ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *CENTAUREA SENEGALENSIS* [DC]

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February, 2020

DECLARATION

I declare that the work in this dissertation entitled "**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *CENTAUREA SENEGALENSIS [DC]***" has been performed by me in the Department of Chemistry, Ahmadu Bello University, Zaria, under the supervision of Dr. A. B. Aliyu and Prof. A. O. Oyewale. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any institution.

Ahmed IDRIS ……... …….

Signature Date

CERTIFICATION

This dissertation entitled "**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *CENTAUREA SENEGALENSIS* [DC]**" by Ahmed Idris meets the regulations governing the award of the degree of Master of Science in Organic Chemistry of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Chairman supervisory committee _____ _____

Signature Date

Prof. A. O. Oyewale

Member supervisory committee _____ _____

Signature Date

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Signature Date

DEDICATION

This research work is dedicated to my parents, and late Hajiya Rahmatu Shehu of blessed memory.

ACKNOWLEDGEMENTS

My acknowledgement goes to Allah for good health and other privileges My profound gratitude goes to my supervisors Dr. A. B. Aliyu and Professor A. O. Oyewale for their time and support throughout the period of this research. I am grateful. I appreciate the effort of the head of the department, teaching and non-teaching staff of Department of Chemistry for their understanding throughout the period of this work. I also wish to appreciate Mr. Kutman Nanpan Ali of Nigerian Institute of Leather and Science Technology (NILEST) for his assistance in antimicrobial analyses.

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ABSTRACT

Centaurea senegalensis DC.is a plant commonly used in Nigeria traditional medicine for treatment of stomach ache and pains. The aim is to isolate and characterize bioactive compound from the aerial part of *Centaurea senegalensis.*The plant was extracted using maceration method to give the crude methanol extract which was sequentially fractionated using hexane, dichloromethane and ethyl acetate respectively. The crude methanol extract and solvent fractions were subjected to phytochemical screeningand found to contain alkaloids, flavonoids, terpenes, tannins, saponins and glycosides. Purification of the n-hexane (CSH) and dichloromethane (CSD) fractionsusing silica gel column chromatography led to the isolation of taraxasteroland eupatorinrespectively. The structural elucidations of the compounds were determined using FT-IR,1D and 2D-NMR.The crude methanol extract, fractions and the isolated compounds were subjected to antimicrobial activity against selected gram-positive and gram-negative bacteria (*Shigella dysenteriae,Salmonella typhi,Escherichia coli,Staphylococcus aureus*, Vancomycin Resistant*Enterococci*, Methicillin Resistant*Staphylococcus aureus*) results indicated varying growth inhibitions with zones of inhibition of 16-28 mm for crude methanol extract,13-31 mm for solvent fractions and 10-15 mmfor isolated compounds. The minimum inhibitory concentration (MIC) of the fractions was12.5 mg/ml on the Methicillin Resistant*Staphylococcus aureus* andVancomycin Resistant*Enterococci*. However, the isolated compounds were found to demonstrate activity on Methicillin resistance *Staphylococcus aureus* and *Shigelladysenteriae* with 25 mg/ml as the MIC and 50 mg/mlas the MBC. In conclusion, the results of this study have shown the importance of *Centaurea senegalensis* as a source of bioactive compounds with potential for the treatment of bacterial infections.

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LIST OF ABBREVIATIONS

Abbreviations	Meanings
MP	Melting point
MRSA	Methichilin Resistant *Staphylococcus aureus*
VRE	Vancomycin resistant *enterococci*
COSY	Correlation spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
DEPT	Distortionless Enhancement by Polarization Transfer
NMR	Nuclear Magnetic Resonance
FT-IR	Fourier Transform Infra-Red
GCMS	Gas Chromatography-Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
1D	One Dimensional
2D	Two Dimensional
CSH	*Centaurea senegalensis* hexane
CSD	*Centaurea senegalensis*dichloromethane
SLs	Sesquiterpene Lactones
TLC	Thin Layer Chromatography

CHAPTER ONE

1.0 Introduction

Natural products chemistry has undergone tremendous growth owing to advances in isolation techniques, synthetic methods, physico-chemical measurements, and new concepts, as well as for the wide range of biological properties exhibited by the substances biosynthesized by living organisms (Cragg *et al*., 1997). Extracts from the various plant parts (leaves, stem bark and roots) of various higher plants are used in herbal medicine production (Sofowora, 1993). Plants extracts are given ordinarily or as concoctions for the treatment of various ailments. In actual sense more than 75% of the world population depends on these various forms of concoctions and herbal decoctions for the treatment of infections (Robenson and Zhang, 2011).

Plants are natural reservoir of medicinal agents and are increasingly gaining acceptance, even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhoea and tuberculosis as well as increase in resistance by several bacterial to various antibiotics and the increasing cost of prescription drugs, for maintenance of personal health (Smolinski *et al*., 2003).

Plants as a potential source of drugs are of great relevance, especially with the current global shift to obtain drugs from plant sources. Several ailments including fever, asthma, constipation, diarrhoea, oesophageal cancer and hypertension have been treated with traditional medicinal plants. Different plant parts and components have been employed in the treatment of infectious diseases (Dutta *et al*., 2013).

The rapid increase in the antibiotic resistance of microorganism to available synthetic drugs and their discomforting side effects necessitates the search for alternative source of antibiotics. Antimicrobial resistance is an increasingly problematic issue that leads to millions of deaths

every year (WHO, 2013). Most bacteria and fungi always develop resistance to antibiotics that are used to kill them. This is a big challenge in controlling infectious diseases and hence, a need to search for new substances, especially from natural sources to solve the problem.

Many species of the genus *Centaurea* (Asteraceae) have been used in traditional medicine since ancient times. These plants are generally rich in flavonoids and sesquiterpene lactones, which are the main constituents responsible for biological activity (Akkal *et al*., 1997).

Sesquiterpene lactones (SLs) demonstrate a broad spectrum of biological activity including disrupting the cell wall of fungi and invasive bacteria, whereas others protect the plant from environmental stress that would otherwise cause oxidative damage. Many of the compounds are effective due to their bitter flavor, which has obvious implications for human consumers (Chadwick *et al*., 2013). Plants of *Centaurea* genus are used as remedy against infectious diseases in Nigeria traditional medicine. Preliminary phytochemical screening of *Centaurea senegalensis* extracts indicated the presence of spots on TLC. However, previous isolation and structural elucidation has been reported on flavonoids only (Aqil *et al*., 1998).

1.1Statement of Research Problem

Antibacterial drug resistance is an increasingly problematic issue that leads to numerous deaths yearly (WHO, 2013). It is a global challenge to antibiotic chemotherapy and hence, the need to search for natural sources for more effective compounds or molecular scaffold for the synthesis and development of new antibacterial agents.

1.2 Aim of the study

The aim of the study was to isolate and characterize bioactive compounds from *Centaurea senegalensis* and evaluation of antibacterial activity of the pure compounds.

1.3 **Objectives of the study**

The objectives of the study were to:

i. extract the pulverized plant using methanol and fractionate the extract using hexane, dichloromethane and ethyl-acetate respectively.

ii. purification of the extract/fractions using chromatographic technique for the isolation of bioactive compounds.

iii. evaluate antibacterial activitiesof the crude extract/fractions and the isolated compounds.

iv. characterization of isolated compounds using IR, 1Dand 2D-NMR spectroscopy.

1.4 Justification for study

The genus *Centaurea* has been known to be used in traditional medicine. The need to scientifically establish the medicinal claims on the plant. This may result to discovery of more potent compounds against drug resistance pathogenic microorganisms. However, although flavonoids have been reported from the plant, there is no documented report to the best of my knowledge on the isolation, characterization and antibacterial activity of other class of compounds from *Centaurea senegalensis.*

CHAPTER TWO

2.0Literature Review

2.1 Botanical information

Centaurea senegalensis [DC] is an annual branched scabrid herb, erect 1.2 ft high or prostrate stem-leaves semi-amplexical upto 2 inch long, the basal leaves of early growth larger; floret purple in spiny heads about half inch broad terminating leafy branches (Hutchison, 1963). It is used in the traditional medicine in Northern Nigeria as a crude drug for the treatment of stomach ache, diuresis, dropsy, gout and pains (Burkill, 1985). It is commonly known as Dayi in Hausa language across Northern Nigeria.

Kingdom: *Plantae*

Division: *Tracheophyta*

Subdivision: Spermatophytina

Class: Magnoliopsida

Order: Asterales

Family: Asteracea

Genus: *Centaurea*

Species: *senegalensis*

Plate I: *Centaurea senegalensis* from natural habitat.

2.2 Ethnomedicinal Uses of *Centaurea* Genus

The decoction of *C.senegalensis* leaves are used for treating fever in Funtua, Nigeria.It was also reported by Burkill (1985) for the treatment of stomach ache and pains. *C. polyclada* leaves are used for inflammatory conditions such as abscesses, asthma, hemorrihoids and peptic ulcer (Demir *et al*., 2017). In China, the leaves of *C. aspera* are consumed as tea for hypoglycemic while leaves of *C. pallascens* are used in Egypt for treatment of diuretics (Kamanzi *et al*., 1983). In Turkey, *C. scabiosa* flowers are used locally for treatment of cough, itch and eye remedies (Chicca *et al*., 2011). In Spain, *C. ornato* leaves are used for tonic and hypotensive and roots as antispasmodic (Bastos *et al*., 1994). A survey of the reported traditional uses of plants of the*Centaurea* family is presented in Table 2.1.

Table 2.1: Some of the *Centaurea* species used in traditional medicine

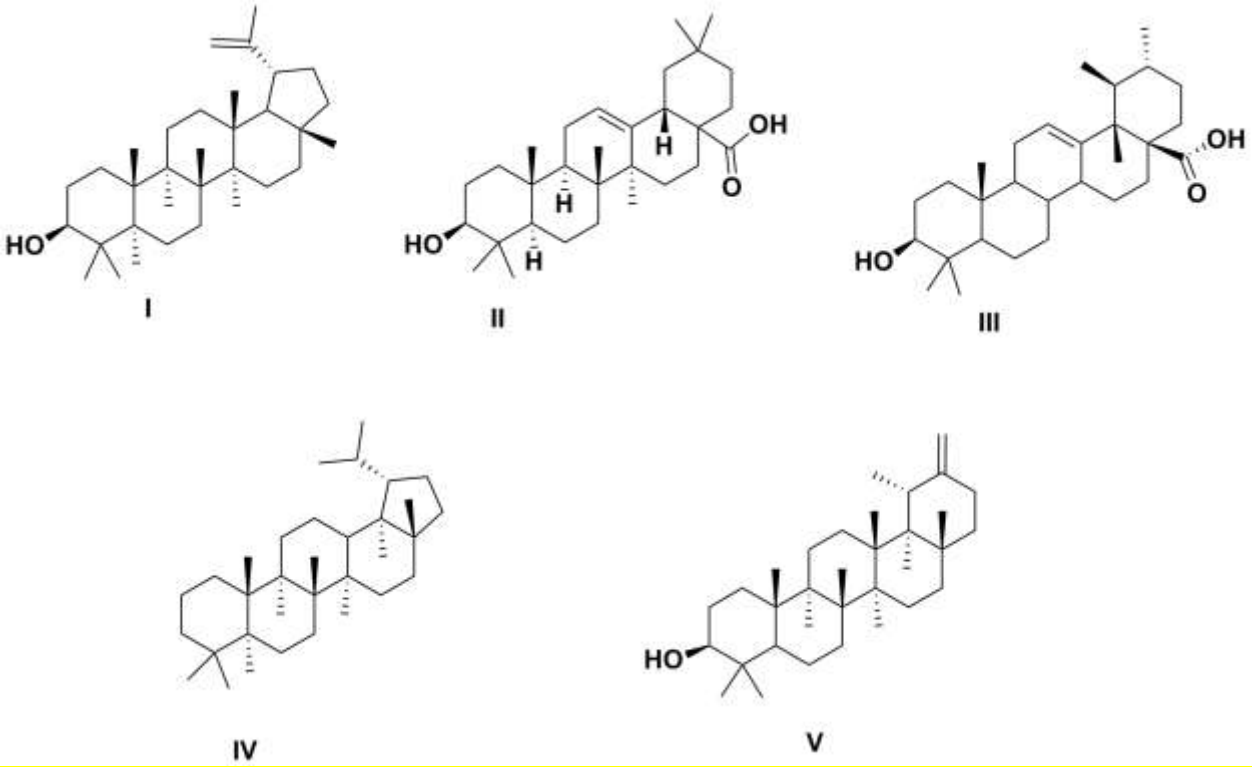
S/N	Plant species	Part(s) used	medicinal uses	Reference
1	*C. montana*	Root, leaves and flower	Astringent, purgative, diuretic and ophthalmic	Ahmad *et al*. (2015)
2	*C. solstitialis*	Flower	Anti-ulcerogenic activities	Yesilada (2004)
3	*C. iberica Trev*	Flower	Wound healing and asthma	Yesilada (2004)
4	*C. drahifoliasin*	Flower	Haemorrhoids	Yesilada (2004)
5	*C. virgate lam*	Flower	Wound healing	Yesilada (2004)
6	*C. pterocaula*	Flower	Wound healing	Yesilada (2004)
7	*C. calcitrapa*	Leaves	To reduce fever	Yesilada (2004)
8	*C. jacea*	leaves	To reduce fever	Yesilad (2004)
9	*C. uniflora*	Root	Fever and for detoxication	Wei *et al*. (1997)
10	*C. chilensis*	Leaves	Fever and rheumatic pain	Sepulveda *et al*. (1994)
11	*C. ornate*	Flower	Rheumatic pain	Bastos *et al*.(1994)
12	*C. sinaica*	Leaves	Fever	Al-Eass *et al*. (1992)
13	*C. melitensis*	Root	Digestive tonic	Bruno *et al*. (2013)
14	*C. uniflora*	Flower	Fever and detoxification	Wei *et al*. (1997)
15	*C. aspera*	Leaves	Tea as hypoglycaemic	Bruno *et al*. (2013)
16	*C. pallascens*	Leaves	Diuretic	Bruno *et al*. (2013)
17	*C. ornato*	Leaves	Pain and hypotensive	Bastos *et al*. (1994)
18	*C. scabiosa*	Flower	Cough, itch, and as liver strengthening	Chicca *et al*. (2011)
19	*C. cyanus*	Flower	Ophthalmic remedies	Chicca *et al*. (2011)
20	*C. polyclada*	Leaves	Inflammatory conditions, asthma and peptic ulcer	Demir *et al*. (2017)
21	*C. senegalensis*	Leaves	Rheumatic pain and fever	Aqil *et al*. (1998)

2.3 Phytochemistry of *Centaurea* Genus

Phytochemical studies of *Centaurea* genus have been explored across the world with abundant information in the literature. Yet several *Centaurea* species have not been extensively studied and *Centaurea senegalensis* is one of them. The report on isolation and characterisation of six (6) flavonoids is currently the only the phytochemistry on *C.senegalensis* (Aqil *et al*., 1998). Previous reports have shown that flavonoids and sesquiterpene lactones are dominant secondary metabolites in addition to triterpenoida as chemical makers from the *Centaurea* genus.

2.3.1 Triterpenoids

Triterpenoids are compounds based on C-30 skeleton consisting of five six-membered rings and a five membered ring. They are a wide spread group of natural terpenoids dominant in plants. They are classified into oleane, ursane, taraxastane, friedelane and lupine types (Gangwal, 2013). Pentacyclic triterpenoids consisting of lupine and taraxastane types have been largely reported from *Centaurea* genus (Chadwick *et al*., 2012). It is the largest class of triterpenoids consisting of lupane, frudaline, taraxasterane, oleanane and ursane. The representative class of triterpenoids isolated from *Centaurea* includes lupane and tarxasterane (Yin *et al.,* 2012).The chemical structure of some triterpenoids reported from the *Centaurea*genus are lupeol (I), oleanolic acid (II), ursolic acid (III), lupane (IV) and taraxasterane (V).

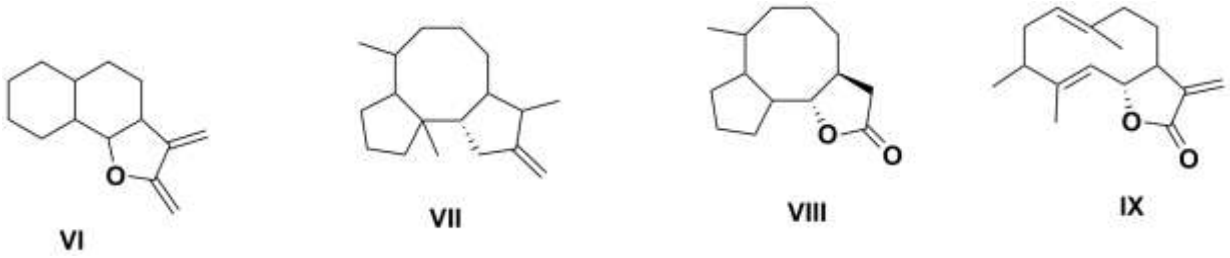


2.3.2 Sesquiterpene lactones

Sesquiterpene lactones are terpenoid compounds characteristic of the Asteraceae. They are found in other angiosperm families as well as from some gymnosperms.Sesquiterpene lactones act as plant growth regulators and are responsible for allelopathic properties of many plants.An important common feature of sesquiterpene lactones is the presence of γ-Iactone ring, in many cases, an α-methylene group. A few sesquiterpene lactones occur in glycosidic form (Herz *et al*., 1970; Kisiel and Davie, 1975) and some contains halogens (Gonzalez *et al*., 1974) or sulphur (Kondo *et al*., 1977). Various activities of sesquiterpene lactones suggest their evolutionary significance in plants as deterrents against herbivores, antifungal and anti-bacterial (Picman, 1986).

