PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF ETHANOL EXTRACT OF *CHROZOPHORA SENEGALENSIS* (LAM) A. JUSS

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AHMADU BELLO UNIVERSITY, ZARIA

NIGERIA

MARCH, 2016

DECLARATION

I declare that the work in this thesis entitled" Phytochemical and Biological studies of Ethanol Extract of *Chrozophora senegalensis* (LAM) A. JUSS (Euphorbiaceae)" 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 list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Isyaku Ibrahim	----------	----------
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE ETHANOL EXTRACT OF *CHROZOPHORA SENEGALENSIS* (LAM) A JUSS" by ISYAKU IBRAHIM meets the regulations governing the award of the Doctorate Degree of Pharmaceutical Chemistry of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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Abstract

The ethanol extract of root of *Chrozophora senegalensis* use in ethanomedicine to treat diarrhea, rheumatism, taeniasis, stomach ache, inflammation and veneral diseases was subjected to Phytochemical and Biological studies. Phytochemical analysis was carried out using standard phytochemical procedures which revealed the presence of flavonoids, tannins, alkaloids, saponins. Chromatographic separation of the ethylacetate fraction using silica gel led to the isolation of three compounds, identified as 5-hydroxy – 2- (4-hydroxyphenol) – 4 – chromenone – 7- glucose (Apigenin – 7- O- glucoside); 5,7 – dihydroxy-2- (4 hydroxyphenyl) – 4 – chromenone – 3 – glucose (Aromadendrin -3-O-glucoside) and 2 – (3,4 – dihydroxy phenyl) – 3,5,7 – trihydroxy 4 H – chromen 4 – one (Quercetin) using spectrocopic analysis. The mean number of writhing in acetic acid induced-writhing in mice is 18.8 with normal saline while at dose of 600, 300 and 150mg/kg of ethanol extract has 1.3,1.7 and 3.7 respectively. The standard drug (ketoprofen, 10mg/kg) has 0.6 mean number of writhing.The Anti-infammatory studies showed that at 135min, the mean volume of paw oedema is 0.43 with normal saline which is control while the standard which is ketoprofen (10mg/kg) has 0.29. The ethanol extract at dose of 600, 300 and 150mg/kg has mean volume of 0.26,0.30 and 0.31 respectively.The extract in analgesic and anti-inflammatory studies showed statistically significant activities when compared to control at p less than 0.05 limits. Biological studies conducted on the ethyl acetate include: acute toxicity using Lorke method and was found to be 2154.1 mg/kg. Analgesic using acetic acid induced writhing, anti-inflammation using carrageenan induced paw oedema test in rats. Antimicrobial was conducted using Agar well diffusion and tube dilution technique with *Escherichia coli*, *Staphylococcus aureaus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albica* and *Trichopyton mentaagrophyte* as the test organisms with inhibition zones ranging from 15-22mm at 20mg/ml while at concentration of 50mg/ml gave range of 19-25 mm. Ethyl

acetate extract (20mg/ml) produce zones of inhibition which ranges from 15-22 mm but using 50mg/ml the zone of inhibition ranges between 19-25 mm. The n-butanol fraction also has activity against the microorganisms tested at 20mg/ml with zone of inhibition between 15-23 mm and at 50mg/ml the range was found to be between 16-26mm. The MIC of ethanol extract, ethylacetate and n-butanol soluble fraction ranges from 0.06 to 0.25mg/ml while the MBC ranges from 0.25 to 1.0mg/ml. This study showed that the plant has analgesic, anti-inflammatory and antimicrobial activity. The study validate the ethnomedicinal claim of the plant

TABLE OF CONTENTS

Title	Page
Title page	i
Declaration	ii
Certification	iii
Acknowledgement	iv
Abstract	v
Table of content	vii
List of Tables.	xi
List of Figures	xiii
Abbreviations	xiv

CHAPTER ONE: INTRODUCTION

1.0 Introduction 1

1.1 Statement of Research problem 11

1.2 Justification of Research 11

1.3 Aim 12

1.4 Objective 12

1.5 Hypothesis 13

CHAPTER TWO:

2.0 Literature Review of the plant 14

2.1 Ethnomedicinal uses of *Chrozophora senegalensis* 14

2.2 Phytochemistry and Pharmacological activities of *Chrozophora senegalensis* 15

CHAPTER THREE: 17

3.0 Materials and Methods 17

3.1 Materials 17

3.1.1 Chemicals and equipments 17

3.1.2 Identification, collection and preparation of plantmaterial 17

3.1.3 Standard drugs and sample used 17

3.1.4 Animal used 17

3.1.5 Microorganisms 18

3.2 Methods 18

3.2.1 Extraction procedure 18

3.2.2 Preliminary Phytochemical screening 18

3.2.3 Thin layer Chromatography (TLC) 21

3.2.4 Column Chromatography (CC) 22

3.2.5 Spectroscopic Analysis of Isolated Compounds 24

3.3 Pharmacological Studies 24

3.3.1 Acute toxicity studies (LD_{50}) in mice 24

3.3.2 Analgesic studies of ethanol extract 24

3.3.3 Effect of the ethanol extract on carrageenan induced oedema in rats 25

3.3.4 Determination of zone of inhibition 26

CHAPTER FOUR:

4.0 Result 27

4.1 Yield Of Crude extract and fraction 27

4.2 Result of Phytochemical Screening 29

4.3 Column chromatography of ethyl acetate fraction 30

4.4 TLC of isolated compound 31

4.5 Spectral Analysis of compound A4 32

4.5.1 UV spectroscopy of compound A4 31

4.5.2 Proton NMR of compound A4 32

4.5.3 ^{13}CNMR of compound A4 33

4.5.4 Mass spectrum of A4 34

4.6 Spectral Analysis of Compound B1 36

4.6.1 UV Spectroscopy of compound B1 35

4.6.2 proton NMR of compound B1 36

4.6.3 ^{13}CNMR of compound B1 37

4.6.4 Mass spectroscopy of compound B1 38

4.7 Spectral Analysis of Compound C1 40

4.7.1 Proton NMR of compound C1 39

4.7.2 Mass spectra of compound C1 40

4.8 Pharmacological Studies 42

4.8.1 Acute Toxicity Studies of Ethanol extract of *Chrozophora senegalensis* root in mice 41

4.8.2 Analgesic Study of Ethanol Extract of *Chrozophora senegalensis* root 41

4.8.3 Anti-inflammatory Study of Ethanol Extract of *Chrozophora senegalensis* root 42

4.8.4 Anti microbial Study of Ethanol Extract, Ethyl acetate and n-butanol fractions 44

CHAPTER FIVE;

5.0 Discussion 47

CHAPTER SIX:

6.0 SUMMARY 52

6.1 CONCLUSION AND RECOMMENDATION 58

REFERENCES 58

LIST OF TABLES

Table	Title	page
3.1	Column chromatography of ethyl acetate soluble fraction	23
4.1	Yield of crude extract and fraction	27
4.2	Result of preliminary phytochemical screening	28
4.3	TLC Properties of ethyl acetate fraction	29
4.4	Column chromatography	30
4.5	TLC of isolated compound	31
4.6	Effect of ethanol extract(EE) of root of *Chrozophora senegalensis* on Acetic acid induced writhing in mice	42
4.7	Result of Anti-inflammatory studies	44
4.8	Antimicrobial studies of ethanol extract, ethyl acetate and n-butanol soluble fraction	45
4.9	Minimum inhibitory Concentration (MIC) of Ethanol Extract, Ethyl acetate and n-Butanol soluble Fraction	46
4.10	Minimum Bactericidal/Fungicidal concentration (MBC/MFC) of ethanol extract, ethyl acetate and n-Butanol soluble Fraction	47

LIST OF FIGURES

Figure		Page
2.1	*Chrozophora senegalensis*	14
4.1	UV Spectrum of compoundA4	31
4.2	Proton NMR spectrum of compound A4	32
4.3	^{13}CNMR Spectrum of compound A4	33
4.4	Mass spectrum of compound A4	34
4.5	UV Spectrum of compound B1	35
4.6	Proton NMR Spectrum compound B1	36
4.7	^{13} CNMR Spectrum of compound B1	33
4.8	Mass spectrum of compound B1	38
4.9	Proton NMR Spectrum of compound C1	39
4.10	Mass spectrum of compound C1	40

ABBREVIATIONS

Analar	=	Analytic Reagent
^0C	=	Degree Centigrade (Celsius)
CC	=	Column chromatography
$CHCl_3$	=	Chloroform
cm	=	Centimeter
DMSO-d6	=	Deutrated Dimethyl sulfoxide
Eth	=	Ethanol Extract
Fig	=	Figure
g	=	Gram
H	=	Hertz
HCl	=	Hydrochloric acid
H_2SO_4	=	Sulphuric acid
mm	=	Millimeter
ml	=	Milliliter
MV	=	Mean Volume
%II	=	Percentage increase in inflammation

%I	=	Percentage inhibition
NB	=	n-Butanol
mg	=	Milligramme
ppt	=	Precipitate
ppm	=	Parts per million
PE	=	Petroleum ether
LD	=	Lethal dosage
TLC	=	Thin layer chromatography
Kg	=	Kilogram
μg	=	Microgram
NMR	=	Nuclear magnetic resonance
$CaCl_2$	=	Calcium chloride

CHAPTER ONE

1.0 INTRODUCTION

The term “traditional medicine” refers to ways of protecting and restoring health that existed before the arrival of modern medicine (WHO,1996). As the term implies, these approaches to health belong to the traditions of each country, and have been handed down from generation to generation. In practice, the term “traditional medicine” refers to the following components: acupuncture, traditional birth attendant, mental healers and herbal medicine (WHO, 1996). Traditional medicines are relatively inexpensive, locally available and are usually readily accepted by the local populace. It was reported that about 60 – 85 percent of the population in developing countries depend on traditional or indigenous form of medicine (African Pharmacopoeia, 1985; Farnsworth, 1988). It is in recognition of these facts that the WHO has been attempting to incorporate traditional medicine officially into the health care systems of developing countries.

