to possess significant anti-inflammatory effect (Rajnarayana *et al.,* 2001; Singh and Pandey, 1997; Kumar *et al.,* 2011). It has been reported that Kaempferol, Quercetin, Myricetin and Fisetin possess COX and LOX inhibitory activity (Kumar *et al.*, 2011).

The inhibitory effect of the n-butanol fraction on the carrageenan was found to be significant and the anti-inflammatory potential observed might be attributable to its flavonoids, saponins or tannins constituents shown to be positive during phytochemical screening.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Phytochemical screening of n-butanol soluble fraction of the methanol extract of *Indigofera hirsuta* revealed the presence of Flavonoids, phenolic, saponins and tannins.

Extensive phytochemical investigation of n-butanol soluble fraction resulted in the isolation of two flavonol glycosides (Kaempferol-3-O-β-D-glucopyranoside and Kaempferol-7-O-β-D-glucopyranoside).

Anti-inflammatory studies on the n-butanol soluble portion showed that the fraction possessed significant anti-inflammatory activity in acute model of inflammation.

6.2 Conclusion

From the findings of this research, the use of *Indigofera hirsuta* in the treatment of inflammation and related inflammatory disorders has scientific basis. The significant anti-inflammatory activity of n-butanol fraction observed might be linked to the flavonoid glycosides isolated; kaempferol-3-O-β-D-glucopyranoside and kaempferol-7-O-β-D-glucopyranoside. To the best our knowledge, this is the first report of isolation of kaempferol glycosides from the n-butanol soluble fraction of *Indigofera hirsuta*.

6.3 Recommendations

i. Further bioassay guided investigations of the constituents of the plant *I. hirsuta* with a view of isolating bioactive compounds be carried out. There is need to screen the plant for anticancer and antiviral activities.

ii. Further studies need to be carried out on the isolated Kaempferol glycosides.

iii. There is also need to carry out chronic toxicity studies on the plant so as to rule out the possibility of any deleterious effect that may arise as a result of prolonged use of the plant.

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Appendix I: Anti-inflammatory effect of n-butanol fraction of the methanolic extract of *Indigofera hirsuta* and Ketoprofen in Rats.