All the sesquiterpene lactonnes have an α-oxygenated function at C-6 that is involved in the formation of the C-6/C-12 γ- lactones but present as a free hydroxyl group in some cases. In

contrast to other genera of the Asteraceae family, belonging to different subtribes, the oxygenated function at C-8 is almost always α-oriented and if present, the ester side chains are often linked at this carbon, although it is possible to find the ester moiety in other positions (Bruno *et al*., 2013). The common skeletal types include eudesmanolides (VI), germacranolides (VII), guainolides (VIII) and pseudoguainolides (IX) isolated from *Centaurea aspera* (Cardona *et al*., 1991),*Centaurea sphaerocephala ssp*. and *Centaurea harmanii* (Sevil *et al*., 1994) respectively. The structures of the skeletal frame work for these compounds are shown as eudesmanolides (VI), germacranolides (VII), guainolides (VIII) and pseudoguainolides (IX).

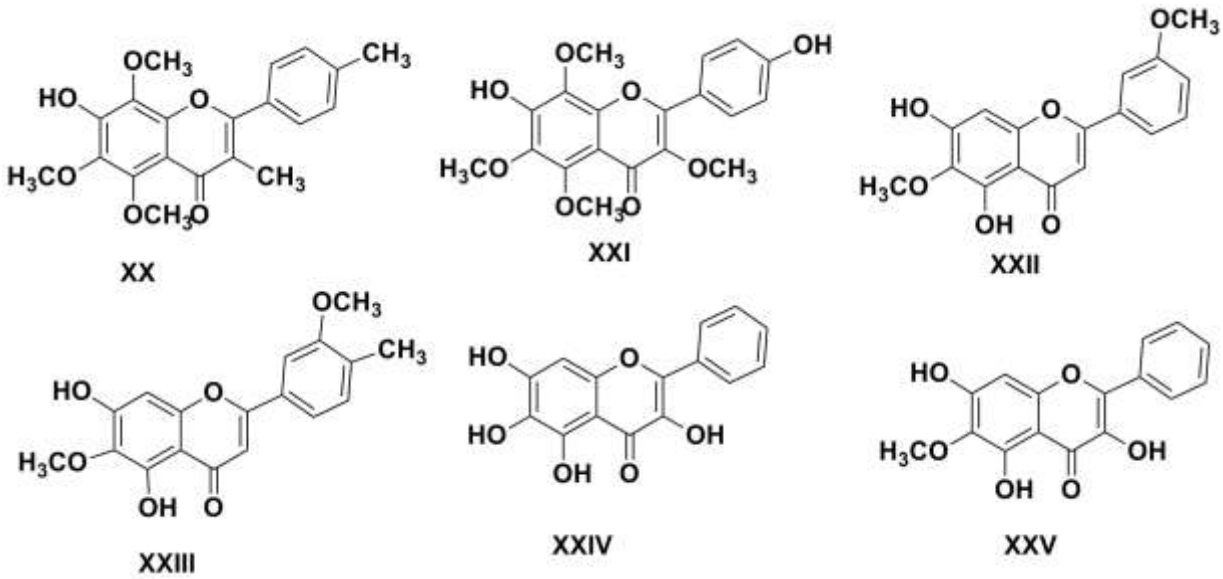


VI VII VIII IX

2.3.3 Flavonoids

Flavonoids are aromatic secondary metabolites naturally synthesized by plants and fungi from aromatic amino acids L-phenylalanine and L-tyrosine. The structure of these phytochemicals is characterized by two phenolic rings and one heterocyclic ring. The main differences between flavonoids are related to the hydroxylation patterns, the position of the second aromatic ring and the saturation of the heterocyclic ring (Grotewold, 2006). Bioactive polyphenols such as flavonoids play a fundamental role in the physiology of plants; their natural functions includes: UV protection, reduction of oxidative damage in cells, and antibacterial effect (Cushnie and Lamb, 2011). Kaempferol, quercetin andfisetin showed anti-cancer, cardio-protective and anti-inflammatory effects (Chou *et al.,* 2013; Chen and Chen, 2013). Previous studies on *Centaurea senegalensis* have reported the isolation of flavonoids 7-hydroxy-3,5,6,8,4-pentamethoxyflavone (centaflavone A) (**XX**), 7,4-dihydroxy-3,5,6,8 tetramethoxyflavone (centaflavone B) (**XXI**),

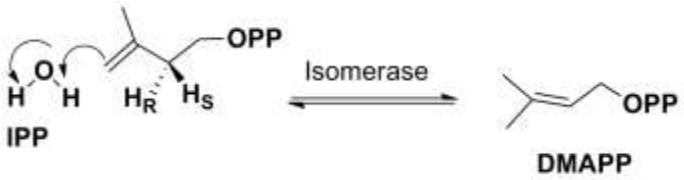
jaceosidin (**XXII**), eupalitin (**XXIII**), 6-methoxykaempferol (**XXIV**) and 6-hydroxykaempferol (**XXV**) (Aqil *et al*., 1998).



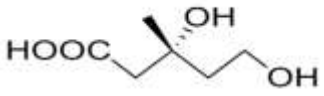
2.4 Biosynthesis of Triterpenoids and Flavonoids

2.4.1 Biosynthesis of triterpenoids

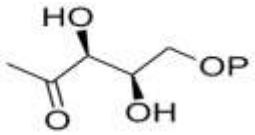
Terpenoids are produced by rearrangement of squalene epoxide which is believed to have been biosynthesized by the mevalonate pathway but another route via the deoxyxylulose phosphate pathway (DXP) is reported to be widely accepted (Dewick, 2006). The main precursor of terpenoids is the isoprene unit, which is formed via isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the latter being formed from IPP with the isomerase enzyme (Dewick, 2006).



This unit may be derived from mevalonic acid (MVA) or deoxy xylulose phosphate (DXP).



MVA



DXP

In plant, the enzymes from the mevalonate pathway are found in the cytosol, therefore triterpenoids and steroids (cytosolic product) are thought to be formed through this pathway, whereas the other terpenoids are derived (Dewick, 2006). IPP isomerises to DMAPP (Figure 2.4) via the isomerase enzyme which stereospecifically removes the pro-R proton from C-2 and incorporates a proton from water onto C-4. Although the isomerisation reaction of IPP to DMAPP is reversible. The equilibrium favours the formation of DMAPP

When phosphate (a good leaving group) of DMAPP leaves, it yields an allylic carbocation that is stabilised by charge delocalisation, therefore making it electrophilic. IPP on the other hand is a strong nucleophilic due to the terminal double bond. This differing reactivity of DMAPP and IPP forms the basis of terpenoid biosynthesis in the linkage of isoprene units in a head-to-tail manner (Dewick, 2006; Zulak and Bohlman, 2010).

The combination of DMAPP and IPP through prenyl transferase mediation, results in the formation of geranyl diphosphate (GPP) the fundamental precursor for monoterpenoids (C10) compounds. This involves ionisation of DMAPP to the allylic cation to which the double bond of IPP adds followed by the stereochemical loss of the pro-R proton. GPP possesses the reactive allyl diphosphate group and the reaction mediated by prenyl transferase continues by addition of an IPP (I) unit to the geranyl cation which results in the formation of farnesyl diphosphate (FPP). Triterpenoids are formed by joining two FPP units in a tail-to-tail manner to yield the hydrocarbon squalene, formed from the precursor presqualene diphosphate by a reaction that follows a different path from the previous condensation reactions. The formation of presqualene

diphosphate is by attack of the double bond of one FPP molecule. The resulting tertiary cation is discharged by loss of a proton with the formation of a cyclopropane ring to yield presqualene phosphate. This is followed by loss of diphosphate to form an unstable primary cation that undergoes rearrangement to generate a more favourable secondary carbocation and less strained cyclobutane ring. Bond cleavage produces an allylic cation, stabilised by charge delocalisation, which is quenched by attack of a hydride ion from NADPH to form squalene, an important precursor of cyclic triterpenoids (Figure 2.1).

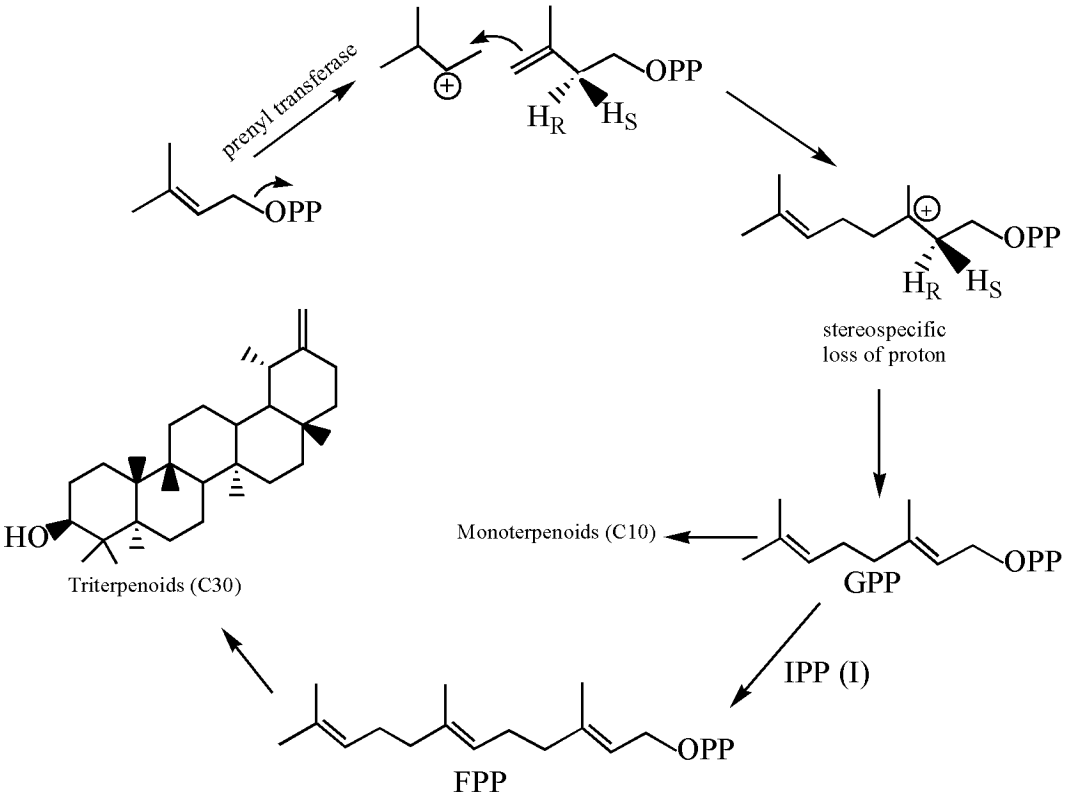
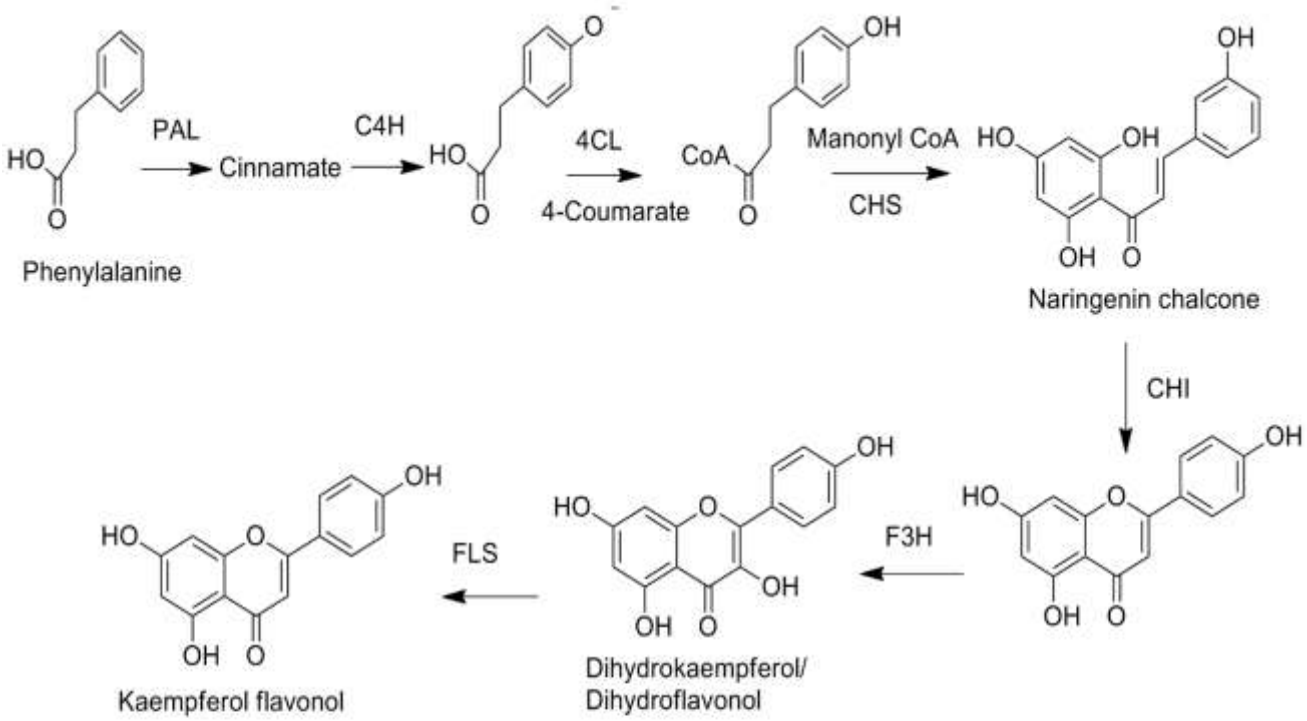


Figure 2.1: Formation of Triterpenoids precursor GPP and FPP

2.4.2 Biosynthesis of flavonoids

The key flavonoid precursors are phenylalanine and malonyl-CoAderived from the shikimate/arogenate pathway and the TCA cycle, respectively. The first flavonoid are the C15 chalcone which are formed by chalcone synthase (CHS), a member of polyketide synthase group of enzymes. CHS takes hydroxyl cinnamic acid-CoA ester unit, usually P-coumaroyl-CoA and carries out three sequential additions of the ‘’extender’’ molecule malonyl-CoA. The chalcones are the first coloured flavonoid and provide yellow colouration to petals of a few plant species, most notably carnation. The chalcones are converted by stereospecific isomerization to the corresponding 2S-flavanone by chalcone isomerase (CHI) establishing the heterocyclic C-ring. Two types of CHI have been identified, the CHI-I can use only 6’-hydroxy chalcone substrate whereas CHI-II which is found mostly in legumes can catalyze isomerization of both 6’-hydroxy- and 6’-deoxychalcones due to the intra-molecular hydrogen bond between the 2’-hydroxyl and the carbonyl group. The isomerization reaction can occur non-enzymatically to form racemic (2R, 2S) flavone and this may occur *in-vivo* to enable formation of anthocyanins in plants with mutations for CHI (Koes *et al*., 1994). The reaction scheme for the enzymatic synthesis of flavonoids is presented as figure 2.2.



Enzyme catalysing some key reaction abbreviations.

PAL = phenylalanine Ammonia-Lyase
C4H = cinnamate-4-hydroxylate
4CL = 4-coumaroyl-coenzyme A ligase
CHS = chalcone synthase
CHI = chalcone isomerase
F3H = flavone 3B-hydroxylase
FLS = flavone synthase

Figure 2.2: Biosynthesis of flavonoids

2.5Antibacterial Activity of Compounds from *Centaurea* Genus

Plants, as with animals, must withstand attacks from microbes. Being sessile, they make easy targets for pathogens and are unable to avoid stressful climates, which may reduce their ability to withstand infections. It comes then as no surprise that they are capable of producing a range of chemicals, which help them withstand attack from fungi, bacteria, and viruses. Typically, these compounds take the form of alkaloids, phenolics, and terpenoids. In the Asteraceae especially, sesquiterpene lactones are one of the main mechanisms for this defense. Sesquiterpenes reduce harm by microbial attack by disruption of a microbe's cell membrane, an effect attributable to the polar groups on these anti-microbial compounds disrupting the phospholipid membrane (Cowan, 1999). Often, and as is the case with lettucenin A, the phytoalexins are especially potent compounds (Bennett, 1994).