Traditional medicine is plagued with disadvantages that include lack of scientific proof of efficacy of its remedies, lack of precise diagnosis of ailments by the traditional practitioners and lack of precise dosage of their medication that are not standardized (D’Arcy, 1991; WHO Drug Information, 1995). It is erroneously believed that herbal medicines as natural products are safe and thus lack toxic effects (D’Arcy, 1991; WHO Drug Information, 1995). Several reports and reviews have recently shown that these so-called safe herbal preparations are either toxic or have several side effects (D’Arcy, 1991; WHO Drug Information, 1995). Plants are regarded as nature’s bio-chemical factory because they serve as source of various types of chemical compounds. Prominent among these compounds are

the plant secondary metabolites which are diverse complex molecules that are valued and exploited by man for their pharmacological and other properties (Miller, 1973).

Over the years, the World health assembly has adopted a number of resolutions drawing attention to the fact that most of the populations in various developing countries around the world depends on traditional medicine for primary health care, that the work force represented by practitioners of traditional medicine is a potentially important resource for the delivery of health care and that medicinal plants are of great importance to the health of individuals and communities.(WHO,1996)

Through its traditional medicine programmed, the World Health Organization (WHO) supports, member States in their efforts to formulate national policies on traditional medicine, to study the potential usefulness of traditional medicine including evaluation for practices and examination of the safety and efficacy of remedies, to upgrade the knowledge of traditional and modern health practitioners as well as to educate and inform the general public about proven traditional health practices (WHO 1996), it is working closely with 19 collaborating centers in ten countries (Belgium, China Democratic People Republic of Korea, Italy, and Japan, Romania, Sudan, United State of America and Vietnam).

A genuine interest in various traditional practices now exist among practitioners of modern medicine and growing number of practitioners of traditional indigenous or alternative system are beginning to accept and use some of the modern technology (WHO,1996). This will help foster teamwork among all categories of health workers within the framework of primary health care. The reason for the inclusion of traditional healers in primary health care are manifold; the healers know the socio cultural background of the people; they are highly respected and experienced in their work, economic considerations; the distance to be

covered in some countries; the strength of traditional belief the shortage of health professionals, particularly in rural areas to name just a few. (WHO, 1996).

Herbal medicine is assuming greater importance in the primary health care of individuals and communities in many developing countries and there has been an increase in international trade in herbal medicines. However, in most countries the herbal medicines market is not adequately regulated, and the products are therefore unregistered and often not controlled by regulatory bodies. The establishment of regulation and registration procedures has become a major concern in both developed and developing countries (WHO, 1996).

Traditional medicine has maintained its popularity in a number of Asian countries, such as China India, Japan and Pakistan. In China for example traditional medicines (herbal preparations) account for 30% to 50% of the total medicinal consumptions. In 1993, the total sales of herbal medicines amounted to more than US $ 2.5 billion. In Japan from 1974 to 1989, there was a 15- fold increase in Kampoh (Chinese method) medicinal preparations in comparison with only 2.6 -fold increase in the sales of main stream pharmaceutical products. The Japanese per capital consumption of herbal medicine appears to be the highest in the world (WHO 1996).

There has also been a growing interest in traditional and alternative system of medicine in many developed countries. One- third of American adult have used alternative treatment and 60% of the public in the Netherlands and Belgium, and 74% in the United Kingdom are in favor of complementary medicine being available within the framework of the National Health Service. A survey among Member States of the European Union in 1991 identified about 1400 herbal drugs used in the European Economic Community (WHO 1996).

Unregulated or inappropriate use of traditional medicines and practices can have negative or dangerous effects. For instance, the herb Ephedra is traditionally used in China to treat respiratory congestion. In the United States, the herb was marked as dietary aid, whose over dosage led to at least a dozen of deaths, due to heart attacks and strokes. In addition to patient safety issues, there is the risk that growing herbal market and its great commercials benefit might pose a threat to biodiversity through the over harvesting of the raw material for herbal medicines and other natural health care products. These practices, if not controlled, may lead to the extinction of endangered species and the destruction of natural habitats and resources (WHO, 2003).

Plants are indispensible sources of medicine since time immemorial. Studies on natural product are aimed to determine medicinal values of plants by exploration of existing scientific knowledge, traditional uses and discovery of potential chemotherapeutic agents. Phytochemicals are used as templates for lead optimization programs, which are intended to make safe and effective drugs (Balunas and Kingbour , 2005)

Plant secondary metabolites can be classified under groups of chemical compound such as steroids, alkaloids, flavonoids, saponins, and tannins etc, many of which have been used by man as drugs, dyes and many other purposes since prehistoric times. Some of these secondary metabolites have been known to have a prominent effect on living cells. Some have important therapeutic properties and have been utilized in the treatment and cure of ailments. Examples of such therapeutically active plant constitutes are the Cinchona alkaloids, cardiac glycosides from *Digitalis* species, *Veratrum* alkaloids and the anticancer agents (Vincristine and Vinblastine) from *Catharanthus roseue.* Plants containing therapeutically active constituents are known as medicinal plants. It has been recorded that

about 25 percent of the active components of currently prescribed medicines were first identified in higher plants (Balandrin *et al.*, 1985; Farnsworth, 1988).

There has been an increasing interest worldwide on therapeutic values of natural products. Nature provides mankind with vast therapeutic flora with a wide variety of medicinal potential. The revival of interest in plant derived drugs is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs many of which have adverse side effects. The need of the hour is to screen a number of medicinal plants for promising biological activity. (Parekh and Chand. 2006)

Theories have been proposed to explain the acquisition of knowledge of medicinal plants. One such theory states that man deliberately select specific plant materials for the treatment of his ailments by rationalizing, while another theory states that the knowledge of medicinal plants was gained by accident. However, it is known that early man could have gain some scientific knowledge by watching the effect produced by medicinal plants on domestic animals, on themselves and on their relatives. These explain why most hunters in Africa and other parts of the world are known to be custodians of some effective traditional herbal recipes (Sofowara,1982)

Medicinal plants constitute an effective resource for both traditional and modern medicines,

and herbal medicine has been shown to have genuine utility. In Nigeria, many plants are used

in traditional medicine as antimicrobial agents but only few are documented. Plants based system of traditional medicine has continued to play an essential role in health care in many

cultures. The increased use of plant derived products as alternatives to orthodox or synthetic

drugs and increasing awareness of beneficial effects of natural products has resulted in increased interest in alternative therapies. Extracts from plants have been utilised for their antifungal, antiviral and antibacterial activities globally.(Odeh and Tor-Anyin. 2014).

Medicinal plants are the oldest known health care products. Their importance is still growing although it varies depending on the ethnological, medical and historical background of each country. Medicinal plants are also important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents but also when they are used as basic materials for the synthesis of drugs or as models for pharmacologically active compounds.(WHO, 1996)

Legislative controls in respect of medicinal plants have not evolved around a structured control model. There are different ways in which countries defined medicinal plant or herbs. As a result different approaches have been adopted with regard to licensing, dispensing, manufacturing and trading in order to ensure the safety, quality, and efficacy of medicinal plant preparations.(WHO,1996)

WHO has compiled a list of medicinal plants which are widely used in primary health care, in July 1996, a WHO scientific group involving 100 experts from various countries around the world adopted the list which includes 28 monographs of 28 medicinal plants originally prepared by the WHO collaborating centre for Traditional Medicine in Chicago, Illinois, United States of America (WHO,1996).

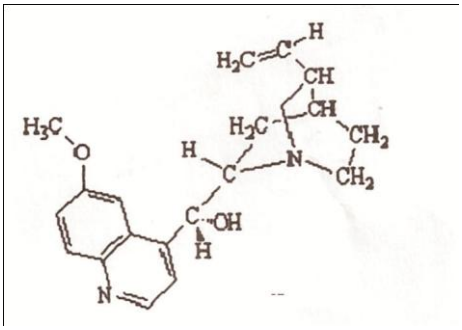
Medicinal plants with anti-inflammatory properties are considerably employed in the traditional treatment of several disorders of inflammation. The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane and Booting, 1987) which are aimed at host defense and usually activated in most disease conditions. These different reactions in the inflammatory response cascade are therapeutic targets which anti-inflammatory agents including medicinal plants interfere with to suppress exacerbated inflammatory responses usually invoked in such disorders as rheumatoid arthritis, in infection or injury. Inhibition of the synthesis of pro-inflammatory prostaglandins is one of such therapeutic targets to which some of the potent anti-inflammatory agents of clinical relevance (e.g. NSAIDs) owe their activity. Several anti-inflammatory medicinal plants have also demonstrated the ability to inhibit the synthesis of prostaglandins (Vane and Booting, 1987).

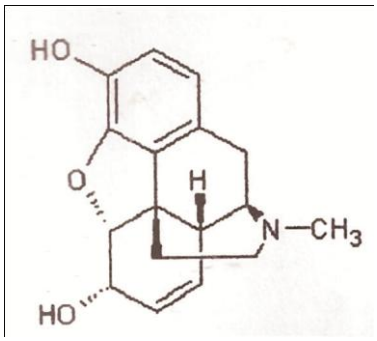
Phytochemical and pharmacological screenings are two important procedures used in the study of plant constituents and their therapeutic or pharmacological effects. It is the presence of bio-active plant secondary metabolites in plant that confers medicinal properties; therefore that study of the properties of these compounds is very important in establishing or confirming the claims of these plants (Farnsworth, 1966)

Phytochemcial screening is the application of simple chemical test to determine or detect the presence of accumulated natural plant product or secondary metabolites of plant such as alkaloids, tannins, steroids/terpenes, glycosides, flavonoids, carbohydrates, resin, gum and volatile substance in the plant extract. These metabolites are responsible for therapeutic properties of the pharmacologically active compounds which are referred to as “active constituents” (Harbone, 1973).