Sesquiterpene lactones of a guaianolide structure are considered to have the greatest antimicrobial potency. while constitutive volatile sesquiterpene lactones work to prevent less severe surface infections from becoming severe or invasive, providing a baseline defence. While anti-fungal sesquiterpene lactones are generally broad spectrum, many microbes are resistant, or even increase growth rate in response to sesquiterpene lactones, and consequently there is a complex system involving species specificity and adaption to particular sesquiterpene lactones.

Three sesquiterpene lactones (Centaurepensin = Chlorohyssopifolin-A, Chlorojanerin and 13-acetyl solstitialin A) were isolated from the aerial parts of *Centaurea solstitialis L. ssp.* and was investigated for antimicrobial activities.13-Acetyl solstitialin-A displayed remarkable antibacterial activity against isolated strains of *E. faecalis* (Zelik *et al*., 2009). A summary of various classes of compounds and their microbial activities from the *Centaurea* genus is presented in Table 2.2.

Table 2.2: Chemical Compounds from *Centaurea genus* with antimicrobial properties

S/N	Compound	Species	Microbial Target	MIC/MBC (µg/ml)	Ref.
1	Dehydrozaluzanin C,	*C. acutatum, C. fragariae*	Colletorichum, Botrytis, Fusarium and Phomopsis	7-8	Wedge *et al*. (2000)
2	Dehydrocostuslactone,	*C. acutatum, C. fragariae*	Colletorichum, Botrytis, Fusarium and Phomopsis	4-8	Wedge *et al*. (2000)
3	5α-hydroxydehydrocostus lactone C	*C. acutatum, C. fragariae*	Colletorichum, Botrytis, Fusarium and Phomopsis	6-7	Wedge *et al*. (2000)
4	Zaluzanin C	*C. acutatum, C. fragariae*	Colletorichum, Botrytis, Fusarium and Phomopsis	4	Wedge *et al*. (2000)
5	8α-(3,4-dihydroxy-2-methylene-butanoyloxy),	*C. deusta*	*P. ochrochloron, A. niger, P. aeruginosa,A. Flavus, E.coli, S. aureus*	0.075-5.0	Karioti *et al*. (2002)
6	8α-(4-acetoxy-3-hydroxy-2-methylene-butanoyloxy)dehydromelitensine,	*C. deusta*	P. ochrochloron, A. niger, *P. aeruginosa, A. Flavus, E. coli, S. aureus*	0.075-2.4	Karioti *et al*. (2002)
7	Methyl 8α-(3,4-di-3-hydroxy-2-methylene- butanoyloxy)-6α, 15-dihydroxyelema-1,3,11(13)-trien-12-oate,	*C. deusta*	*P. ochrochloron, A. niger, P. aeruginosa, A. Flavus, E. coli, S. aureus*	1.25-7.5	Karioti *et al*. (2002)
8	8α-(4-acety-3-hydroxyl-2-methyl- buten-2-oyloxy)salonitenolide	*C. deusta*	*P. ochrochloron, A. niger, P. aeruginosa, A. Flavus, E. coli, S. aureus*	0.075-0.3	Karioti *et al*. (2002)
9	8α-(4-acety-3-hydroxyl-2-methylenebutanoyloxy)-sonchucarpolide,	*C. deusta*	*P. ochrochloron, A. niger, P. aeruginosa, A. Flavus, E. coli, S. aureus*	0.15-2.4	Karioti *et al*. (2002)
10	3S, 5R-loliolide	*C. deusta*	*P. ochrochloron, A. niger, P. aeruginosa, A. Flavus, E. coli, S. aureus*	3.75-12.0	Karioti *et al*. (2002)
11	Salograviolide A,	*C. nicolai*	*A. niger, A. ochraceus, P. ochlocloron*	6.25-25	Vajs *et al*. (1999)
12	3-deacetyl-9-O-acetylsalograviolide	*C. nicolai*	*A. niger, A. ochraceus, P. ochlocloron*	6.25-25	Vajs *et al*. (1999)
13	9-O- acetylsalograviolide A	*C. nicolai*	*A. niger, A. ochraceus, P. ochlocloron*	6.25-25	Vajs *et al*. (1999)
14	8α-0-(4-hydroxy-2-methylenebutanoyl-	*C. pullata*	*B. subtilis*	0.1-0.3	Djeddi *et al*. (2008)

	oxy)melitensin				
15	Melitensin	*C. pullata*	*M. flavus*	0.3–0.7	Djeddi *et al*. (2008)
16	11β, 13- dihydrosalonitenolide	*C. pullata*	*S. epidermidis*	0.3–0.7	Djeddi *et al*. (2008)
17	8α-hydroxy-11β, 13-dihydro-4-epi-sonchucarpolide	*C. pullata*	*B. subtilis*	0.3–0.7	Djeddi *et al*. (2008)
18	8α-hydroxy-11β, 13-dihydro-onopordaldehyde	*C. pullata*	*S. epidermidis*	0.2–0.4	Djeddi *et al*. (2008)
19	Arctii	*C. schleropsis*	*M. leteus, S. aureus, B. cereus and E. coli*	62.5–250	Arslanyolu and Erdemgi (2006)
20	Scopoletin	*C. spuneri*	*P. mirabilis, P. aeroginosa, S. typhimurium and B. cereus*	0.655–4.15	Ciric *et al*. (2011)
21	Eriodictyol	*C. spuneri*	*P. mirabilis, P. aeroginosa, S. typhimurium and B. cereus*	0.655–4.15	Ciric *et al*. (2011)
22	Isovanillic acid	*C. spuneri*	*P. mirabilis, P. aeroginosa, S. typhimurium and B. cereus*	0.655–4.15	Ciric *et al*. (2011)
23	Protocatechuic acid	*C. spuneri*	*P. mirabilis, P. aeroginosa, S. typhimurium and B. cereus*	0.655–4.15	Ciric *et al*. (2011)
24	Isoscopoletin	*C. spuneri*	*P. mirabilis, P. aeroginosa, S. typhimurium and B. cereus*	0.655–4.15	Ciric *et al*. (2011)
25	cynaropicrin	*C. zuvandica*	*S. aureus E. coli, B. cereus*	0.29-1.37	*Baharfar et al. (2009)*
26	Costunolide	*C. acaulis*	*P. solanacearum, S. lutea*	2-16	Bentamane *et al*. (2005)
27	Santamarine	*C. acaulis*	*P. solanacearum, S. lutea*	2-16	Bentamane *et al*. (2005)
28	Reynosin	*C. kurdica*	*P. solanacearum, S. lutea*	32–64	Fisher *et al*. (1998)
29	8α (3', 4'-dihydroxy-2'-methylenebutanoyl-dihydromelitensin:isocnicin	*C. aegialophilla*	*B. subtilis, S. aureus, E. coli*	6.25–12.25	Bruno *et al*. (2013)
30	8α (3', 4'-dihydroxy-2'-methylenebutanoyl-dihydromelitensin:isocnicin	*C. attica*	*B. subtilis, S. aureus, E. coli*	6.25–12.25	Skaltsa *et al*. (1999)

CHAPTER THREE

3.0Materials and Methods

3.1Materials

All organic solvents used were of general-purpose grade (Sigma Chemical Co., U.S.A) and distilled before used. All equipment and capacity are quoted accordingly and operated with standard procedures as a guide. The silica gel for column chromatography is of mesh size (60 – 120) and that of thin layer chromatography is 0.25 mm thickness. Solvents (methanol, ethyl acetate, dichloromethane and n-hexane), TLC plates (Silica gel coated), Column (100 × 4 cm), Burette (50 cm^3), Foil paper, Distilled water, Cotton wool, Syringes (2 ml, 5 ml, 10 ml and 20 ml), Capillary tube, Mueller-Hinton agar medium, Mueller-Hinton broth, dimethyl sulfoxide (DMSO), 37°C ambient-air incubator, Incandescent light, Vortex mixer, Weighing balance, Sterile cotton-tipped swabs, Biohazard waste bag, 13 × 100 mm test tubes with snap caps, Test tube racks, Millimeter ruler, Sterile transfer pipette, Forceps and Inoculating loop or needle.

3.2 Instrumentation

1.The melting points of the isolated compounds were determined using Stuart automatic melting point/SMP40 obtained from Department of Chemistry, Faculty of Physical Sciences, Ahmadu University Zaria.

2.Fourier Transform Infrared (FT-IR) absorption spectra were recorded using an infrared spectrophotometer Agilent Technology Cary 630 FT-IR.

3. The NMR spectra was obtained on a Bruker AVANCE (600 MHz for ^{1}H and ^{13}C) spectrometer from Department of Pure and Applied Chemistry, University of Strathclyde, Scotland-United Kingdom.

3.3 Methodology

3.3.1 Plant collection

The plant sample was collected at Funtua (11^{o} 32N 7^{o} 19E and 11.533^{o} N 7.317^{o} E), Katsina State, Nigeria in November, 2017. It was authenticated by Mallam Nnamadi Sambo at the Herbarium, Department of Botany, Faculty of Life Science,Ahmadu Bello University Zaria, where a Voucher number (2052) was deposited. The plant was air-dried and pulverized using mortar and pestle.

3.3.2 Extraction

The powdered sample (980 g) was extracted with methanol (7.5 L) using cold maceration method for 13 days. The extract was filtered and evaporated to dryness with a rotary evaporator as described by Albyrak *et al.* (2010).The extract was green gummy material (274.60 g) and referred to as the methanol extract of *Centaurea senegalensis*. The methanolcrude extracted (160.0 g) was partitioned using hexane (2.5 L), dichloromethane (2.0 L) and ethyl acetate (2.0 L) by maceration.

3.3.3 Phytochemical Screening

The n-hexane, dichloromethane, ethyl acetate fractions and methanol extracts were subjected to phytochemical screening for plant secondary metabolites using standard techniques (Trease and Evans, 2009).The following procedures used are:

3.3.3.1 Test for flavonoids

(a) Shinoda Test: A small quantity of magnesium powder and a few drop of conc. HCl were added to an alcoholic solution of each plant extract (2 ml). The appearance of an orange or pink or red to purple colour indicates the presence of flavonoids (Sofowora, 1993).

(b) Sodium Hydroxide Test: The extract (2 ml) was dissolve in 10% aqueous sodium hydroxide solution and filtered to give yellow color, a change in color from yellow to colorless on addition of dilute HCl indicates the presence of flavonoids (Cannel, 1998).

3.3.3.2 Test for alkaloids

(a) Mayer's reagent: Freshly prepared Mayer's' reagent (Potassium mercuric iodide solution) was added drop wise to each acidified solution of the extracts (1 ml). A white or creamy precipitate was observed indicating the presence of alkaloid (Silva *et al*., 1998).

(b) Dragendorff's reagent test: An acidified solution of the extract was treated with Dragendorff's reagent (Bismuth nitrate and potassium iodide solution). An orange-brown precipitate was formed indicating the presence of alkaloid (Silva *et al*., 1998).

3.3.3.3 *Test for tannins*

(a) $FeCl_3$ test: A small quantity of each extract (0.5 g) was dissolved in distilled water (10 ml), boiled and filtered. To the filtrate was added few drops of 1% ferric chloride solution, a formation of blue black or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 2009).

(b) Lead sub-acetate Test: To a small quantity of the extract (1 ml), three drops of lead sub-acetate was added. The solution was observed for the presence of green precipitate as evidence for the presence of tannins (Trease and Evans, 2009).

3.3.3.4 Test for saponins

Frothy test: Distilled water (5 ml) was added to 0.3 g of each extract in a test tube and shaken vigorously for 30 sec then allowed standing for 45 min. A honey-comb froth that persisted for 30 min was observed indicative of the presence of saponins (Silva *et al*., 1998)**.**

3.3.3.5 Test for glycosides

$FeCl_3$ test: To the extract/fraction (0.5 g), conc. H_2SO_4(5 ml)was added and boiled for 15 min. This was then cooled and neutralized with 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution were added to one of the portions and a green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycoside (Silva *et al*., 1998).

3.3.3.6 *Test for steroids*

Salkowski Test: The plant extracts were dissolved in 1 ml chloroform respectively and 1 ml conc. H_2SO_4 was added. The formation of a reddish-brown interphase was taken as an indication for the presence of was steroids (Silva *et al*., 1998).

3.3.3.7 Test for Anthraquinones

(a) Free Anthraquinones

Small quantity of the extracts was shaken with 10 ml of benzene, the contents were filtered and subjected to the following test independently and respectively. 10% ammonia solution (5 ml) was added to the filtrate, the mixture was shaken. No colour change was observed in the ammoniacal layer (Lower phase) that indicates the absence of free anthraquinone in all the extracts (Trease and Evans, 2009).

(b) Combined Anthraquinones

The extract was boiled with 10 ml of aqueous Sulphuric acid, filtered hot and subjected to the following test independently and respectively. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and to half its own volume, 10% NH_4OH was added. A pink colouration was observed in the ammonia phase (lower phase) which indicates the presence of

combined anthraquinone or anthraquinone derivatives in methanol, ethyl acetate and n-hexane extracts (Trease and Evans, 2009).

3.3.4 Thin Layer Chromatography

Thin layer chromatography was carried out on silica gel coated aluminum sheet with layer thickness of 0.25 mm. One dimensional TLC technique was employed. The spots were applied manually using capillary tubes and the plates developed at room temperature using a Shandon chromatotank. Mobile phase solvent systems include n-hexane with ethyl acetate 8:2 and n-hexane with ethyl acetate 2:8. Detection of spots on TLC plates were by UV light (254 and 366 nm) and spraying with 10% Sulphuric acid or p-anisaldehyde, followed by heating at 110^{o}C using hair dryer for 2-3 min.

3.3.5 Column Chromatography

The following column conditions were employed in running the column chromatography. Gradient elution technique was employed. Glass column (100 × 3 cm) with sintered disc at the bottom of various dimensions was selected. Silica gel, 60 – 120 mesh size 34 was used as the stationary phase with wet slurry method of packing. The sample was applied using dry loading method (Cannell, 1998), which was dissolved in small amount of suitable organic solvent, and it was then mixed with a small quantity of silica gel, dried, evaporated and then loaded on top of the column. n-hexane, DCM and ethyl acetate were used 100% and in combination with varying polarity as solvent elution.

3.3.6 Column chromatographic separation of n -hexane fraction (CSH).

The n-hexane fraction (7.1 g) was chromatographed over silica gel (60 – 120 mesh size) packed column of dimension (100 x 3 cm), the column was eluted with 100% n-hexane then followed by hexane: ethyl acetate in the ratio of 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40 to

30:70. Seventy one fractions (100 ml each) were collected and were pooled together based on their TLC profiles to give five major sub-fractions (B1, B2, B3, B4 and Y1). Sub-fraction B2 was packed in a burette using hexane: ethyl acetate (8:2) as TLC mobile phase solvent system showed one major spot with R_f value 0.55 which was coded CSH_5.

3.3.7 Column chromatographic separation of dichloromethane fraction (CSD).

The dichloromethane fraction (5.5 g) was chromatographed over silica gel (60 – 120 mesh size) packed column of dimension (100 x 3 cm), the column was eluted with 100% DCM then followed by hexane : ethyl acetate in the ratio of 1:9, 2:8, 2:8, 2:8 2:8, 2:8, 3:7, 4:6 and 5:5. Eleven fractions (100 ml each) were collected, based on their TLC profiles they showed similar spot. Fraction CSD_5 showed one spot with R_f 0.78.

3.4 Melting Point Determination

The melting points of the isolated compounds were determined using Stuart automatic melting point/SMP40 obtained from Department of Chemistry, Faculty of Physical Sciences, Ahmadu University Zaria.

3.5 Spectral Analysis

The NMR spectra was obtained on a Bruker AVANCE (600 MHz for ^{1}H and ^{13}C) spectrometer from Department of Pure and Applied Chemistry, University of Strathclyde, Scotland-United Kingdom. Dimethyl sulphoxide (DMSO) was used as solvent. Chemical shift values (δ) were recorded in parts per million (ppm) relative to appropriate internal solvent standard. Infrared (IR) absorption spectra were recorded using an infrared spectrophotometer Agilent Technology Cary 630 FT-IR.

3.6.Antibacterial Susceptibility Test

3.6.1 Test organisms

The microorganisms used in this study include *Staphylococcus aureus,* Methicillin resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis,Salmonella typhi, Shigella dysenteriae,* Vancomycin resistant *Enterococci* (VRE) and *Escherichia coli.*

3.6.2 Preparation of culture media

Culture media was be prepared by suspension of 38 g of the medium in one litre of distilled water with frequent agitation and boiled to aid dissolution, autoclaved at 121^{o}C for 15 min. The cultured media was then cooled to 45^{o}C and 25 ml of the sterilized media was aseptically dispensed into sterilized petri dishes. The media was covered and allowed to solidify.

3.6.3 Preparation of the plants extracts for antimicrobial screening

Stock solution of the plant extracts was prepared by dissolving 1.0 g of the n-hexane, dichloromethane, ethyl acetate and methanol extracts and the isolated compounds in 10 ml of dimethyl sulphoxide (DMSO) to obtain a concentration of 100 mg/ml. From the stock solution, serial dilution of the extract and isolated compounds of concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml was obtained for the extracts.

3.6.4 Susceptibility test

The antimicrobial screening was carried out using agar diffusion method as described by Nostro *et al*. (2000). The antimicrobial activities of the n-hexane, dichloromethane, ethyl acetate and methanol extractwere determined using stock concentration of 100 mg/ml. 0.1 ml standard inoculums of the test organisms was uniformly streaked unto freshly prepared Mueller Hinton Agar plates with the aid of a sterile swab stick. Using a sterile cork borer (6 mm) 5 appropriately labelled wells was punched into each agar plate. Thereafter, 0.2 ml of the appropriate extract concentration was placed in each well and allow to diffuse into the agar. An extra plate was also streak with the innocula and Ciprofloxacin as standard (5 µg/disc) was placed in it. The plates

will be incubated at 37^{o}C for 24 h. The antimicrobial activities were expressed as diameter of zones of inhibition produced by the plant extracts and reported in millimetre (mm).

3.6.5 Minimum inhibitory concentration (MIC)

The antimicrobial activities of all the extracts, isolated compounds and control drugs (Ciprofloxacin and Erythromycin) was used for the studies. The agar well diffusion method of Nostro *et al.* (2000) was used in the antimicrobial screening of the extract while disc dilution will be used for the positive control.

Minimum inhibitory concentrations of the extract and fractions were carried out on the microorganisms that was sensitive to the four crude extracts and the isolated bioactive compounds was done using broth dilution method. Nutrient broth was prepared according to the manufacturer's instructions as recommended by Nostro *et al*. (2000).The 10 ml solution was dispensed into test tubes and was sterilized at 121^{o}C for 15 min and the broth was allowed to cool. McFarland's turbidity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared and dispensed into test tube and the test microorganism then be inoculated into the normal saline, incubation will be done at 37^{o}C for 6 h, dilution of the microorganism in the normal saline was performed until the turbidity marched that of the McFarland's by visual comparison at this point the microorganism had a concentration of about 1.5×10^{8} CFU/ml. Two-fold serial dilution of the extract in the broth was performed to obtain the concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml. The initial concentration was obtained by dissolving the extract (0.1 g) in the extracts in the broth (10 ml), 0.1 ml of the standard inoculated into the different concentrations of the extracts in the broth, was inoculated at 37^{o}C for 24 h after which the test tubes was observed for turbidity (growth).

The lowest concentrations of the extract which shows no turbidity was recorded as the minimum inhibitory concentrations.

3.6.6 Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration of the extract and fractions was carried out to confirm whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar was prepared according to the manufacturer's instruction, boiled to dissolve and was sterilized at 121^{o}C for 15 min. The media was cooled to 45^{o}C and the medium (20 ml) was poured into sterile Petri dishes, the plates was covered and allowed to cooled and solidified. The contents of the MIC in the serial dilution was inoculated on to the media, the media was incubated at 37^{o}C for 24 h for the bacteria after which the plates were observed for colonies growth. The MBC is the plate with lowest concentrations of the extract without colony growth.

CHAPTER FOUR

4.0 Results

4.1 Extraction and Phytochemical Screening

The extraction of *Centaurea senegalensis*(980 g) by simple maceration using methanol(7.5 L) recovery 274.6 g representing 5.9 % extracted. The partition of the methanol crude extract (160.0 g) using hexane, DCM and ethyl acetate yielded 26.31 g (16.40%), 20.50 g (12.81%) and 14.45 g (9.03%) respectively.Phytochemical screening on the methanol extract and solvent fraction showed the presence of alkaloid, glycosides and terpenes present in all fractions and extract. Flavonoids was absent in hexane fraction. Tannins and saponins were not detected in both hexane and DCM fractions. Anthraquinone was absent in all fractions and crude extract. The results are presented in Table 4.1.

Table 4.1: Percentage Extracted and Phytochemical Screening of *C. senegalensis.*

	CSH	CSD	CSE	CSM
Percentage extracted (%)	16.40	12.81	9.03	5.90
Alkaloids	+	+	+	+
Flavonoids	-	+	+	+
Glycosides	+	+	+	+
Saponins	-	-	+	+
Terpenes	+	+	+	+
Anthraquinone	-	-	-	-
Tannins	-	-	+	+

+ = present, - = absent, CSH = hexane fraction, CSD = dichloromethane fraction, CSE = ethyl acetate fraction and CSM = methanol extract.

4.2 Chromatographic Purification of the Isolates

4.2.1 Purification of hexane fraction

Table 4.2: Column Chromatographic Separation of the CSH Fraction of *C. senegalensis*.

Collections	Sub-fraction	Eluents	Number of spot(s)	R_f Value
1-4	B_1	H 100%	-	-
5-8	B_2	H : E 95:5	3	(0.43, 0.55, 0.68)
9-13	B_3	H : E 90:10	3	(0.43, 0.55, 0.68)
14-18	B_4	H : E 85:15	4	(0.43, 0.55, 0.68, 0.69)
19-22	B_5	H : E 80:20	4	(0.43, 0.55, 0.68, 0.69)
23-27	B_6	H : E 75:25	4	(0.43, 0.55, 0.68, 0.69)
28- 32	B_7	H : E 70:30	2	(0.43, 0.55)
33-37	B_8	H : E 65:35	2	(0.43, 0.55)
38-42	B_9	H : E 60:40	2	(0.43, 0.55)
43-47	B_{10}	H : E 55:45	2	(0.43, 0.55)
48-52	**B_{11}**	**H : E 50:50**	**2**	(0.43, 0.55)
53-57	B_{12}	H : E 45:55	2	(0.43, 0.55)
58-62	B_{13}	H : E 40:60	3	(0.43, 0.55, 0.68)
63-65	B_{14}	H : E 35:65	3	(0.43, 0.55, 0.68)
66-67	B_{15}	H : E 30:70	3	(0.43, 0.55, 0.68)
68-70	B_{16}	H : E 30:70	2	(0.55, 0.68)
71-72	**B_{17}**	**H : E 30:70**	**2**	(0.55, 0.68)
73-74	B_{18}	H : E 30:70	0	-

Key: H= hexane, E= ethyl acetate

Table 4.3:Repeated Column Chromatographic Separation of Column Fraction

Collections	Sub-fraction	Eluenting solvent	Number of Spot(s)
1-3	CSH_1	100 H	-
4-5	CSH_2	H:E 95:5	-
6-7	CSH_3	H:E 90:10	-
8-10	CSH_4	H:E 85:15	1
11-12	**CSH_5**	**H:E 80:20**	**1**
13-15	CSH_6	H:E 79:21	1
16-17	CSH_7	H:E 78:22	1

Key: H = hexane, E = ethyl acetate, CSH = *Centaurea senegalensis* hexane

4.2.2 Purification of dichloromethane fraction

Table 4.4: Column Chromatographic Separation of the CSD fraction of *C. senegalensis*.

Eluent	Fraction	Eluent solvent	Number of spot
1	CSD_1	D 100%	3
2-4	CSD_2	H : E 10:90	3
5-8	CSD_3	H : E 20:80	3
9-10	CSD_4	H : E 20:80	3
11-12	**CSD_5**	**H : E 20:80**	1
13-14	CSD_6	H : E 20:80	1
15-16	CSD_7	H : E 20:80	2
17-18	CSD_8	H : E 30:70	2
19-20	CSD_9	H : E 40:60	2
21-22	CSD_{10}	H : E 50:50	2

Key: H = hexane, E = ethyl acetate, CSD = *Centaurea senegalensis* dichloromethane.

4.2.3: Thin-Layer Chromatography (TLC) of the Isolated Compounds.

Table 4.5: TLC profile of isolated Compounds: CSH_5 and CSD_5

Compound code	Solvent system	Observed spot(s)	Colour of spot on heating	R_f value	Melting point (^oC)	Weight of isolated compound (g)
CSH_5	HEX:EA (80:20)	1	Purple	0.55	231 - 233	2.10
CSD_5	HEX: EA (20:80)	1	Yellow	0.78	185 - 187	1.90

Compound CSH_5 and CSD_5 were soluble in methanol, ethyl acetate and dichloromethane, HEX = hexane and EA = ethyl acetate. CSH = *Centaurea senegalensis*hexane, CSD = *Centaurea senegalensis* dichloromethane