One of the major set backs often encountered with drugs of plant source is that, most of the active compounds occur in minute quantity which requires tedious extraction procedures. In order to overcome this limitation the synthetic chemist takes the approach of determining the structure activity relationship of the isolated, biologically active constituents to synthesize simpler analogues, some of which might be more active and less toxic than the natural products (Trease and Evans, 1983). A good example is the anti malarial drug quinine (I), obtained from cinchona bark and its synthetic analogue, Chloroquine. Structural modification of pure bioactive molecule of plant origin . morphine (II) isolated from *Papavera somniferum*) has presented a major breakthrough (Trease and Evans, 1983).

(I)

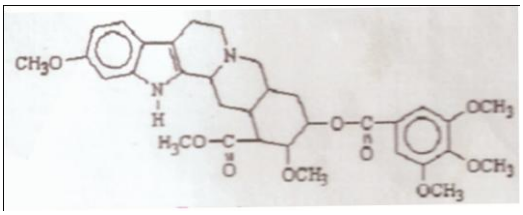




(II)

Some of the most useful compounds obtained from plants are of such complex structure that man is still unable to synthesize them from simpler compounds. And even where such complex compounds have been synthesized, the synthetic procedures are often so difficult and expensive that large –scale synthesis for commercial purpose is quite uneconomical. (Bankole and Ogunkoya, 1977) Man still relies entirely on the natural source for the commercial supply of some compounds. Typical of such cases are the antibiotic, penicillin(III) and the anti-hypertensive agent reserpine (IV) Besides being the main source of certain drugs, chemical compounds extracted from plant sources have also provide very

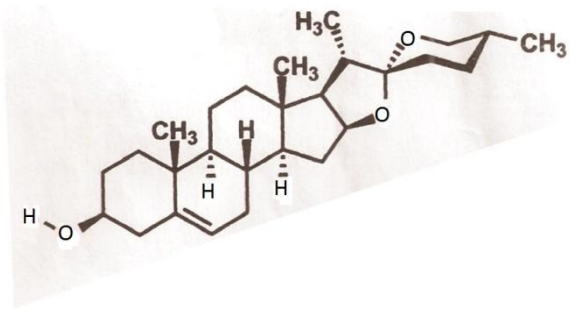
useful intermediates in the synthesis of other commercially important compounds.. For example, diosgenin (V), obtained from some species of yam, has proved very useful in the synthesis of sex hormones which help to cure certain diseases.



(III)

(IV)

(V)



Effect of plant extract

Some of the effects elaborated by extracts of plants used in traditional medicine include antiviral, antitumor, antibacterial, antifungal, analgesic, anti-inflammatory, e.t.c (Sofowora, 1982).

Cyclooxygenase and lipoxygenase play an important role as inflammatory mediators. They are involved in the release of arachidonic acid, which is a starting point for a general

inflammatory response. The exact mechanisms by which flavonoids inhibit these enzymes is not clear. Quercetin , in particular, inhibit both cyclooxygenase and lipoxygenes activities, thus diminishing the formation of these inflammatory metabolites (Robak*, et al..,*1996*)*

Species of *Chrozophora genus* (Euphobiaceae family) are distributed in West African and Asia. These species are monoecious, shrubby herb and annual plants and their leaves, stems and fruits besides the whole plant have been used in food and traditional medicine for the treatment of infectious diseases. Many of these species showed high content of protein and oil with high percentages of fatty acids. The extracts from different parts of the *Chrozophora* plants showed the presence of diterpenoids, triterpenoids, flavonoids and chromone glucosides, Alkaloids, coumarins, chromones xanthones, diterpenoids and phenylpropanoid glycosides. However, there are many investigations showed this genus have antimicrobial, anticancer and antioxidant, antiplasmodial, antidiabetes, and anthelmintic property agents, and phytotoxic activity (Manar, A, *et al.,* 2014).

Importance of flavonoids

Flavonoids are group of polyphenolic compounds, diverse in chemical structure and characteristics, mainly found in fruits, vegetables and cereals. Therefore, flavonoids are part of the human diet; over 4000 different flavonoids have been identified within the major flavonoid classes which include flavonols, flavones, flavanones, catechins, antho-cyanidins, isoflavones, dihydroflavonols, and chalcones.

Flavonoids are potentially antioxidants, metal chelators and inhibit lipid peroxidation (Leopoldini *et al.*, 2006).

Flavonoids are polyphenolic in nature therefore they can behave as antioxidants. These properties depend on the number of hydroxyl groups present on the flavonoid structure. It is said if the number of hydroxyl groups in flavonoids is higher; the antioxidant capacity is also better (Cao *et al*., 1997).

Flavonoids are divided into eight different groups: flavonoids (quercetin, myricetin, kaemferol and rutin) flavonones (taxifolin) flavones (luteolin and apigenin) isoflavones (daidzein and genistein)Catechins, anthocyanidins, dihydroflanols and chalcones (Lu *et al.,* 2006). The function of flavonoid in flowers is to provide colors attractive to plant pollinators (Harbone *et al*.,1992). In leaves, these compounds are increasingly believed to promoted physiological survival of the plant, protecting it from, for example, fungal pathogens and U-V radiation (Harbone *et al*., 1992).

1.1 Statement of Research problem

Disease related with pain and inflammations are the most important symptoms that bring the patient to physician. Pain is frequently associated with inflammation. It is an ill-defined, unpleasant sensation and may be caused by nociceptive or inflammatory agents. There are several kinds of drugs currently available as anti-inflammatory and anti-nociceptive agents to relieve pain but unfortunately, the uses of these drugs are not fully satisfactory in all cases due to their adverse side effects, for example gastric lesions, ulcers and cardiac abnormalities are caused by non-steroidal anti-inflammatory drugs and opiates (Allura *et al*., 2009, Mapphsa *et al*., 2009). Therefore, development of newer, more powerful and relatively cheap anti-inflammatory and analgesic drugs with lesser side effects is necessary for human welfare.

1.2 Justification of Research

The plant *Chrozophora senegalensis* has been used in ethnomedicine for the treatment of pain and inflammation, therefore this reseach work is desinged to authenticate this traditional claim

1.3 Aim

The aim of this research work is to isolate some of the bioactive constituents and validate the ethnomedical claim that *Chrozophora senegalensis* has Analgesic, Anti-inflammatory and antimicrobial activities.

1.4 Objectives

The specific objectives are:

Collection and identification of the plant material followed by extraction of the root portion of the plant using petroleum ether (defatting) than with ethanol.The crude extract will be fractionated with ethyl acetate and n-Butanol. Preliminary phytochemical screening using the crude extract and the fractions.Chromatographic separation of the ethyl acetate fraction, Followed by spectroscopic analysis of the isolated compounds. Analgesic and anti-inflammatory investigation of crude extract and isolated compounds using acetic acid induced whrithing and paw oedema respectively. Antimicrobial evaluation using clinical isolates

1.5 Hypothesis

$H_{1;}$ The plant *Chrozophora senegalensis* contain secondary metabolite with Analgesic, Anti-inflammatory and Antimicrobial activity.

H_{O} ; The plant *Chrozophora senegalensis* does contain secondary metabolite with Analgesic, Anti-inflammatory and Antimicrobial activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

Chrozophora senegalensis belongs to Euphorbiaceae family .The plant is known locally as; Damagi, Damangi or Bauren Kiyashi, also a Walkin Maciji in "Hausa" Language. *Chrozophora senegalensis* occurs from Mauritania east to Nigeria. (Burkill, 1994).



Fig 2.1 : *Chrozophora senegalensis*

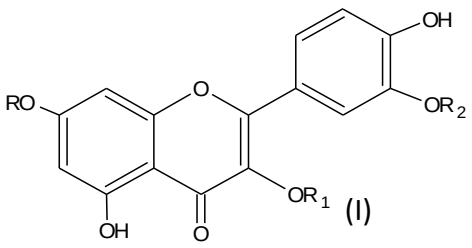
2.1 Ethno medicinal uses of *Chrozophora senegalensis*

The maceration of the leaf is taken to treat tapeworm and roundworm. In Senegal a root decoction is used to treat diarrhoea. A maceration of leaves and roots is drunk to treat loss of hair and as antidiabetic. A maceration of the aerial parts is use in treatment of wound, diarrhea, rheumatism, teniasis, stomachache, and venereal disease. An infusion of the aerial parts is taken to treat stomach-ache and venereal diseases. A fruit maceration is taken to treat conjunctivitis and night blindness (Burkill,1994).

Original and Geographical Distribution ; *Chrozophora senegalensis* occurs from Mauritania east to Nigeria.