4.3 One Dimensional (1D) NMR Analysis of Compound CSH_5

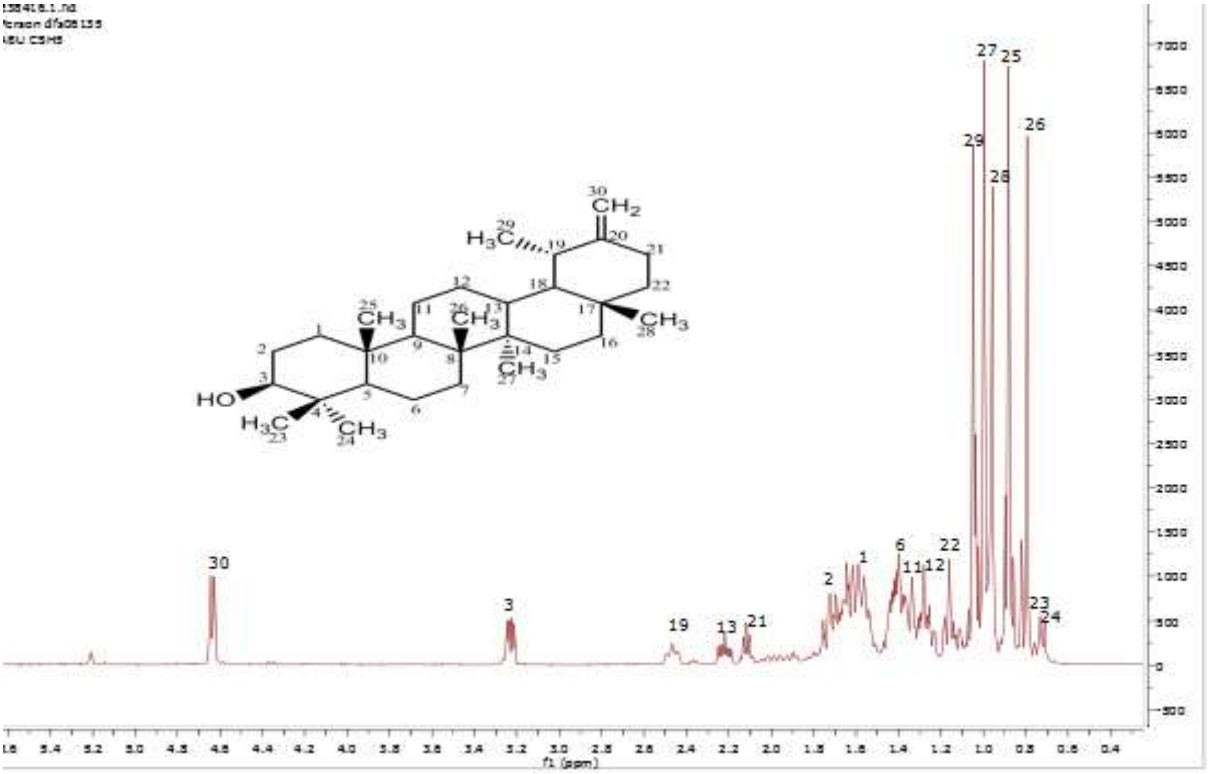


Figure 4.1: ^{1}H-NMR Spectrum of Compound CSH_5

4.4 The DEPT Spectrum of Compound CSH_5

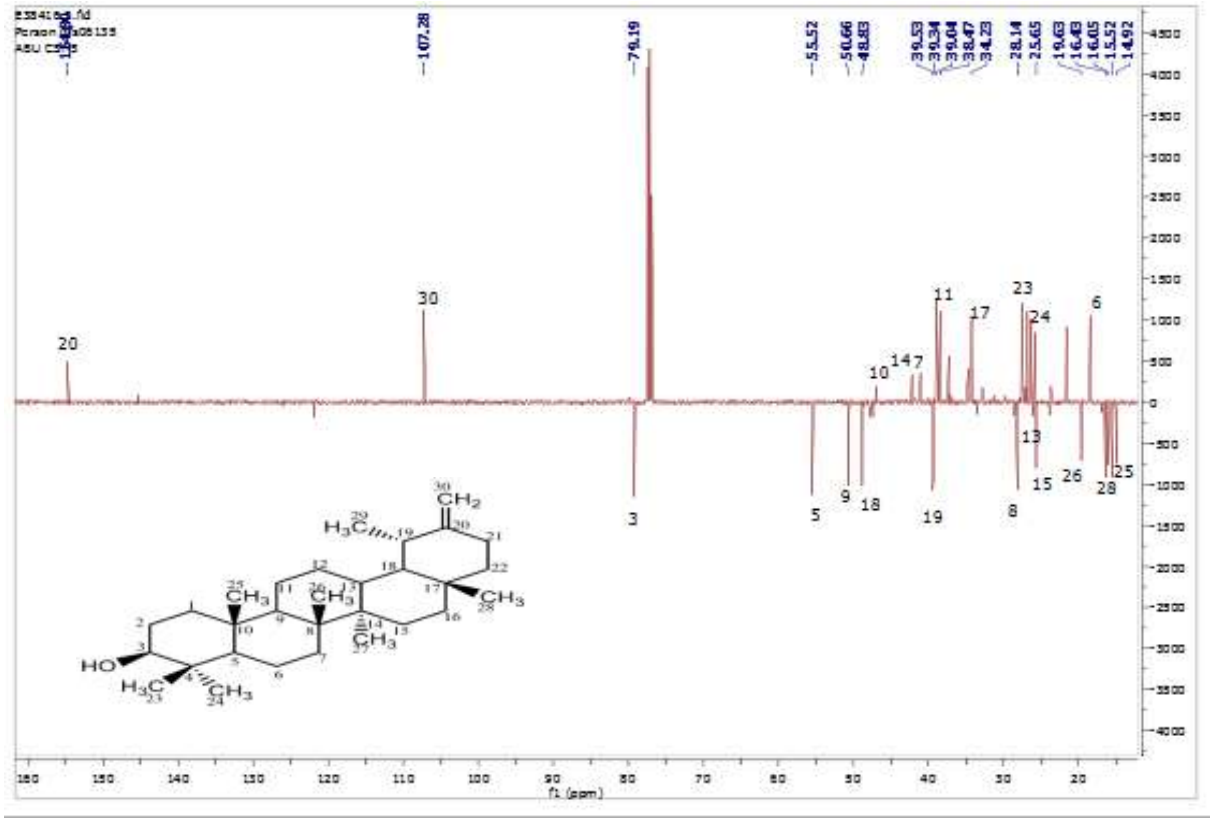


Figure 4.2: DEPT Spectrum of Compound CSH_5

4.5 Correlation Spectrum (COSY) of Compound CSH_5

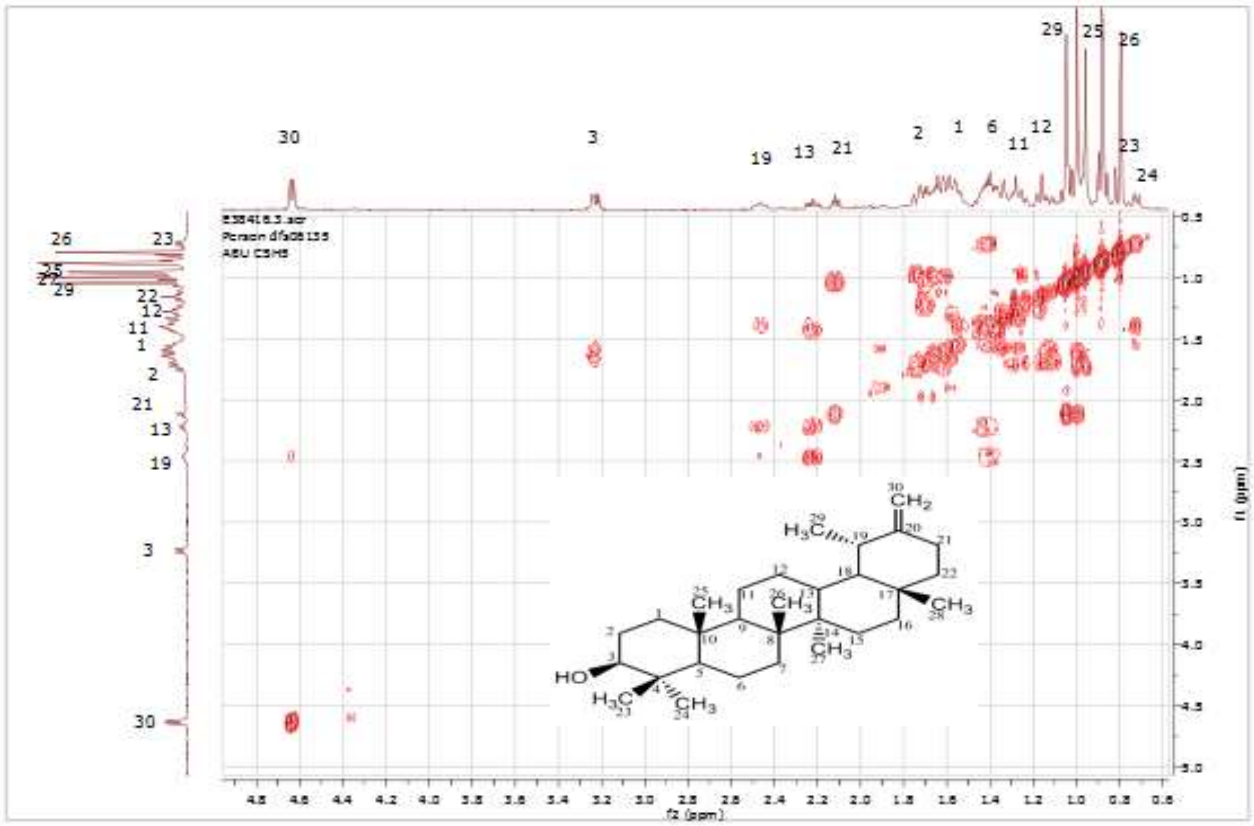


Figure 4.3: COSY Spectrum of Compound CSH_5

4.6 The Heteronuclear Multiple Bond Correlation (HMBC) of Compound (CSH_5)

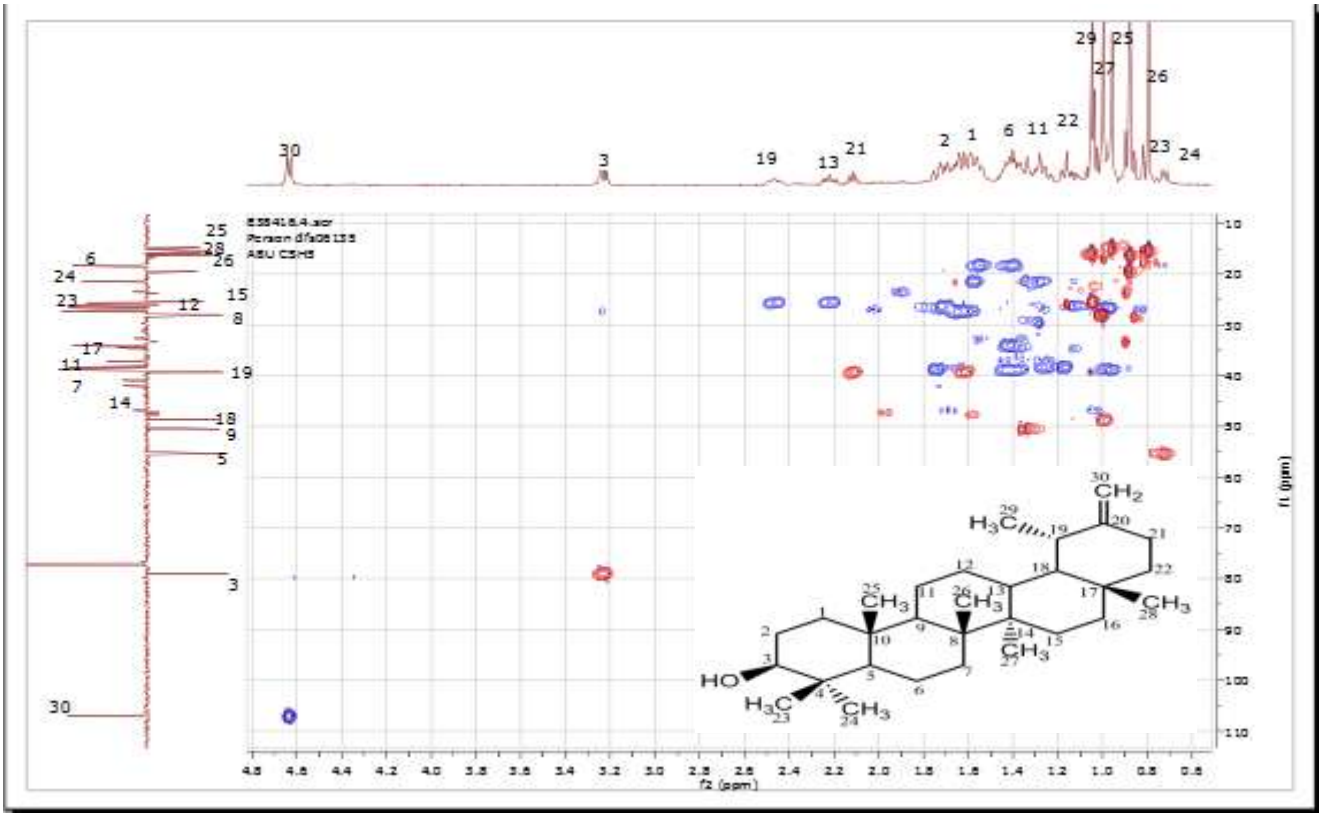


Figure4.4: HMBC Spectrum of Compound CSH_5

4.7 The Heteronuclear Simple Quantum Correlation (HSQC) of Compound CSH_5.

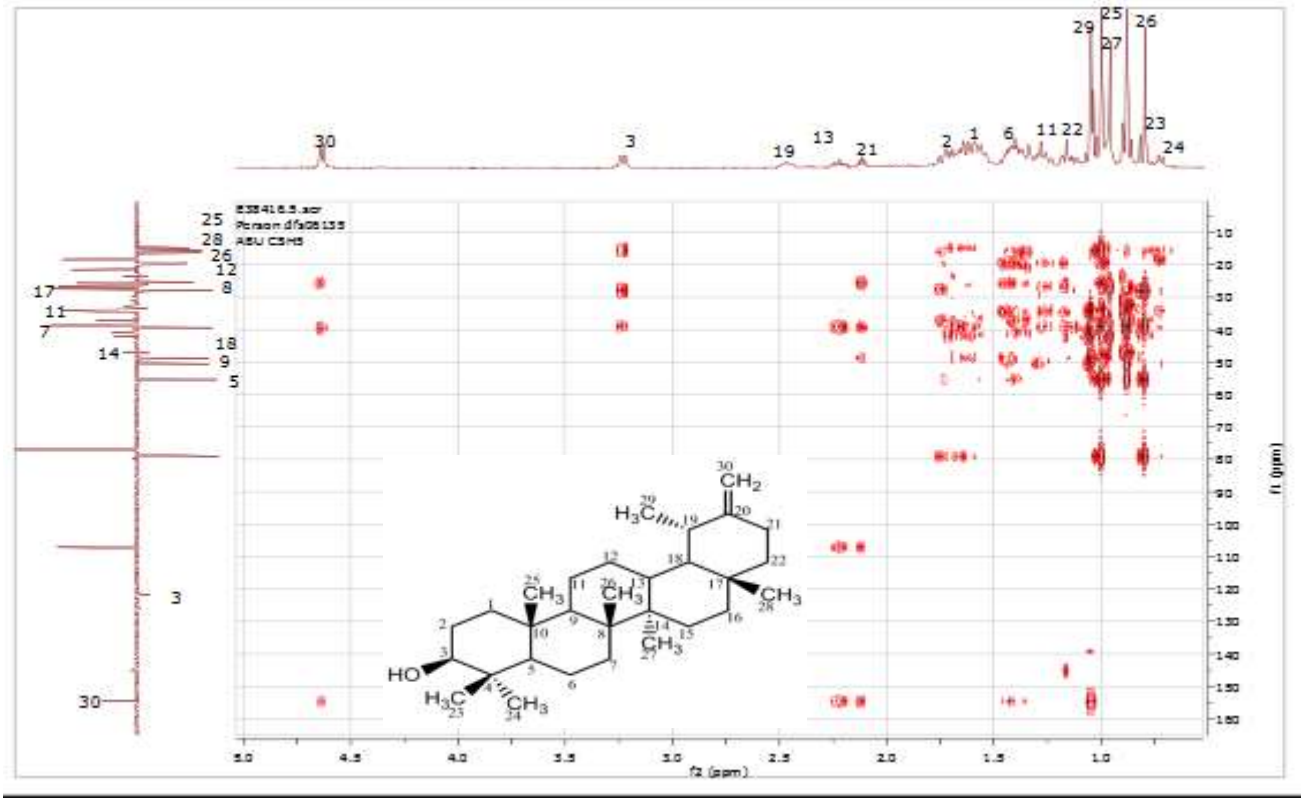


Figure4.5: HSQC Spectrum of Compound CSH_5

4.8: Infrared Spectral Analysis of Compound CSH_5

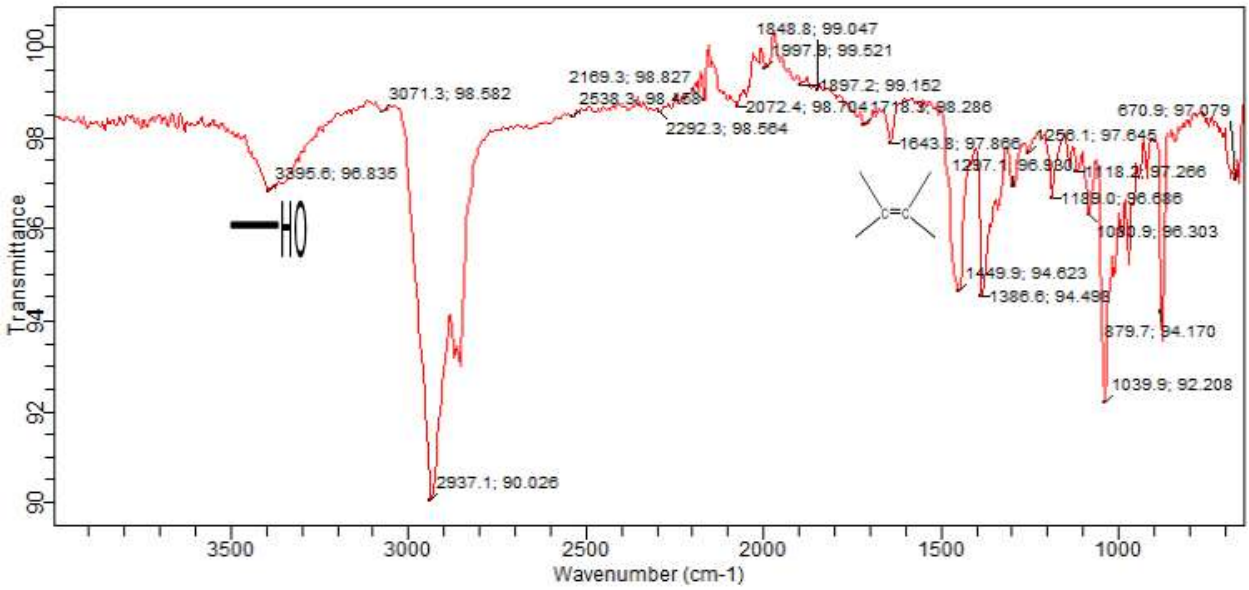


Figure 4.6: FT-IR Spectrum Compound of CSH_5

4.10 One Dimensional (1D) NMR Analysis of Compound CSD_5

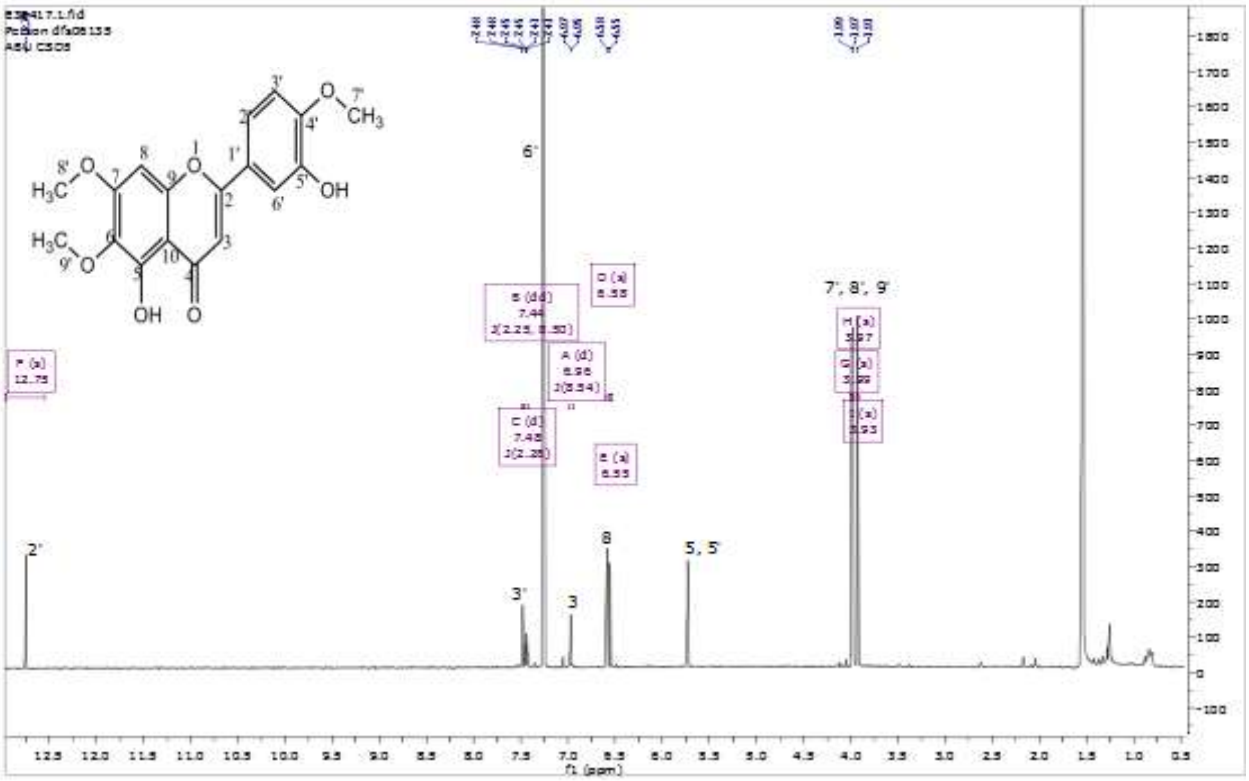


Fig 4.8: ^{1}H-NMR Spectrum of compound CSD_5

4.11: The ^{13}C-NMR Spectrum of Compound CSD_5

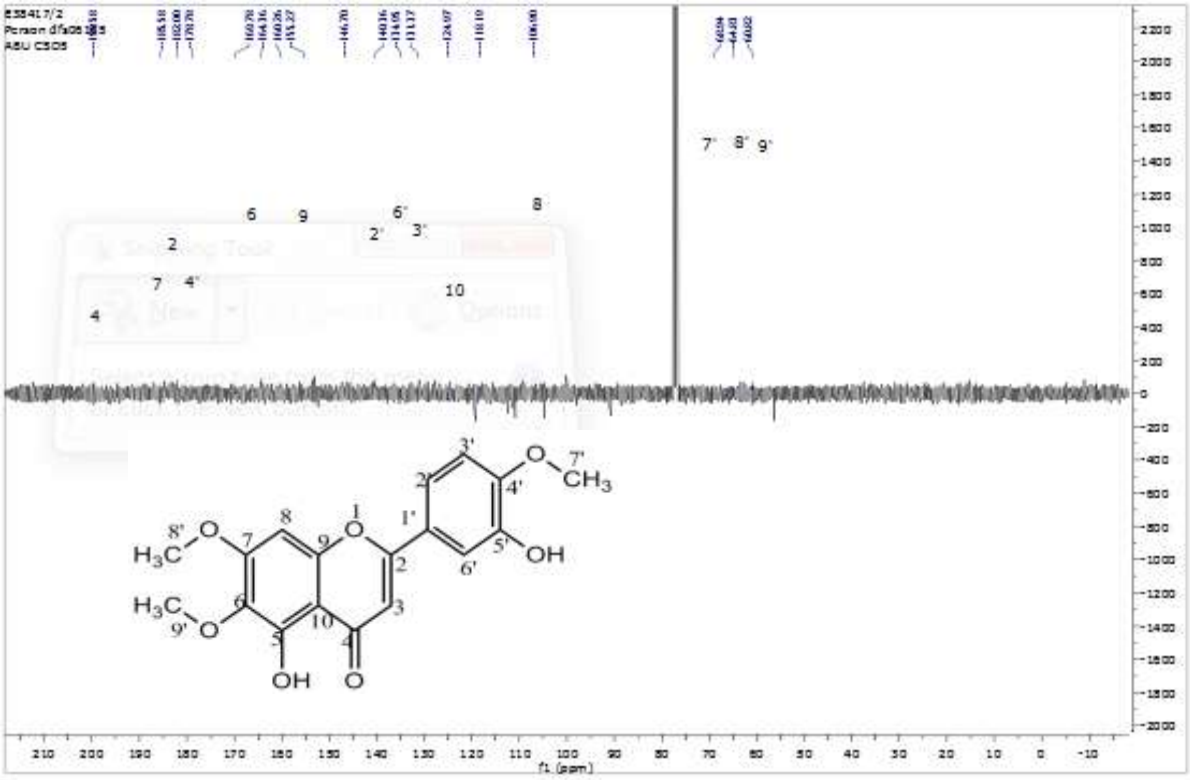


Figure4.9: ^{13}C-NMR spectrum of compound CSD_5

4.12 Correlation Cpectrum (COSY) of Compound CSD_5

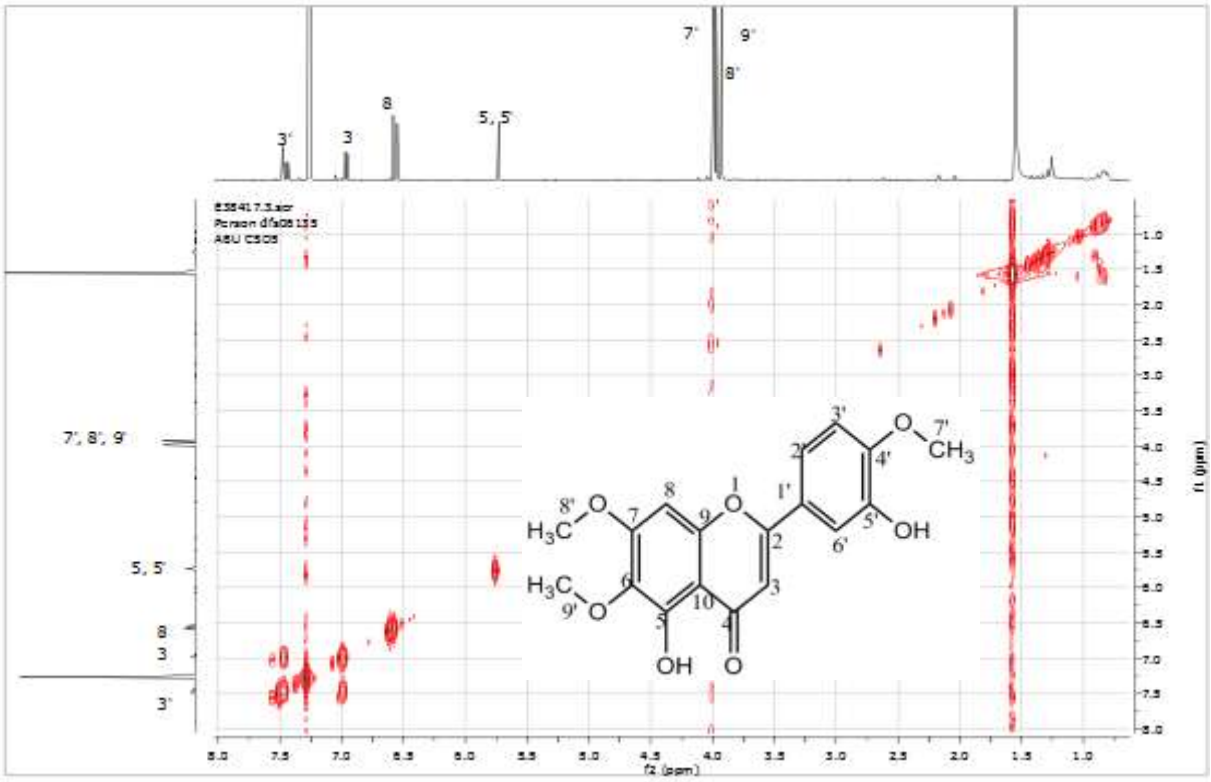


Figure4.10: ^{1}H-^{1}H-COSY Spectrum of compound CSD_5

4.13 The Heteronuclear Multiple Bond Correlation (HMBC) of Compound (CSD_5)

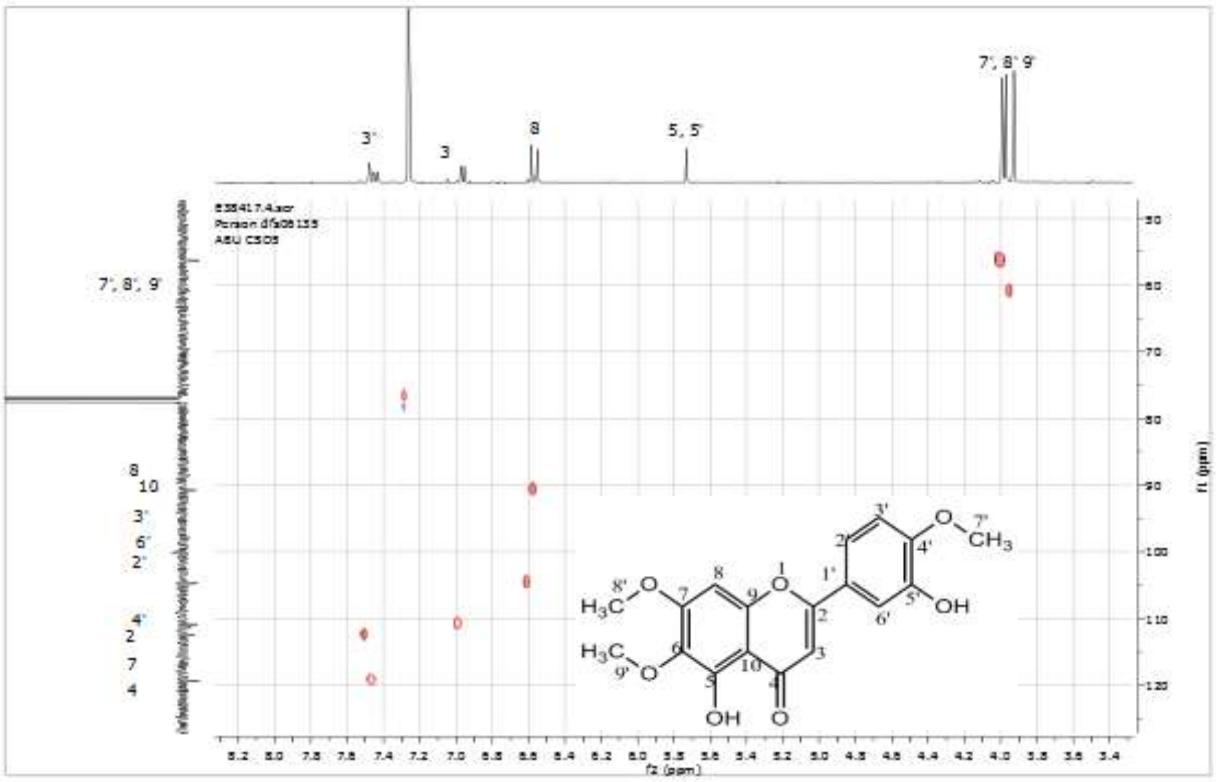


Figure 4.11: HMBC Spectrum of compound CSD_5

4.14 The Heteronuclear Simple Quantum Correlation (HSQC) of Compound CSD_5.

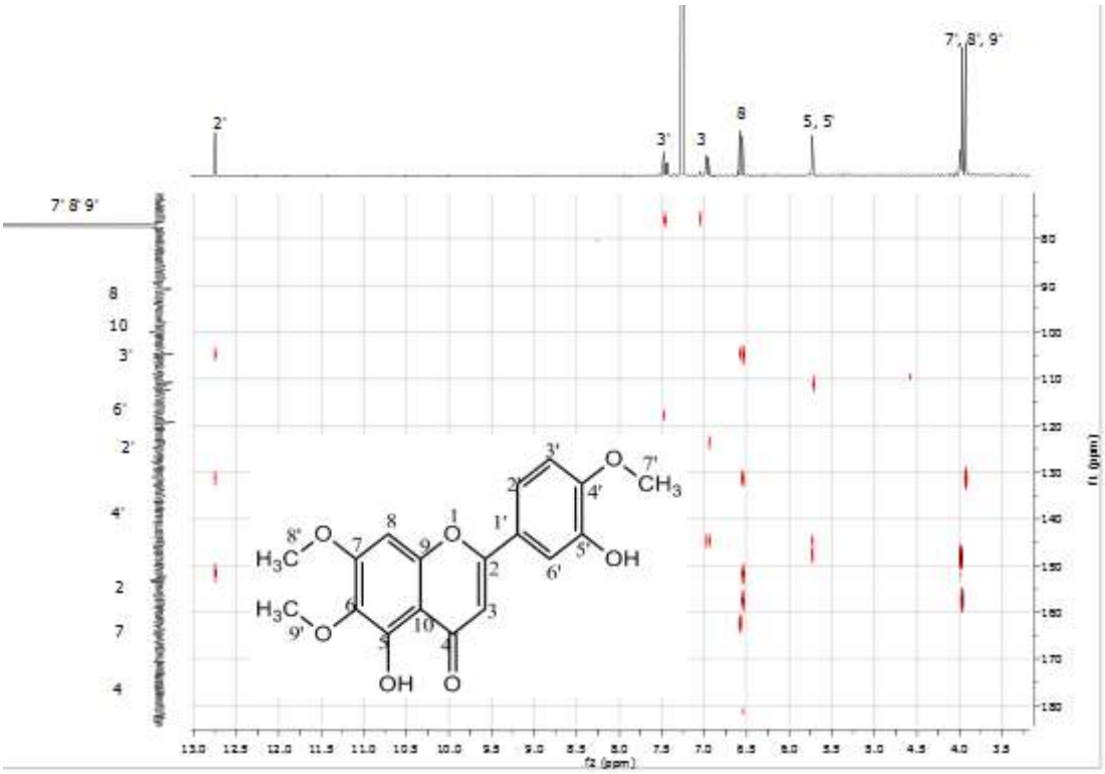


Figure 4.12: HSQC Spectrum of compound CSD_5

4.15: Infrared Spectral Analysis of Compound CSD_5

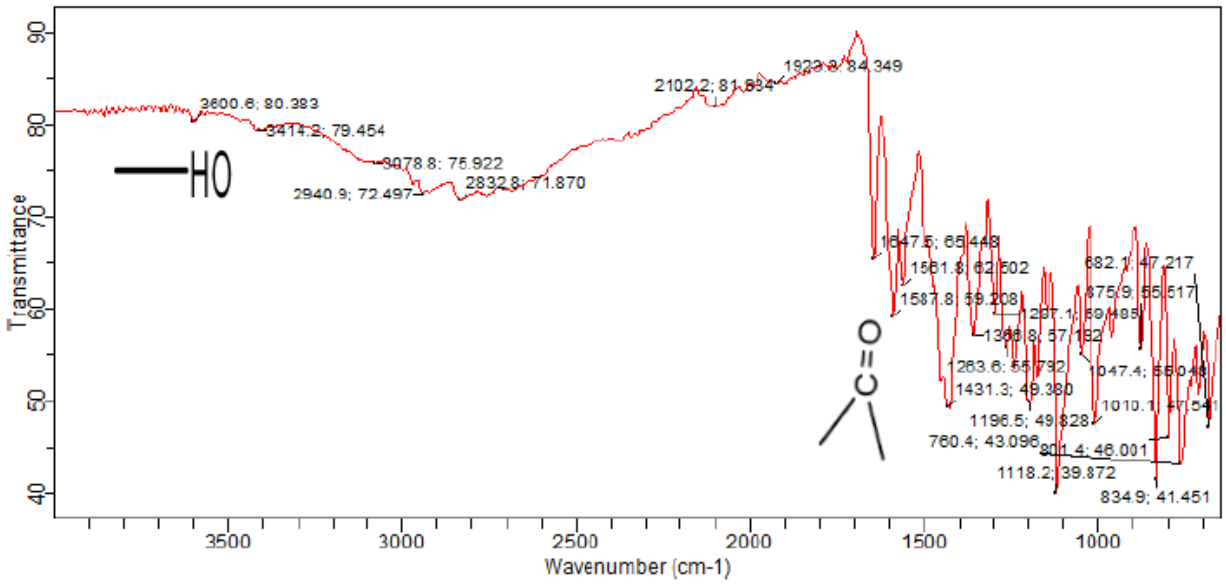


Figure 4.13: FT-IR Spectrum of compound CSD_5

4.16 Antimicrobial Studies

4.16.1: The susceptibility test of the fractions of *Centaurea senegalensis* and standard antibiotics against selected microorganisms.

The antibacterial activities (zones of inhibition) of extract /fractions CSH, CSD, CSE and CSM of the extract at 200 mg/mlconcentration and the reference drugs Ciprofloxacin and Erythromycin at a concentration of 100 μg/ml on the test microorganisms measured in millimeter (mm) are shown in Table 4.8

Table 4.8:Antibacterial activity of *C. senegalensis* extract and fractions

Pathogens	Zone of inhibition (mm)					
	CSH	CSD	CSE	CSM	CFX	ERT
S. aureus	13	13	21	16	33	23
MRSA	18	28	26	23	24	31
VRE	27	28	24	26	31	-
B. subtilis	16	11	17	20	-	29
E. coli	15	25	31	20	24	33
S. typhii	17	26	28	22	-	27
S. dysenteriea	21	28	24	28	33	-

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant *enterococci*, CFX = Ciprofloxacin, ERT = Erythromycin, CSH = hexane fraction, CSD = dichloromethane fraction, CSE = ethyl acetate fraction and CSM = methanol extract.

4.16.2 Zone of Inhibition of compounds CSH_5 and CSD_5

Table 4.9: Antibacterial activity of isolated compounds

Microorganism	Zone of inhibition (mm)			
	CSH_5	CSD_5	CFX	ERT
S. aureus	13	15	33	23
MRSA	10	13	24	31
VRE	10	10	31	-
B. subtilis	10	11	-	29
E. coli	11	15	24	33
S. typhii	10	15	-	27
S. dysenteriea	11	13	33	-

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant*enterococci,* CSH_5 and CSD_5

4.16.3 Minimum Inhibitory Concentration Determination

The minimum inhibitory concentration of extract/fractions CSH, CSD, CSE and CSM at 200 $mgmL^{-1}$ concentration on the test microorganisms revealed the smallest concentration that can inhibit the microbes. All fractions/extract showed 12.5 mg/mlas the MIC against VRE as presented in Table 4.10.

Table 4.10: Minimum Inhibitory Concentration of extract/fractions of *Centaurea senegalensis.*

Microorganisms	MIC (mg/ml)			
	CSH	CSD	CSE	CSM
S. aureus	50	50	25	50
MRSA	25	12.5	12.5	25
VRE	12.5	12.5	12.5	12.5
B. subtilis	50	100	50	25
E. coli	50	12.5	12.5	25
S. typhi	12.5	12.5	12.5	25
S. dysenteriae	25	12.5	12.5	12.5

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant*enterococci*.

4.16.4 The Minimum Inhibitory Concentration of Isolated Compounds

Table 4.11: Minimum Inhibitory Concentration of Isolated compounds from *Centaurea senegalensis.*

Microorganism	MIC (mg/ml)	
	CSH_5	CSD_5
S. aureus	-	-
MRSA	50	25
VRE	100	50
B. subtilis	100	100
E. coli	100	100
S. typhii	100	50
S. dysenteriea	100	25

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant*enterococci*, CSH_5= *Centaurea senegalensis* hexane and CSD_5 = *Centaurea senegalensis*dichloromethane.

4.16.5 Minimum Bactericidal ConcentrationDetermination

The minimum bactericidal concentration of extract/fractions CSH, CSD, CSE and CSM on the test microorganisms reveals the smallest concentration that can kill the microbes. All fractions/extract showed 25 mg/mlas the MBC against S. *dysenteriae* as presented in Table 4.14.

Table 4.12: The Minimum Bactericidal Concentrationof Extract/Fractions

Microorganisms	MBC (mg/ml)			
	CSH	CSD	CSE	CSM
S. aureus	100	100	50	100
MRSA	50	25	25	50
VRE	25	25	25	25
B. subtilis	100	-	100	50
E. coli	100	50	50	50
S. typhii	50	50	50	100
S. dysenteriae	25	25	25	25

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant*enterococci*.

4.16.6 The Minimum Bactericidal Concentration of Isolated Compound

Table 4.13: Minimum Bactericidal Concentration of Isolated Compound

MicroorganismsMBC (mg/ml)		
	CSH_5	CSD_5
S. aureus	-	-
MRSA	100	50
VRE	-	100
B. subtilis	-	-
E. coli	-	-
S. typhii	-	100
S. dysenteriae	-	50

(-) No inhibition zone, MRSA = Methicillin resistant *Staphylococcus aureus*, VRE = Vancomycin resistant*enterococci*, CSH_5 = Taraxasterol and CSD_5= Eupatorin.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Extraction and Phytochemical Screening.