2.2 **Previous Phytochemistry and pharmacological activities on *Chrozophora senegalensis***

Vassallo *et al* (2006) reported the isolation of three flavonoids from methanol leaves extract these are Quercetin -3-0-(6″-caffeoyl)-β-D-glucopyranoside-3-0-β-D-glucopyranoside(I), Quercetin- 3-methyl ether -7-0-α-L-rhamnopyranosyl (1→6)- (2″-P-coumaroyl)-β-D-glucopyranoside (II) and Acacetin 7-0-(6″-P-coumaroyl)-β-D-glucopyranoside) (III) and Quercetin Rhamnoside (IV) (Ibrahim,2006)

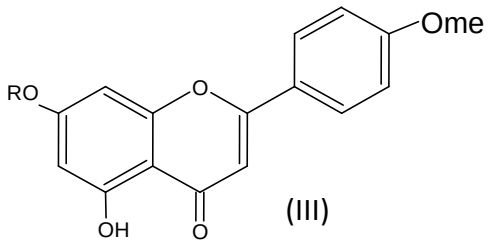


(I)

R = H

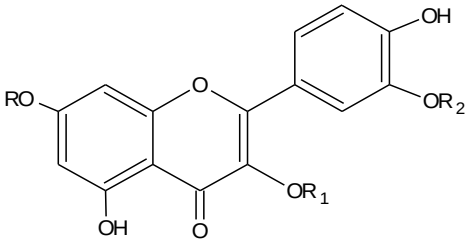
R_1 = (6″ – caffeoyl) glc

R_2 = glc



(III)

R= (6″ –P- coumaroyl) glc

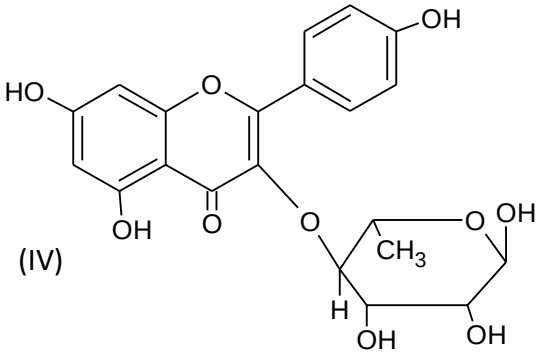


(II)

R = (2″ -P- coumaroyl)-glc-(6-1) rha

R_1 = Me

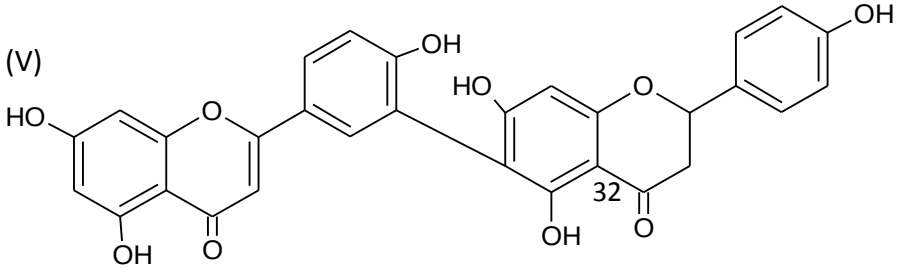
R_2 = H



(IV)

The isolatiom of Biflavonoid amentoflavone (V) have been reported and were shown to have anti-tumor activity. (Valeria, *et al.,* 2011).

(V)



Amentoflavone

Ethanol extract of leaves of *Chrozophora senegalensis* and its fractions have been shown to have antimicrobial activity (Usman *et al.,*2007).

It is reported that the aceton and n-hexane extract of leaves of *Chrozophora senegalensis* has antimicrobial activity against clinical bacteria isolate of *E. coli, Klebsiella spp, Proteus spp,* and *Salmonella spp* (Yusha'u,2011).

Report also shows that the methanol extract of the leaves of *Chrozophora senegalensis* have antiplasmodial activity (Ali *et al*, Benoit,2008) analgesic and antiinflammatory effect on mice (Ali *et al*., 2011).

The n-butanol fraction was also shown to have anti diarrheal activity. (Ibrahim,2006)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials .

3.1.1 **Identification, Collection and Preparation of plant materials;**

The plant, *Chrozophora senegalensis* was collected from Tudun Wada Local Government Area of Kano State in August, 2008. The plant was authenticated at the Herbarium by Musa Muhammad of Department of Biological Sciences, Ahmadu Bello University, Zaria Nigeria by comparison with Herbarium specimen (Voucher number 159). The plant material was air dried under shade for a week. The root removed, crushed and then pounded into coarsed form.

3.1.2 Chemicals and Equipment

All chemicals used are of analar grade and were distilled before use. The silica gel for column chromatography is of mesh size (60-120) Merck and that for thin layer chromatography is 0.25mm thickness. Silica gel, precoated aluminum sheets (0.25mm) Merck.

^{1}HNMR spectroscopy was recorded on Bruker DRX 400 MHZ Spectrophotometer using $DMSOd_6$ as solvent The mass spectrometry was carried out on mass spectrometer. The ^{13}C

NMR Spectroscopy was carried out at 100 MHz spectrometer, the UV spectroscopy was obtained in methanol on a Helios-zeta, UV-VIS Spectrophotometer.

3. 1.3 Standard drugs and sample used

The standard drugs (Ketoprofen and Carrageenana), and aqueous ethanol extract of the plant were prepared by dissolving a known weight of the drug and the extract in required volume of distilled water.

3.1.4 Animals Used

The animals used for experiments were mice (18-22g) and rats (133-164g). They were obtained and kept in the Animal House, Department of pharmacology and therapeutics Faculty of Pharmaceutical Science A.B.U, Zaria.

3.1.5 Microorganism

The microorganism (standard cultures) used were obtained from Department of Pharmaceutics and Pharmaceutical Microbiology A.B.U Zaria. The organisms used were *Escherichia coli* (ATCC 11775), *Staphylococcus aureus* (ATCC 021001), *Pseudomonas aeruginosa* (ATCC 104145) *and Bacillus subtilis* (NCTC 10342 A76) while the fungi includes *Candida albicans* and *Trichophyton mentagrophyte* were laboratory isolates.

3.2 Methods

3.2.1 Extraction procedures

The powdered part of plant material (1.5 kg) was extracted to exhaustion with petroleum ether (60-80^{0}C) using soxhlet extractor and the solvent evaporated to give a brownish oily mass (10g), The defatted marc was air-dried and extracted with 95% ethanol to exhaustion. The ethanol extract was concentrated at room temperature to give a brownish gummy mass (150 g), coded ethanol extract. (EE) 100 g of the crude ethanol extract was suspended in water and partitioned with ethyl acetate (2.5L) and then n-butanol (2.5L) to give EA and NB fractions respectively.

3.2.2 Preliminary phytochemical screening

The crude ethanol extract, ethyl acetate and n-butanol soluble fractions were subjected to preliminary phytochemical screening using standard procedures.

Test for Alkaloids

Each weighing 0.5g extract was stirred with 5ml of 1% aqueous hydrochloric acid on a steam bath, 1ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1ml portion was treated similarly with Dragendroffs reagent, turbidity or precipitation with either of these reagent was taken as preliminary evidence for the presence of alkaloid . (Harbone 1973,).

Test for Tannins

About 0.5g of sample was stirred with 10ml of distilled water, filtered and ferric chloride reagent added to the filtrate. A blue black, green or blue green precipitate was taken as evidence for the presence of tannin (Trease and Evans, 1989).

Test for Saponins

To a portion of the extract in a test-tube, 10ml of distilled water was added and shaken vigorously for 30 seconds. The tube was allowed to stand for 30minutes.(Trease and Evans,1983)

Test for phlobatanins

Deposition of a red precipitate when an aqueous extract of the plant part was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins (Trease and Evans, 1989).

Test for anthraquinones

Bontrager's test was used for the detection of antraquinones. 0.5g of each plant extract was shaken with 10ml benzene, filtered and 5ml of 10% ammonia solution added to the filtrate. The mixture was shaken and the presence of a pink red or violet colour in the ammoniacal (lower) phase indicates the presence of free hydroxyl anthraquinone (Trease and Evans 1989). For combined anthraquinones 0.5g of each extract was boiled with 10ml aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5ml of benzene, the benzene layer separated and half its own volume of 10% ammonia solution added. A pink, red or violet coloration in the ammonia phase (Lower layer) indicate the presence of anthraquinone derivatives in the extract (Trease and Evans, 1989).

Test for flavonoids

a. Shinoda test

A little quantity each of the extract was dissolved in ethanol, warmed and filtered, three pieces of magnesium turning were added to the filtrate followed by the addition of a few drops of concentrated Hydrochloric acid; organge, pink, red to purple colors indicate the presence of flavones, the corresponding 2,3, dihydro derivatives and/or xanthone are presentred red to crimson indicated flavonoids, crimson to magenta indicated flavonones (Silva *et al.,* 1998).

b. Ferric chloride test

A little quantity of the extract was dissolved in ethanol and boiled with few drops of freshly prepared 10% $FeCl_3$ solution. A violet coloration was an indication of the presence of a phenolic hydroxyl group (Trease and Evans 1983).

c. Sulphuric acid test

Flavones and flavonols dissolve in concentrated sulphuric acid giving a deep yellow solution. Chalcones and aurones produce red or red-bluish solution. Flavanones give orange to red colors (Silva *et al.,* 1998).

Test for cardiac glycoside

a. Legal test

The extract was dissolved in pyridine and few drops of 2% sodium nitropruside together with a few drops of 20% NaOH were added. A deep red colour which faded to a brownish yellow indicated the presence of cardenolides (Silva *et al.,* 1998).

b. Leiberman Buchard test

0.5g of the extract was dissolved in 2ml acetic anhydride and cooled well in ice, H_2SO_4 was then carefully added. A color change from violet to blue to green indicated the presence of steroidal nucleus (i.e aglycon portion of the cardiac glycoside.)

Terpenes and sterols

a Salkowski test

0.5g of the extract was dissolved in 2ml of choloroform. Sulphuric acid was carefully added to form a lower layer. A reddish brown colour at the interface indicated the presence of a steroidal ring . (Silva *et al.,* 1998).

3.2.3 Thin Layer Chromatography (TLC)

Ascending technique was used, The spots were applied manually using capillary tubes. The plates were developed at room temperature in an appropriate solvent system(ethyl acetate ; methanol ; water, 7;3;1) using a Shandom chromo-tank, the developed chromatograms were dried in the air and visualized as follows: -

a. View under sunlight
b. Exposed to iodine vapour in a closed chamber

c. Anilin hydrogen phthalate reagent

3.2.4 Column Chromatography (CC)

The gradient elution technique was employed using sintered glass column (2.5cm x 120cm) packed by slurry method the adsorbent was silica gel (60-120mesh)

Column chromatography of ethyl acetate fraction -

Ethyl acetate fraction (3g) was applied over a well-packed silica gel column, it was eluted gradiently with chloroform, the polarity was gradually increased using methanol the eluents were collected in 10ml aliquots (250 fractions) and subjected to thin layer chromatography using solvent system (ethyl acetate: methanol : water, 7:3:1). Similar aliquots were pooled together based on TLC profile to give 14 pooled fractions and concentrated at room temperature as shown in Table 3.1

Repeated silicagel column chromatography separation of fraction 43-62 led to isolation of three compounds coded A4, B1 and C1.

3.2.5 Spectroscopic analysis of isolated compounds

Compound A4 and B1 were subjected to UV, ^{1}HNMR, ^{13}CNMR; mass spectrometry analysis while compound C1 was subjected to ^{1}HNMR and Mass spectrometry only. The results are presented in tables and figures

3.3 Pharmacological Studies

3.3.1 Acute toxicity studies (LD_{50}) of ethanol extract in mice

An acute toxicity study was conducted using the method of Lorke (1983). In the first phase, 3 groups of three mice each were treated with ethanol extract of the plant at dose of 10, 100 and 1000mg/kg body weight intraperitoneally and observed for 24 hours. In the second phase, 3 groups of one mouse each were injected with the ethanol extract at doses 1600, 2900 and 5000mg/kg intraperitoneally and observed for sign and symptoms for 24 hours.

3.3.2 Analgesic studies on ethanol extract

Acetic acid-induced writhing test:

The method reported by Koster *et al.,*1959 was applied. Five groups each comprises six mice were used. The animals were orally administered normal saline for group I, 600,300 and 150mg/kg body weight of ethanol extract for group II, III and IV respectively. The last group receive 10mg/kg ketoprofen body weight which serve as standard. Thirty minutes after treatment, mice received an intraperitoneal Injection of 0.2ml of 0.6 % v/v acetic acid body weight and placed in transparent boxes for observation. The number of withes was counted for 30 minutes after acetic acid injection and the percentage protection was calculated.

Statistical Analysis; Results were analyzed using One way analysis of varience (ANOVA) expressed as Mean ± SEM. Data was further subjected to Fischer LSD post hoc test and differences between means were regarded sifnificant at $P \leq 0.05$

Percentage inhibition of Acetic acid induced writhing is calculated as:

$$\% \text{ Inhibition} = \frac{\text{Mean control} - \text{Mean Treated}}{\text{Mean Control}}$$

3.3.4 Carrageenaan-induced paw edema test: -

The method described by Winter ,*et al.,* 1962 was employed. Five groups each comprises six rats were used. Group I received norrmal saline and serve as control. Group II, III and IV received 600,300 and 150mg/kg body weight ethanol extract of *Chrozophora senegalensis* while group V receives 10mg/kg ketoprofen and served as standard drug. Acute inflammation was induced in all groups by subcutaneous injection of 0.1ml of 1% carrageenan in the left hind paw of the rats one hour after administration of drugs. Mean paw volume was measured before carrageenan injection (Vo) and then every hour (Vt) for three hours post administration of the test drug and control using vanier caliper.

The percentage oedema and the percentage inhibition were calculated as follows:

$$\% \text{ oedema (E)} = \frac{V t - Vo}{Vo} \times 100$$

$$\% \text{ Inhibition of oedema} = \frac{VC - VT}{VC} \times 100$$

VC = Paw Oedema in control group Animals

VT = Paw Oedema in Test group Animals

Statistical Analysis; Results were analyzed using One way analysis of varience (ANOVA) and expressed as Mean ± SEM. Data was further subjected to Fischer LSD post hoc test and differences between means were regarded sifnificant at $P \leq 0.05$

3.3.4 Determination of zone of inhibition

The pathogenic micro-organisms used were: *Escherichia coli,Pseudomonas aeruginosa,Bacillus subtilis, Staphylococcus aureus, Trichophyton mentagrophyte* and *Candida albicans*. Ethyl acetate, n-butanol (0.2g each) was weighted and dissolved in 10ml of distilled water to obtain a concentration of 20 mg/ml each while 0.5g of each in 10ml of

distilled water to obtained 50mg/ml. This was the concentrations of the extract used to check the antimicrobial activities of the extracts from the plant. The agar well diffusion technique was used to screen the plant extracts for antimicrobial activities. An inoculums (0.1ml) of an overnight broth culture of test organism (1.5 x108 cfu/ml) was spread evenly on a Mueller-Hinton agar plate, and a 6 mm diameter well was cut at the center of the plate using a cork borer. A sample (0.1ml) of extract solution in water (concentration 20 mg/ml and 50mg/ml) was introduced into the agar well. The agar plate was incubated (37ºC, 24h) for bacteria while 27^0 ,30min for fungi and observed for zone of inhibition.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration

Extract solution (concentration 20 mg/ml) was serially diluted two-folds in Muller-Hinton broth to give decreasing concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml. An aliquot (0.1ml) of overnight broth culture of test microorganism (concentration 1.5 x 108 cfu/ml) in sterile normal saline was introduced into each extract dilution. The mixtures in sterile test tubes were incubated (37ºC, 24h) and observed for turbidity (signifying growth) or

absence of it (signifying inhibition). Streptomycin (antibacterial drug) was used as positive controls, and sterile normal saline as negative control. The minimum inhibitory concentration was the lowest concentration of the extract solution that inhibited microbial growth.

Minimum bactericidal/fungicidal concentration

A loopful of the test mixture was removed from each MIC tube that showed no growth, inoculated onto antibiotic-free Mueller-Hinton agar plate, incubated (37ºC, 24h), and inspected for presence of colonies indicating growth. The minimal bactericidal or fungicidal

concentration is the lowest concentration of extract that showed no bacterial or fungal growth.

CHAPTER FOUR

4.0 RESULTS

4.1 Yield of crude extract and Fractions

The percentge of extract and the fractions are shown in Table 4.1

Table 4.1: Yield of crude extract and fractions

Extract/Fraction	Weight (g)	Percentage (%)
Ethanol Extract	150	10
Ethyl acetate fraction	3.5	3.5
N-butanol fraction	4 .0	4.5

4.2 Result of preliminary phytochemical screening.

The Preliminary phytochemical screening of Ethanol extract (EE), Ethyl acetate (EA) and the n-butanol soluble fraction revealed the presence of Alkaloid,flavonoids, cardiac glycosides as shown in Table 4.2

Constituents	Test	Observation	EE	E.A	N-B
Alkaloids	Dragendorff's	Orange red ppt	+	+	-
	Mayer	Buff ppt	-	-	-
Phenolics nucleus	$FeCl_3$	Blue-black	+	-	+
Saponins	Frothing	Frothing	+	-	+
Phlobatannins	1% HCl (aq)	Red ppt	+	+	+
Anthraquinones	Borntrager's	Pink, red or violet colour	_	_	_
Combined Anthraquinones			_	_	_
Flavonoids	Shinoda's	Orange, pink, red, purple	+	+	+
	$FeCl_3$	Violet colour	+	+	+
	H_2SO_4	Deep yellow or red-bluish or organged-red	+	+	+
Cardiac glycoside	Libermann	Colour change from violet to blue	+	-	+
	Legal test	Deep red colour	-	-	-
Terpenes and sterols	Salkowski's Test	Reddish brown ring at interface	-	-	-

Table 4.2; Result of Preliminary Phytochemical Screening

Key: + = Present - = Absent

TLC of ethyl acetate fraction(EA)

The TLC analysis using EtOH, MEOH,H_2O (7:3:1) with Rf value of 0.3, 0.5, 0.6, 0.7 is shown in table 4.3

Table 4.3: TLC properties of ethyl acetate soluble fraction

Spot	visualization in I_2 Vapour	Rf value
A	Dark brown	0.3
B	Dark brown	0.5
C	Dark brown	0.6
D	Dark brown	0.7

4.3 Column chromatography of ethyl acetate fraction

TLC profiles of some collections (43-62) from the olumn chromatography of ethyl acetate soluble fraction using solvent system of ethyl acetate: methanol: water (7:3:1) indicate three spots while others indicate two or one spot

Table 4.4 Column chromatography of ethyl acetate fraction

Solvent System	Ratio	Aliquots	No. of spot	TLC Remark
1.$CHCl_3$	100%	1-15	–	No spot seen
2.$CHCl_3$: MeOH	9:1	16-30	-	No spot seen
3.$CHCl_3$: MeOH	8:2	31-42	Two	Two minor spots not well separated
4.$CHCl_3$: MeOH	8:2	43-62	Three	Three major spots well separated
5.$CHCl_3$:MeOH	8:2	63-74	Three	Three spots note well separated,
6.$CHCl_3$:MeOH	8:2	75 – 88	–	No major spot
7.$CHCl_3$:MeOH	7:3	89-100	Two	Two spots not well separated
8.$CHCl_3$:MeOH	7:3	101-120	Two	Two spots not well separated
9.$CHCl_3$:MeOH	7:3	121-133	Two	Two minor spots no well separated
10.$CHCl_3$:MeOH	7:3	134-156	Two	Two spots not well separated.
11.$CHCl_3$:MeOH	7:3	157-180	-	No major spot
12.$CHCl_3$:MeOH	6:4	181-200	-	No major spot
13.$CHCl_3$:MeOH	5:5	201-220	Two	Two spots well separated.
14.$CHCl_3$:MeOH	5:5	221-240	Two	Two spots well separated.