The result of the percentage extracted using n-hexane, dichloromethane, ethyl acetate fractions and methanol extract as reported in Table 4.1 shows that the n-hexane fraction had the highest yield followed by dichloromethane fraction, ethyl acetate fraction.

The result of the phytochemical analysis of the ethyl acetate fraction and methanol extract revealed the presence of some secondary metabolite (alkaloids, flavonoids, glycosides, saponins, terpenes and tannins) as an evidence of its antimicrobial properties. Specifically, *C. paui*, *C. tweediei* and *C. sulphurea*have been reported to have antimicrobial properties (Bruno *et al*., 2013).

5.2 Antibacterial Activity of Extracts/Fractions

The zones of inhibition (11-31 mm) (Table 4.8), the minimum inhibitory concentration (MIC) (Table 4.10) and minimum bactericidal concentration (MBC) (Table 4.12) of hexane, DCM, ethyl acetate and methanol extract/fractions of plant*C. senegalensis* showed substantive activities on seven organisms tested against (*S. aureus*, MRSA, VRE, *B. subtilis*, *E.coli*, *S.dysenteriae and S. typhi*). This result is similar to those reported on two members of the *Centaurea* genus *C. scabiosa* and *C. nigra*(Kenny *et al*., 2014). The MIC studies of the n-hexane fraction from *C. senegalensis* inhibited the growth of *S. aureus, B. subtilis* and *E. coli* at 50 mg/ml while *S. typhi* and VRE were inhibited at 12.5mg/mlfor the MBC analysis, the best sensitivity 25mg/mlwas

observed. for *Shigella dysentiae* and VRE while 50 mg/mlwas recorded for *Salmonella typhii* and MRSA.

The MIC studies of DCM fraction of *C. senegalensis* (Table 4.10) inhibited the growth of *S. typhii*, *E. coli*, MRSA, *S. dysentariea* and VRE at a concentration 12.5 mg/mlwhile MBC at (25-100 mg/ml) with *E.coli* and *S. typhii* recorded 50 mg/mlexcept for hexane fraction and methanol extract respectively.

The MIC studies of ethyl acetate fraction from *C. senegalensis* (Table 4.10) which inhibited the growth of *S. typhii*, *E. coli*, MRSA, *Shigella dysenteriea*, VRE at a concentration of 12.5 mg/ml. This is similar to the observed inhibition for the dichloromethane fraction.*S. aureus* was at 50 mg/ml and *Bacillus subtilis* at 50 mg/mlfor hexane and ethyl acetate fractions with a corresponding MBC at 25-50 mg/mlwhile *B. subtilis* is at 100 mg/ml.

The MIC studies of crude methanol extract from *C. senegalensis* (Table 4.10) inhibited the growth of *S. typhii*, *B. subtilis, E. coli* and MRSA at a concentration 25 mg/ml with a corresponding MBC at 50 mg/mland 25 mg/ml for *S. dysenteriea* and VRE.

These results when compared to the MIC results of ethanolic extracts of *C. scabiosa* and *C. nigra*reported by Kenny *et al*. (2014) against *S.aureus*, *B. cereus* and MRSA (187.5-365 µg/ml) showed activity.

5.3 Antibacterial Activities of CSH_5 and CSD_5

The sensitivity of several compounds isolated from *Centaurea* genus has been determined. Most of the isolated compounds showed varying sensitivity depending on the extractants in this study. The zone of inhibition of compound CSH_5 (10-13 mm) (Table 4.9) showed some activity. The sensitivity of compound CSH_5 was performed using the disc diffusion method and was active against gram positives bacteria such as *S. aureus,* MRSA, VRE (5 - 10 mm) and gram negative bacteria such as *B.subtilis, S. typhii, S. dysenteriae* and *E. coli* (11-10 mm) respectively which is lower when compared to inhibition zones of taraxasterol isolated from *Strobilanthes callosus* against *E.coli* (17.0 mm) and *S. aureus* (14.0 mm) by Singh *et al*. (2002). The zone of inhibition of compound CSD_5 (10 –15 mm) (Table 4.9),showed inhibition of gram-negative bacteria (*E.coli and S. typhii*) with MIC (50 – 100 mg/ml) in (Table 4.11) and MBC range of (25–50 mg/ml) in (Table 4.12). The findings is also in accordance with that of Bouteghae *et al*. (2013).

Taraxasterol belongs to class terpenoids that has been reported to possess antitumor properties (Jamshieed *et al*., 2010), it shows anti-inflammatory activities in albino rats as well as anticancer activities against sarcoma 180 cell line in mice as reported from the fruit of *Dregas volubilis* by Biswas *et al*. (2009). Taraxasterol is also known to possess antimicrobial and anti-allergic potentials (Liu *et al*., 2013).

Eupatorin is a flavone which has been reported to have potentials such as a meaningful anti-inflammatory property, it inhibits tumor necrosis factor production(Laavola *et al*., 2012). Eupatorin inhibited lipopolysaccharide (LFS) induced activation of transcription 1α and it also inhibited carrageenan induced paw inflammation in mice (Laavola *et al*., 2012). Also, eupatorin isolated from *C. tomorosii,C. soskae* and *C. galicicae* was reported to have been used to treat

various disorders such as lack of appetite, dyspeptic troubles, liver diseases and biliousness (Tesevic *et al*., 2014).

5.4 Structural Elucidation of CSH_5

5.4.1 Structural Elucidation of Compound CSH_5

The suggested structure of the isolated compound is presented as Figure 4.7 based on NMR and IR spectra data. The assignments were presented in the Table 4.6 with a comparison with literature data for the proposed compound.

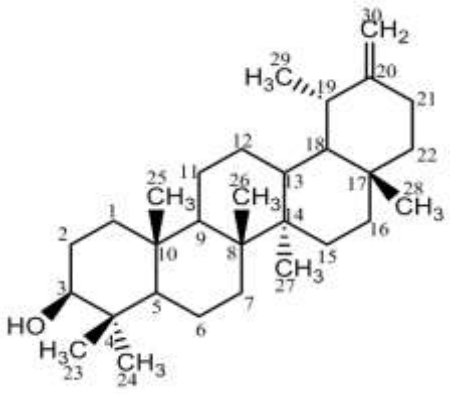


Figure 5.4: Taraxasterol

Table 4.6: Comparison of ^{13}C-NMR and ^{1}H-NMR of CSH_5data with literature values

Position	$\delta_{C\ EXPT}$	$\delta_{H\ EXPT}$ J ($^{r}H_2$)	δ_{CRef}	$\delta_{H\ Ref}$ J ($^{r}H_2$)	DEPT
1	38.70	1.64,0.94	38.71	1.64,0.92	CH_2
2	27.50	1.75,1.67	27.53	1.75,1.67	CH_2
3	79.50	3.22(11.46,4.7)	79.12	4.70(11.8,4.9)	CH
4	39.40	-	38.98	-	C
5	55.50	0.79	55.49	0.78	CH
6	18.50	1.52,1.27	18.41	1.46,1.35	CH_2
7	34.0	1.56,1.27	34.20	1.35,1.35	CH_2
8	41.25	-	41.02	-	C
9	50.66	1.39	50.61	1.33	CH
10	37.5	-	37.26	-	C
11	21.75	1.52,1.27	21.56	1.48,1.21	CH_2
12	25.65	1.64,1.36	25.60	1.65,1.08	CH_2
13	39.04	2.22(15.37,5.5)	38.42	1.56(12.2,3.3)	CH
14	42.50	-	42.15	-	C
15	26.50	1.64	26.78	1.65,0.94	CH_2
16	39.40	1.14	39.26	1.26,1.17	CH_2
17	34.23	-	34.50	-	C
18	48.83	0.72	48.82	0.99	CH
19	39.53	2.24(6.9)	39.49	2.15(6.8)	CH
20	154.80	-	154.79	-	C
21	25.60	2.47	25.60	2.48,2.23	CH_2
22	38.47	1.36	38.43	1.41,1.41	CH_2
23	28.12	0.99	28.08	0.91	CH_3
24	16.08	0.99	16.01	0.91	CH_3
25	15.75	0.88	15.46	0.86	CH_3
26	16.43	1.00	16.38	0.99	CH_3
27	14.92	0.96	14.86	0.96	CH_3
28	14.92	0.94	14.86	0.96	CH_3
29	25.90	1.05	25.92	1.08	CH_3
30	107.28	4.69	107.23	4.79	CH_2

(Ref* = Sabri et al., 2017)

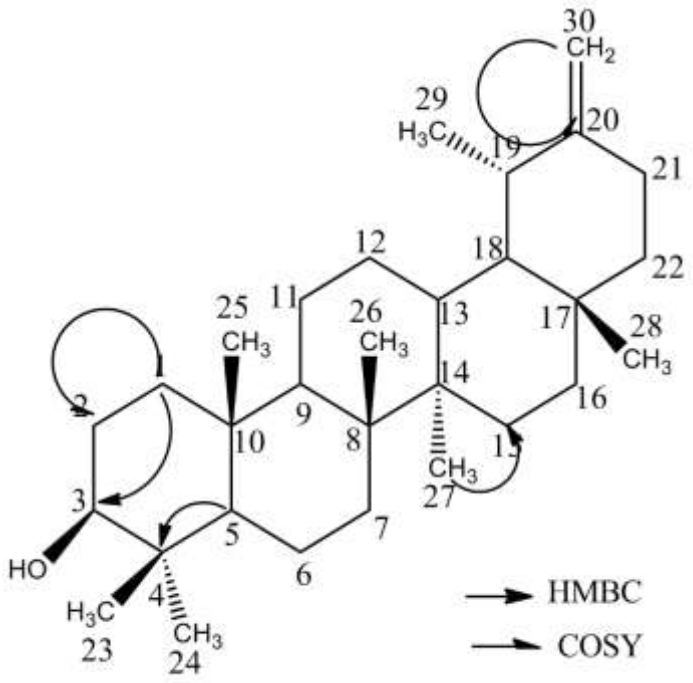


Figure 5.0: 2D Correlation of compound CSH_5

Compound CSH_5 was proposed to be taraxasterol ($C_{30}H_{50}O$, 426.7 g/mol) using 1D and 2D NMR Spectroscopic analyses. Taraxasterol with five cyclohexane rings, seven methyl, one exomethylene group on C-20 and an alcohol on C-3.Signals in the proton (Figure 4.1) and ^{13}C-NMR spectra (Figure 4.2) were assigned completely (Table 4.6) using 1 and 2 D NMR methods (COSY, HSQC and HMBC).The proton spectrum contains signals for three proton at weak field region: two broad singlets at δ 4.64 ppm and δ 4.60 ppm and one doublet of doublet at δ 3.23 ppm based on cross-peaks in the HSQC (Figure 4.5), the first two protons belongs to C-30 with chemical shift δ 107.28 ppm. The doublet of doublet at δ 3.23ppm corresponds with the proton on C-2 (δ 79.19 ppm) of ring A. The large vicinal spin-spin coupling constant $^{2}J_{HH}$ = 11.46 Hz is consistent with an axial position for H-2. Cross-peaks in the COSY spectrum (Figure 4.3) with

H-1 and H-1’ at δ 1.75 ppm and δ 1.67 ppm respectively correspond with the proton cross-peaks with H-6 and H-6’ at δ 1.64 ppm and δ 0.94 ppm are observed in COSY spectrum in addition. Atoms C-1 and C-6 have δ 38.70 andδ 27.50 ppm respectively. Cross- peaks with C-1 and a quaternary C atom at 39.34 ppm (C-3) are found in the HMBC spectrum (Figure 4.4) for H-2.

The proton with δ 0.99 ppm in the HSQC corresponds with signals for C atom with δ 28.12 and δ 16.08 ppm of the gem - dimethyl C-24 and C-28 their protons also correlate with tertiary (δ 55.50 ppm) and quaternary (δ 39.34 ppm) C atom C-4 and C-3 respectively. Signals for H-7 and H-8 of ring B are assigned using their correlations with H-4 in the COSY spectra. Their characteristic atoms C-7 (δ 18.50 ppm) and C-8 (δ 34.0 ppm) are found using the HSQC spectra proton H-6 and H-4 and protons of the methyl with δ 0.88 ppm gives correlations in the HMBC spectrum with a quaternary C atom with δ 37.5 ppm. These are C-5 and methyl C-24. This assumption is also confirmed by HMBC spectra in which correlation of the C-24 proton with C-4, C-6 and methane C-10 with δ 50.66 ppm are observed in the HSQC spectrum, the last signal correlates with H-10 with δ 1.39 ppm which in turn correlates with C-5 and yet another quaternary C atom as shown in (Figure 4.4).

The doublet for methyl protons H-30 at δ 1.05 ppm in the HSQC spectrum corresponds with the C-atom at δ 25.90 ppm (C-30). Corresponding cross-peaks in the HMBC spectrum between methyl proton in ring D at δ 1.05 ppm and C atoms at δ 39.53 ppm (C-22) and δ 154.80 ppm (C-21) in addition to C-30 at δ 25.90 ppm and a proton at δ 2.42 ppm (H-22) confirm that the assignment is consistent (Table 4.6).

Hence, signals for protons and C atoms were assigned completely using one- and two-dimensional proton and ^{13}C-NMR experiments. The spectra information agreed with the report of Khalilov *et al*. (2003) as shown in the Table 4.6.The ^{13}C-NMR spectrum of compound CSH_5

showed thirty (30) major recognizable carbon signals, seven methyl groups at δ 28.12 (C-23), 16.08 (C-24), 15.75 (C-25), 16.43 (C-26), 14.92 (C-27), 19.63 (C-28), 25.90 (C-29) and α hydroxyl bearing carbon 79.19 (C-3) (Table 4.6). It also showed some recognizable signals at δ 154.80 and 107.28 ppm which is assignable to the double bond at C-20 and C-30 respectively. In addition, 11 methylene groups, 6 methyne and 6 quaternary carbon atoms were observed. δ 154.80 and δ 107.28 which are C-20, C-30 and consistent flow of methyl group from C-23 to C-29 were characteristic peaks for Taraxasterol type of skeleton (Sabri *et al*., 2017).

The biological activities shown by compound CSH_5 may be as a result of presence of hydroxyl group at C-3 and the exo-methylene double bond at C-30 as reported by De falco *et al*. (2015).

5.5 Structural Elucidation of CSD_5

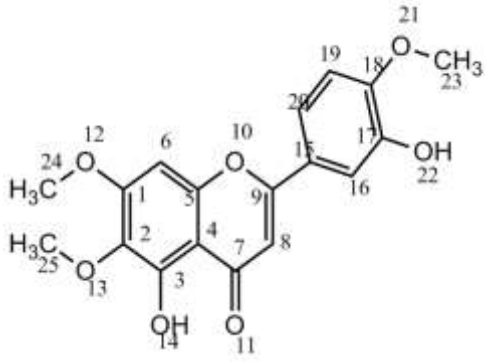


Figure 4.14: Eupatorin

Compound CSD_5 was proposed to be Eupatorin (3',5-dihydroxy-4',6,7-trimethoxyflavone) ($C_{18}H_{16}O_7$, 344 g/mol) using 1D-NMR and 2D-NMR spectroscopic analyses. Eupatorin with two benzene rings with heterocyclic diene, three methoxy, one ketone on C-4 and two hydroxyls on C-5 and C-5' (Table 4.7). The proton spectrum contains signals of methyl which are downfield due to electronegative influence of oxygen at δ 3.93 ppm δ 3.99 ppm and δ 3.93 ppm. The two-hydroxyl proton with singlets at δ 6.58 ppm and δ 6.55 ppm (Figure 4.8). The benzylic region showed signals with doublet of doublet at δ 7.44 ppm with coupling constant (*J* 8.50,2.25), δ

6.96 ppm (*J* 8.54). A singlet at δ 12.75 ppm at C-3, it was deshielded probably due to electronegative effect of oxygen.

The hydroxyl groups at special sites of compound CSD_5 like the aromatic ring at C-5 and C-5', oxygen containing heterocyclic moieties such as position 1 usually enhance the activity for Eupatorin (Gu and Weng, 2001). However, methylation of the active hydroxyl group at C-7', C-8' and C-9'generally decrease the activity. These functional groups present can inhibit the cytoplasmic membrane of the selected bacterial and reduce their energy metabolism (Gu and Weng, 2001), as demonstrated with gram negative (*E. coli* and *S. typhii*) and gram positive (*S. aureus* and MRSA) which were weaker than the selected antibiotics (Table 4.7) (Khalilov *et al*., 2003).

Table 4.7: Comparison of ^{13}C-NMR and ^{1}H-NMR of Compound CSD_5 Data with Literature values.

Position	$\delta_{C\ EXPT}$	$\delta_{H\ EXPT}$J ($^{r}H_2$)	δ_C Ref	δ_H RefJ ($^{r}H_2$)	DEPT
1					
2	162.3	-	164.6	-	C
3	100.0	6.97	104.22	6.82	CH
4	185.0	-	183.01	-	C
5	154.2	12.75	153.40	12.91	C
6	135.2	3.99	132.44	3.73	C
7	157.1	3.93	159.54	3.93	C
8	90.90	6.96(8.54)	92.41	6.91	CH
9	152.1	-	152.91	-	C
10	105	-	105	-	C
1’	124.0	-	128.82	-	C
2’	117.4	7.48(2.26)	119.64	7.60	CH
3’	113.1	7.44(8.50,2.25)			CH
4’	151.2	3.77			C
5’	146.3	6.55			C
6’		6.58		7.47	
7’	61.5		60.90		OCH_3
8’	57.1		57.3		OCH_3
9’	56.70		56.70		OCH_3

(Ref* = Khalilov et al., 2003)

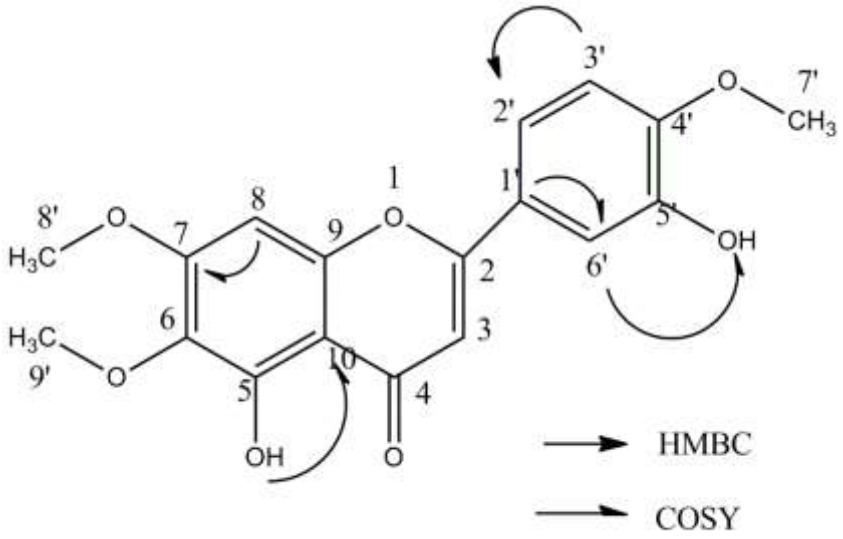


Figure 5.1: COSY and HSQC Correlation of Compound CSD_5

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Preliminary phytochemical screening of crude methanol extract, n-hexane, dichloromethane and ethyl acetate fractions of*Centaurea senegalensis* revealed the presence of alkaloids, flavonoids, glycosides, tannins and terpenes. Column chromatography on the n-hexane and dichloromethane fractions of methanol extract led to the isolation and characterization of Taraxasterol (CSH_5) and Eupatorin (CSD_5) respectively. The antimicrobial activity of methanol extract,fractions and the isolated compounds showed broad spectrum activity against selected bacteria, with low MIC and MBC of extract and fractions indicating potential antibacterial efficacy of the plant *Centaurea senegalensis*.

6.2 Conclusion

Secondary metabolites from plants have provided useful guide to drug discovery and hence the phytochemical studies on *Centaurea senegalensis* has resulted to isolation and characterization of triterpenoid-Taraxasterol and flavonoid-Eupatorin. These compounds are reported for the first time from *C. senegalensis*, to the best of our knowledge. The search for chemotherapeutic agents from *C. senegalensis* extract, fractions and isolated compounds against drug resistant bacteria have shown some activities. This may provide the rationale for use of *C. senegalensis* in traditional medicine against infectious diseases.

6.3 Recommendations

(i) The isolation and characterization of bioactive compounds from *Centaurea senegalensis*may be carried outon polar extract such as methanol.

(ii) Phytochemical analysis through HPLC quantification may be carried out to elucidate the major components of *C. senegalensis*.

(iii) The combination of isolated compounds with standard antibiotics may be studied to establish the interaction as synergistic or antagonistic effect against resistant bacteria.

6.4 Contribution to knowledge

(i) The isolation and characterization of Taraxasterol and Eupatorin from *Centaurea senegalensis* is reported for the first time from the plant.

(ii) The antibacterial evaluation of *Centaurea senegalensis* methanol extract, hexane, dichloromethane, ethyl acetate fractions and isolated compounds have shown broad spectrum activity, indicating potential therapeutic properties with MIC of hexane fraction with 12.5 mg/mlon MRSA and VRE while the MIC of isolated compounds on MRSA and *S. dysenteriea* showed (25 mg/ml) and MBC (50 mg/ml).

REFERENCES

Ahmad, N., Shinwari, Z. K., Hussain, J., Ahmad, I., Perveen, R. and Rehman, S. (2015). Phytochemical screening and biological activities of different parts of *Centaurea montana*. *International Journal of Plant Science and Ecology,1*(6): 237-240.

Akkal, S., Benayache, F., Benayache, S. and Jay, M. (1997). Flavonoids from *Centaurea incana* (Asteraceae). *Biochemical Systematics and Ecology*.92:19 -63

Albayrak S., Aksoy A., Sagdic O.,and Hamzaoglu E. (2010). Compositions, antioxidant and antimicrobial activities of *Helichrysum* (Asteraceae) species collected from Turkey. *Food Chemistry*, 119: 114-122.

Aqil M., Khan I. Z. and Diamari G. A. (1998). Flavonoids from *Centaurea senegalensis* DC (Compositae). *Chemical Society of Ethiopia*, 12(2): 177-180.

Baharfar, R., Khalilzadeh, M. A., Gheibi, S., Jazayeri, O., Azimia, R. and Tajbakhsha, M. (2009). Antioxidant and antibacterial activities of the methanolic extract of *Centaurea zuvandica Sosn*. *Iranian Journal of Organic Chemistry*, *3*: 172-177.

Bastos, M.M.S.M., Kijjoa, A., Pinto, M.M.M., (1994). Constituents of *Centaurea ornate* ssp. *ornata*. *Fitoterapia,* 65: 191.

Bennett, M.H. (1994**).** The phytoalexin response of lettuce to challenge by Botrytis cinerea, *Bremia lactucae*, and *Pseudomonas syringae* pv. *phaseolicola*. *Physiological Molecular Plant Pathology,* 44: 321–333.

Bentamane, A., Benayache, S., Creche, J., Petit, G., Bermejo-Barrera, J., Leon, F., Benayache, F., (2005). A new guaianolide and other sesquiterpene lactones from *Centaurea acaulis*. *Biochemical Systematics and Ecology*, 33: 1061–1065.

Biswas, M., Biswas, K., Ghosh, A. K.and Haldar, P. K. (2009). A pentacyclic triterpenoid possessing anti-inflammatory activity from the fruits of *Dregea volubilis*. *Pharmacognosy Magazine*, *5*(19), 64.

Bruno, M., Bancheva, S., Rosselli, S., and Maggio A. (2013) Sesquiterpenoids in subtribe Centaureinae (Cass.) Dumort (tribe Cardueae, Asteraceae): Distribution, ^{13}C-NMR spectral data and biological properties. *Phytochemistry*, 95:19 -93

Burkill, H. M. (1985). The Useful Plants for West Tropical Africa, Royal Botanical Garden: London; Pp. 738-759.

Cannel, R. J. P. (1998). *Natural Products Isolation*. Humana Press Incorporation; New Jersey, U.S.A. Pp. 165-208.

Cardona, M. L., Fernández, I., Pedro, J. R. and Pérez, B. (1991). Sesquiterpene lactones and flavonoids from *Centaurea aspera*. *Phytochemistry*, *30*(7), 2331-2333.

Chadwick, M., Trewin, H., Gawthrop, F. and Wagstaff, C. (2013). Sesquiterpenoids lactones: benefits to plants and people. *International Journal of Molecular Sciences*, 14: 12780–12805.

Chen, A.Y. and Chen, Y.C. (2013). A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. *Food Chemistry*, 138:2099–2107.

Chicca, A., Tebano, M., Adinolfi, B., Ertugul, K., Flamini, G. and Neri, P. (2011). Antiprolilferative activity of aguerin B and a new rare nor-guaianolide lactone isolated from the aerial parts of *Centaurea deflexa*. *European Journal of Medicinal Chemistry*, 46: 3066 – 3070.

Chou, R.H., Hsieh, S.C., Yu, Y.L., Huang, M.H., Huang, Y.C., and Hsieh, Y.H., (2013). Fisetin inhibits migration and invasion of human cervical cancer cells by down-regulating urokinase plasminogen activator expression through suppressing the p38 MAPK-dependent signaling pathway. *PLOS One*, 8: 1–12.

Cowan, M. M. (1999). Plant products as antimicrobial agents. Clinical microbiology resistance in malaria: Reasons and Solutions. *Phytotherapy Reviews*, 24(7): 1104-1106.

Cragg, G. M., Newman, D. J. and Snader, K. M. (1997) Natural products in drug discovery and development. *Journal of Natural Products*, 60: 52-60.

Cushnie, T.P.T. and Lamb, A.J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, 38: 99–107.

De Falco, B., Incerti, G., Amato, M. and Lanzotti, V. (2015). Artichoke: botanical, agronomical, phytochemical, and pharmacological overview. *Phytochemistry Reviews*, *14*(6), 993-1018.

Demir, S., Karaalp, C. and Bedir, E. (2017). Specialized metabolites from the aerial parts of *Centaurea polyclada* DC. *Phytochemistry*, *143*: 12-18.

Dewick, P. M. (2006). *Essentials of Organic Chemistry: for Students of Pharmacy, Medicinal Chemistry and Biological chemistry*. John Wiley and Sons. Pp. 123-145.

Dutta K. N., Chetia P., Lahkar, S. and Das, S. (2013). Herbal plants used as diuretics: A comprehensive review. *Journal of Pharmacological, Chemical and Biological Sciences*, 2(1):27-32.

Gangwal, A. (2013). Neuropharmacological effects of triterpenoids. *Phytopharmacology*, *4*(4): 354-372.

Gonzalez, A. G., Bermejo, J., Breton, J. L., Massanet, G. M. and Triana, J. (1974). Chlorohyssopifolin C, D, E and vahlenin, four new sesquiterpene lactones from *Centaurea hyssopifolia*. *Phytochemistry*, *13*(7): 1193-1197.

Grotewold E., (2006). The Science of flavonoids, Springer Science Business Media, Inc, New York. Pp. 239-267.

Gu, L. and Weng, X. (2001). Antioxidant activity and components of *Salvia plebeia* R. Br.—a Chinese herb. *Food Chemistry, 73*(3), 299-305.

Herz, A., Albus, K., Metys, J., Schubert, P. and Teschemacher, H. (1970). On the central sites for the antinociceptive action of morphine and fentanyl. *Neuropharmacology*, *9*(6), 539-551.

Hutchison, J. (1963) Flora of West Tropical Africa, 2nd ed., Vol II, crown Agents, Millbank: London; Pp. 291-292.

Jamshieed, S., Das, S., Sharma, M. P. and Srivastava, P. S. (2010). Difference in *in vitro* response and esculin content in two populations of *Taraxacum officinale* Weber. *Physiology Molecular Biology of Plants*, *16*(4), 353-358.

Kamanzi, K., Raynaud, J. andVoirin, B. (1983). The C-glycosyl flavonoids in flowers of *Centaurea melitensis* L,(Compositae).[French]. *Plantes Medicinales et Phytotherapie*.

Khalilov, L. M., Khalilova, A. Z., Shakurova, E. R., Nuriev, I. F., Kachala, V. V., Shashkov, A. S. and Dzhemilev, U. M. (2003). PMR and 13 C NMR spectra of biologically active compounds. XII. Taraxasterol and its acetate from the aerial part of *Onopordum acanthium*. *Chemistry of Natural Compounds*, *39*(3), 285-288.

Kenny, O., Smyth, T. J., Walsh, D., Kelleher, C. T., Hewage, C. M., and Brunton, N. P. (2014). Investigating the potential of under-utilised plants from the Asteraceae family as a source of natural antimicrobial and antioxidant extracts. *Food Chemistry, 161*: 79-86.

Kisiel, W. and Davie, E. W. (1975). Isolation and characterization of bovine factor VII. *Biochemistry*, *14*(22), 4928-4934.

Koes, R. E., Quattrocchio, F. and Mol, J. N. (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioAssays*, *16*(2): 123-132.

Kumarasamy, Y., Cox, P. J., Jaspars, M., Nahar, L. and Sarker, S. D. (2002). Screening seeds of Scottish plants for antibacterial activity. *Journal of Ethnopharmacology*, *83*(1-2), 73-77.

Laavola, M., Nieminen, R., Yam, M. F., Sadikun, A., Asmawi, M. Z., Basir, R.and Moilanen, E. (2012). Flavonoids eupatorin and sinensetin present in leaves inhibit inflammatory gene expression and STAT1 activation. *Planta Medica*, *78*(08): 779-786.

Newman, D. J., Cragg, G. M. and Snader, K. M. (2000). The role of natural product chemistry in drug discovery. *Journal of Natural Product Chemistry*, *17*: 215-234.

Nostro, A., Germano, M.P., Diiangelo, V., Marino, A. and Cannattelli, M.A. (2002). *Letters of Applied Microbiology*. 30: 379-381.

Picman, A. K. (1986). Biological activities of sesquiterpene lactones. *Biochemical Systematics and Ecology*, *14*(3), 255-281.

Robenson, M. M. and Zhang, X. (2011). *The world medicine situation 2011, traditional medicine: global situation, issues and challenges.* Available from http://www.oalib.com/references/8513138.

Sabri, N. A., Ahmad, W. Y. W., Abdullah, N. S. and Ibrahim, N. (2017). The chemical constituents from the fruits of *Johannesteijsmannia altifrons* and antibacterial activity. *Malaysian Journal of Analytical Sciences, 21*(2): 304-311.

Sepulveda, S., Delhvi, S., Koch, B. and Zilliken, F. (1994). Constituents of *Centaurea chilensis*. *Fitoterapia*, 65: 88–89.

Silva, G.L., Lee, I. and Doglas, K.A. (1998). Special problems with extraction of plants. *In Natural Product Isolation. Humana Press Publishers New Jersey*. Pp. 356-357.

Singh, B., Sahu, P. M., and Sharma, M. K. (2002). Anti-inflammatory and antimicrobial activities of triterpenoids from *Strobilanthes callosus* Nees. *Phytomedicine*, *9*(4), 355-359.

Smolinski M. S., Hamburg M. A., and Lederberg J. (2003). Microbial threats to health: Emergency, detection, and response. Washington, DC: Institute of Medicine, National Academies Press. 203-210.

Sofowora, E. A., (1993). Introduction to Medicinal Plants and Traditional Medicine. Spectrum Books Limited; 2: 8-76.

Tesevic, V., Aljancic, I., Milosavljevic, S., Vajs, V., Đorđevic, I., Jadranin, M. and Matevski, V. (2014). Secondary metabolites of three endemic *Centaurea* species. *Journal of the Serbian Chemical Society, 79*(11): 1355-1362.

Trease, G.E. and Evans, W.O. (2009). Trease and Evans *Pharmacognosy*. 16th Edition. New York: Sauders Elsevier Limited. Pp. 104-262.

Wedge, D.E., Galindo, J.C.G. and Macias, F.A. (2000). Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. *Phytochemistry*, 53: 747–757.

Wei, H. X., Gao, W. Y., Tian, Y. J., Guan, Y. K., Huang, M. H., and Cheng, D. L. (1997). New eudesmane sesquiterpene and thiophene derivatives from the roots of *Rhaponticum uniflorum*. *Pharmazie, 52*(3): 245-247.

World Health Organization. (2013) Traditional medicine strategy 2014-2023. Geneva: World Health Organization.

Yesilada, E., Gürbüz, I., Bedir, E., Tatli, I. and Khan, I. A. (2004). Isolation of anti-ulcerogenic sesquiterpene lactones from *Centaurea solstitialis*. ssp. solstitialis through bioassay-guided fractionation procedures in rats. *Journal of Ethnopharmacology*, *95*(2-3): 213-219.

Yin, M. C., Lin, M. C., Mong, M. C. and Lin, C. Y. (2012). Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. *Journal of Agricultural and Food Chemistry*, *60*(31): 7697-7701.

Zelik, B., Gurbuz, I., Karaoglu, T. and Yesilada, E. (2009).Antiviral and antimicrobial activities of three sesquiterpene lactones from *Centaurea solstitialis* L.ssp. *Solstitialis*. *Microbiological Research*, 164: 545-552.

Zulak, K. G. and Bohlmann, J. (2010). Terpenoid biosynthesis and specialized vascular cells of conifer defense. *Journal of Integrative Plant Biology*, *52*(1): 86-97.