4.4 TLC of isolated compounds (A4, B1, and C1)

The TLC profile of isolated compounds A4, B1, C1 shows single spot each using solvent system ethyl acetate: methanol: water (7:3:1) as shown in Table 4.3

Table 4.5 TLC of isolated compounds

Compounds	R_f Value	Solvent system
A4	0.4	Ethyl acetate: methanol: water (7:3:1)
B1	0.5	,,
C1	0. 6	,,

4.5 Spectral Analysis of compound A4

4.5.1 UV Spectral Analysis :

The UV spectral analysis of compound A4 revealed λmax(MeOH) of 268,333nm

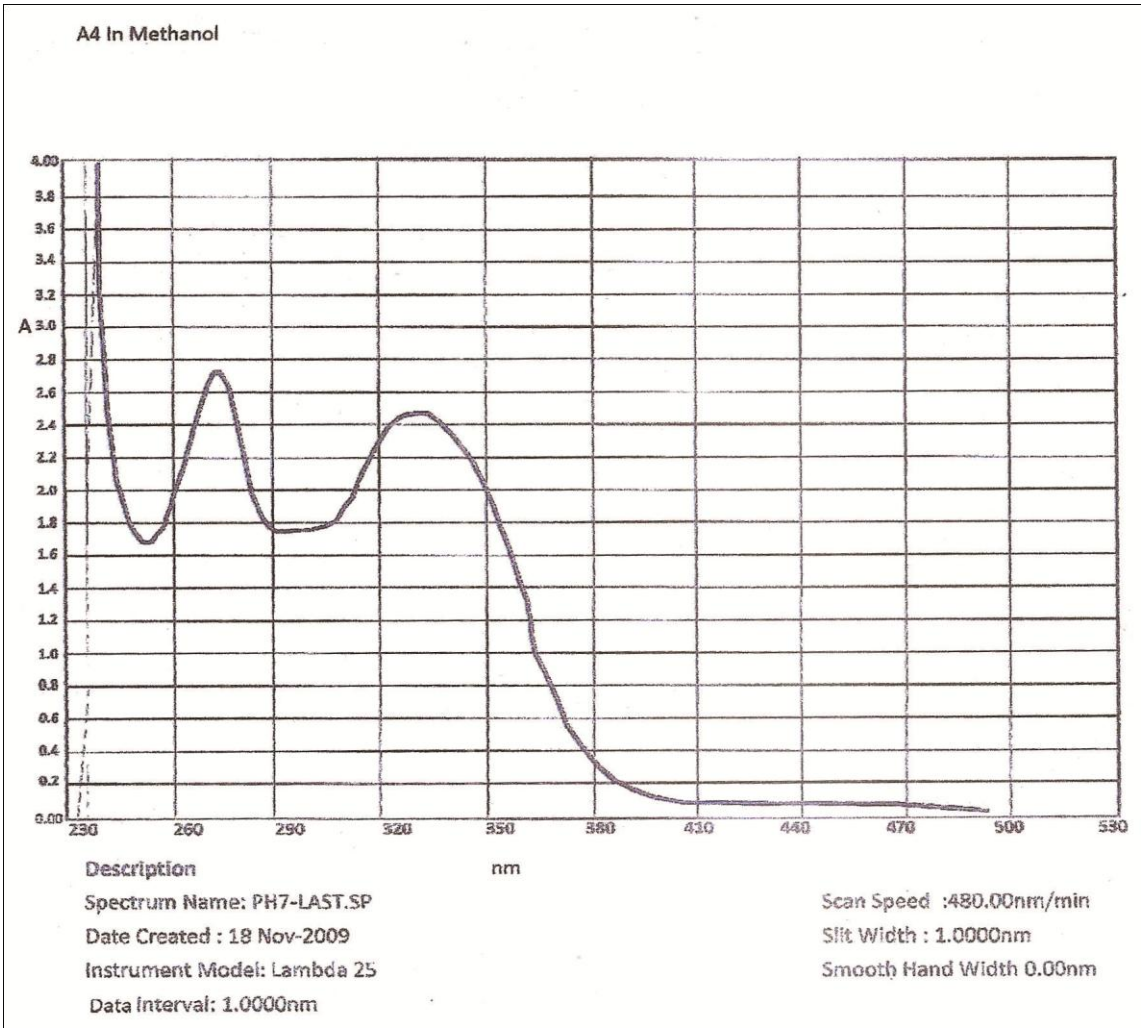


Fig 4.1: UV Spectrum of Compound A4

4.5.2 ^{1}HNMR Spectral Analysis:

The ^{1}HNMR of A4 revealed signals at δ 7.92 (2H, d, *J*= 8.4 Hz) and δ 6.97 (2H, d, *J* = 8.0 Hz) which were assined to aromatic protons. The *J* value of 8.0 and 8.4 Hz indicate an ortho coupling for both protons and disubstituted benzene ring. While signal at δ 5.20 (1H, d, *J* = 7.6 Hz) shows aromatic proton.

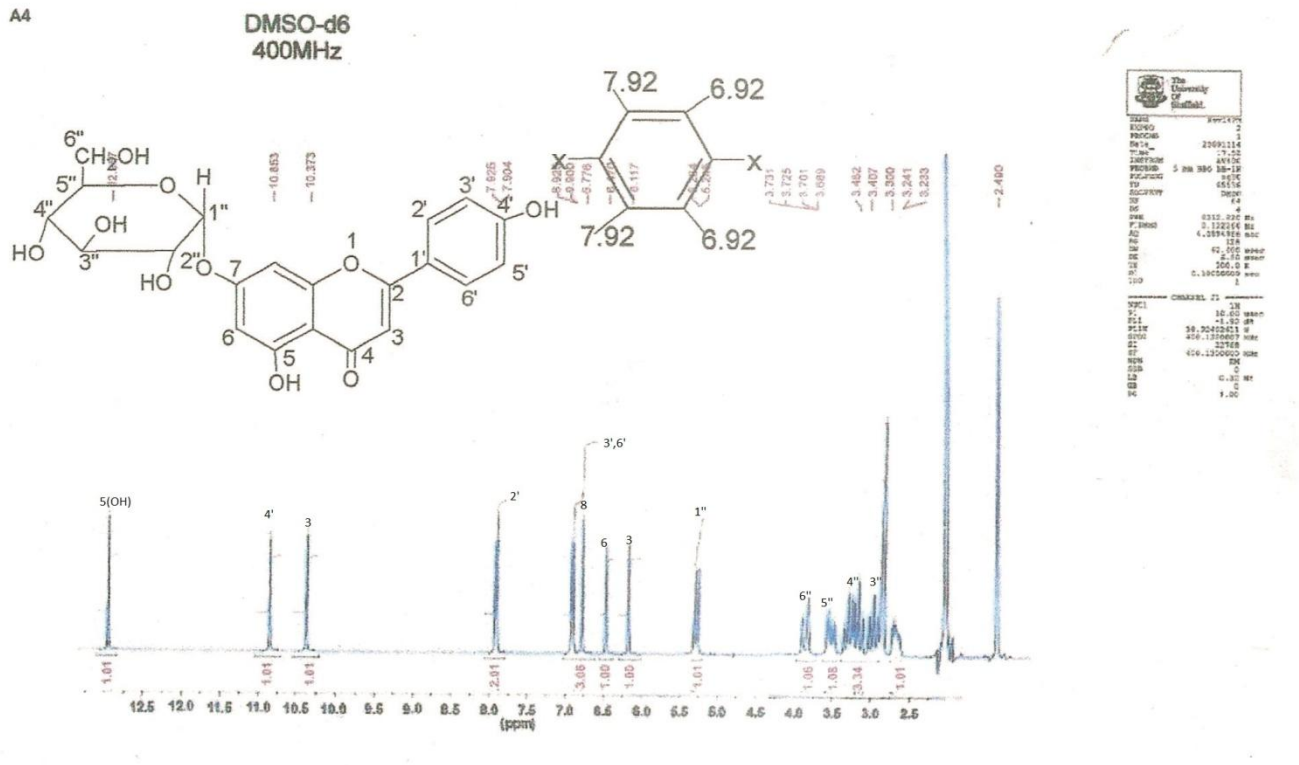
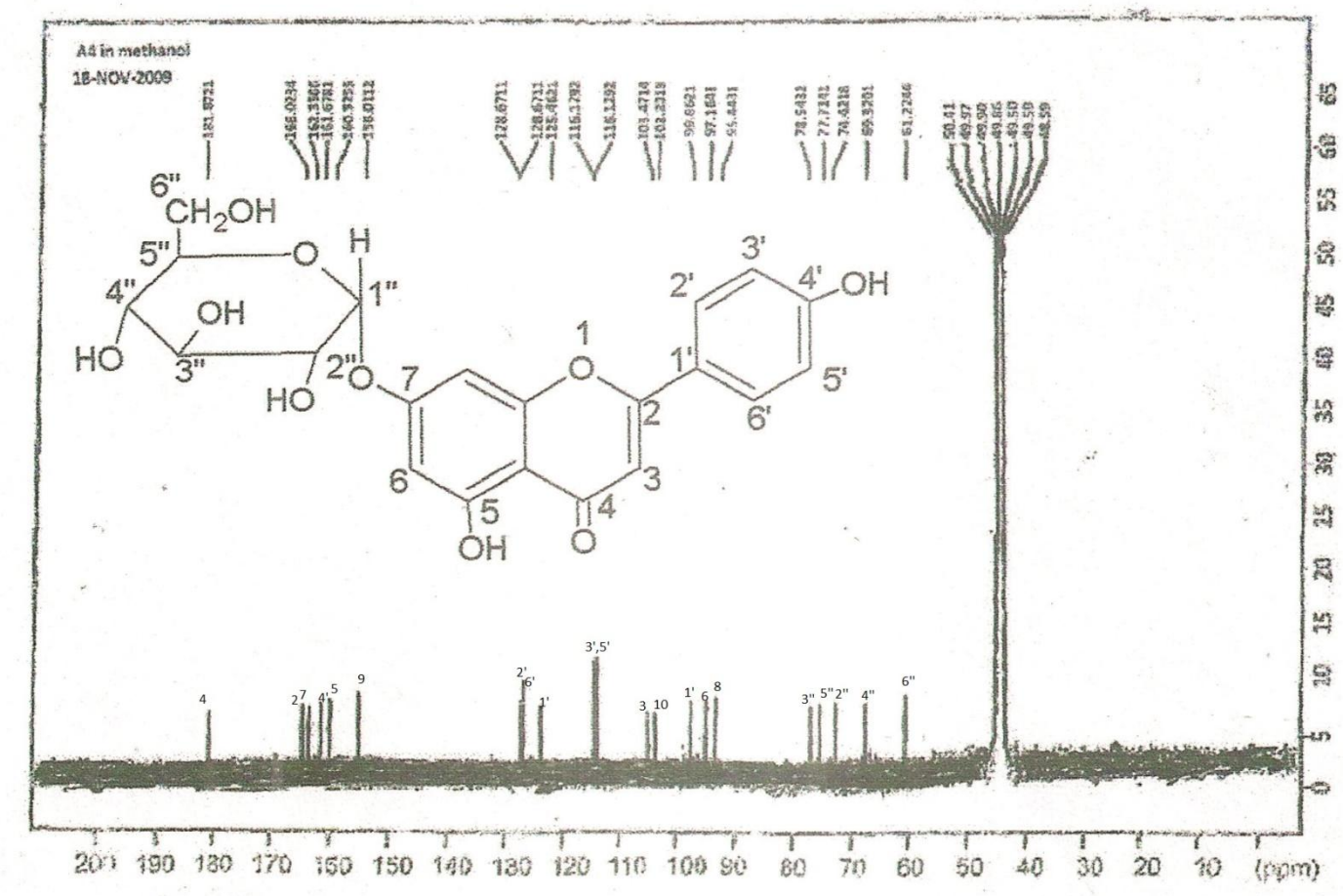


Fig 4.2: Proton NMR Spectrum of compound A4

4.5.3 ^{13}C-NMR Analysis of A4:

The ^{13}C-NMR(δ ppm, MEOH) experiment showed the presence of 21 carbon atoms; 166.0(C-2), 103.4(C-3), 181.8(C-4), 160.3(C-5), 97.1(C-6), 162.3(C-7), 95.4(C-8),156.0(C-9), 102.2(C-10), 125.4(C-1'), 128.6(C-2'), 116.1(C-3'),161.6(C-4'), 116.1(C-5'), 128.6(C-6'), 99.8(C-1''), 74.4(C-2''), 78.5(C-3''), 69.3(C-4''), 77.7(C-5''), 61.2(C-6'')



4.5.4 Mass spectrum Analysis of A4: The Ms spectrum of A4 revealed molecular ion peak at m/z 431(4%), the base peak at 155(100%), other prominent peak include 127(37%), 271(5%), 105(15%), 111(25%), 119(18%)

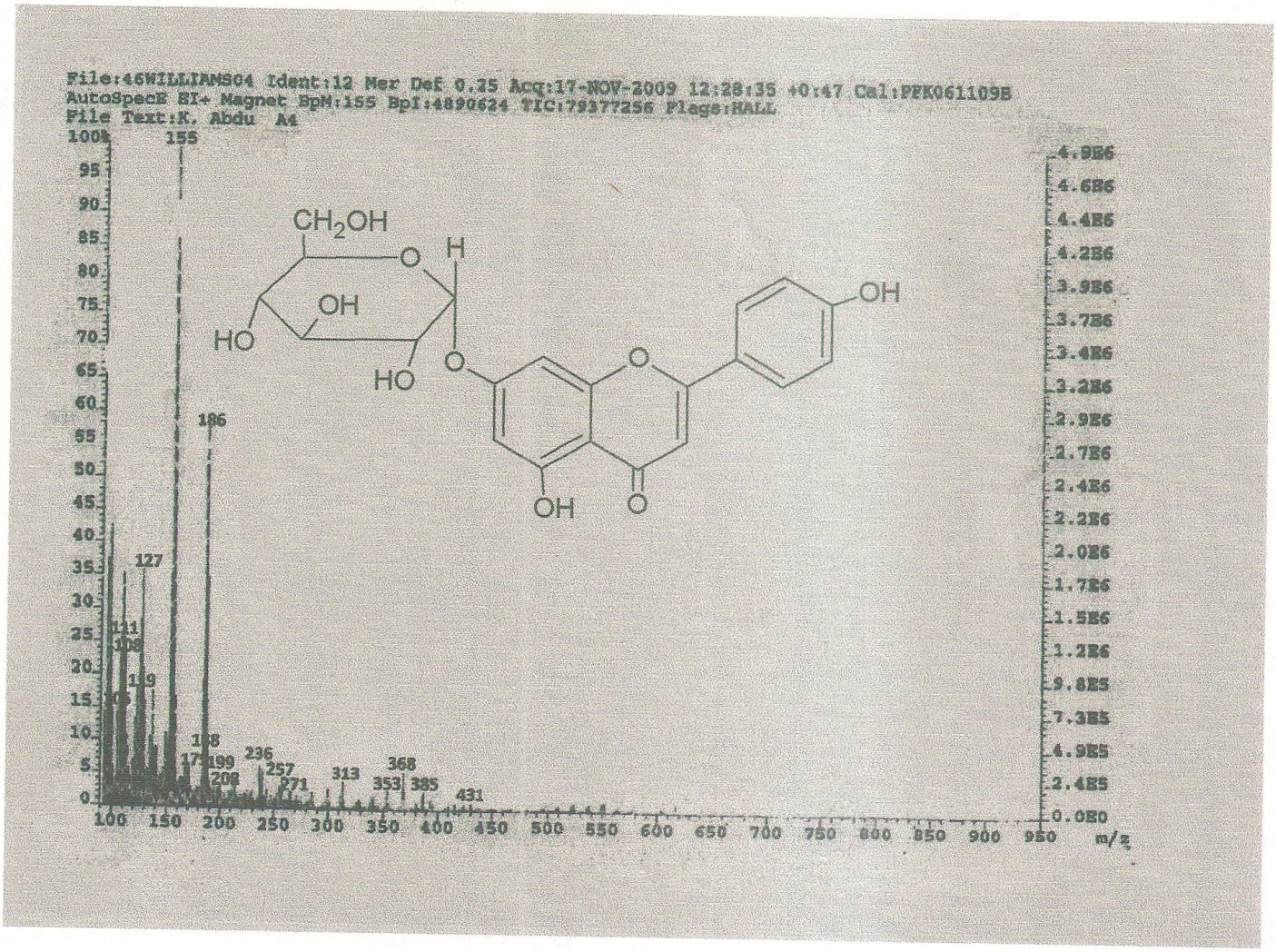


Fig 4.4: Mass spectrum of compound

4.6 **Spectral Analysis of compound B_1**

4.6.1 UV Analysis of B1:

The UV spectral analysis of compound A4 revealed λmax(MeOH) of 265, 353 nm

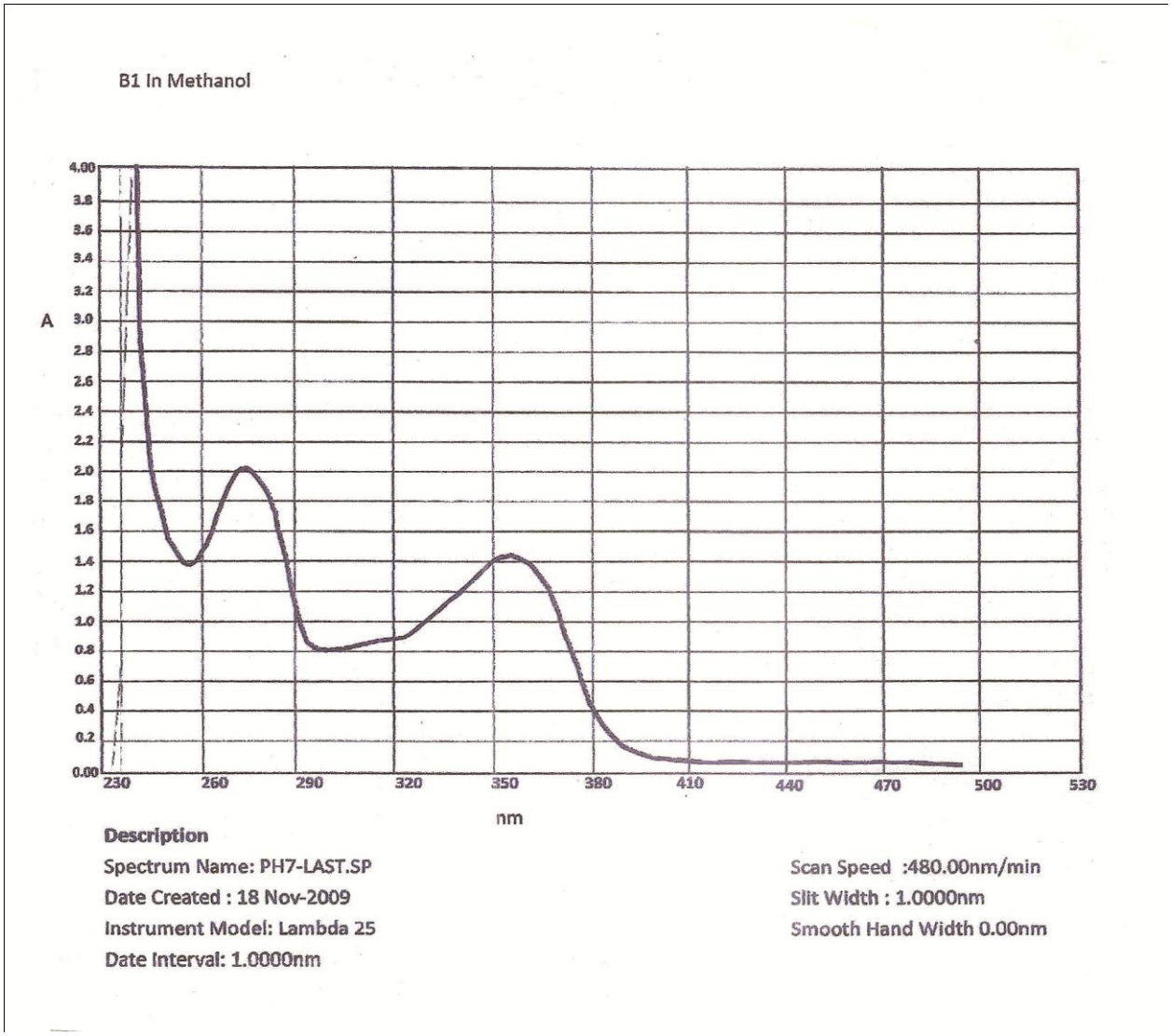


Fig 4.5: UV Spectrum of B1

4.6.2 [1]HNMR Spectral Analysis:

The [1]HNMR of B1 spectrum in DMSO-d6(indicates the aglycon moiety of Aromadendrin and glucose) showed the following characteristic proton: Signals at δ 5.918 (IH, d, *J*= 1.6 Hz) H – 8 assigned to aromatic protons, *J* – value = 1.6 Hz show meta coupling. δ 4.98 (d, j =6.4Hz) H-1'' shows anomeric proton

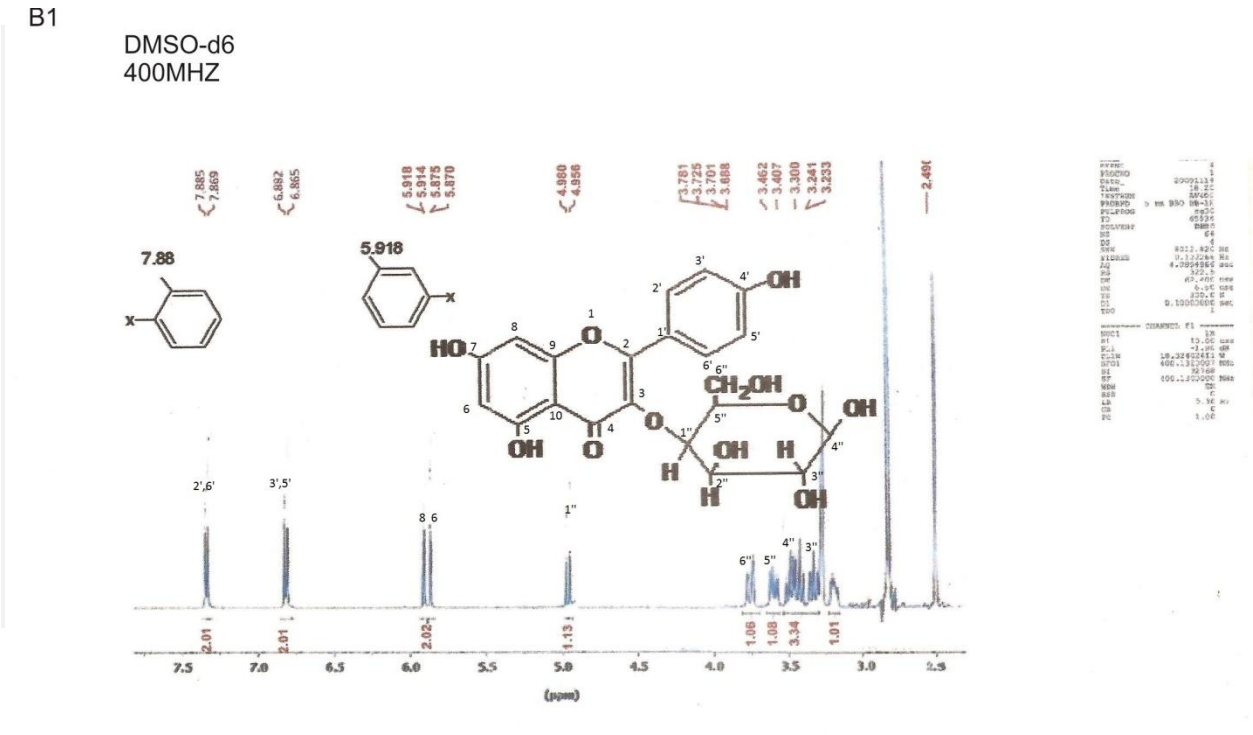


Fig 4.6 : Proton NMR of B1

4.6.3 ^{13}C-NMR Analysis of B1:

The ^{13}C-NMR(δ ppm, MEOH) experiment showed the presence of 21 carbon atoms, 15 carbon atoms for Aromadendrin aglycon while 6 for glucose.

158.5(C-2), 130.2(C-3), 177.3(C-4), 161.5(C-5), 98.8(C-6), 164.4(C-7), 92.8(C-8), 156.7(C-9), 103.3(C-10), 119.4(C-1'), 108.5(C-2'), 145.4(C-3'), 135.5(C-4'), 144.5(C-5'), 108.5(C-6'), 100.8(C-1''), 72.7(C-2''), 76.4(C-3''), 69.8(C-4''), 77.5(C-5''), 60.5(C-6'')

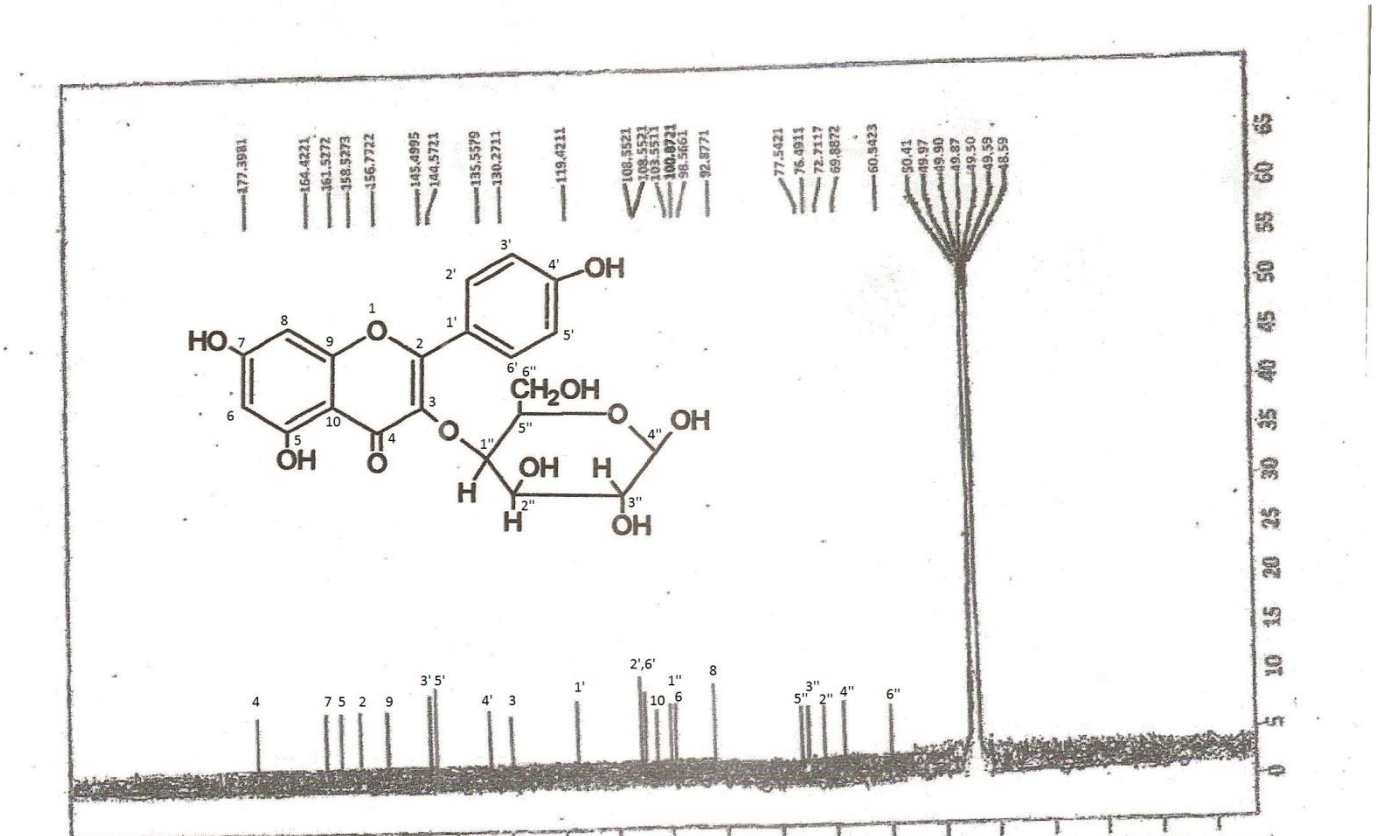


Fig 4.7 ^{13}CNMR spectra of B1

4.6.4 Mass spectrum Analysis of B1 :

The Ms spectrum of B1 revealed molecular ion peak at m/z 479(4%), the base peak at 125(100%), other prominent include 317(3%), 271(5%), 151(5%), 303(25%),119(20%)

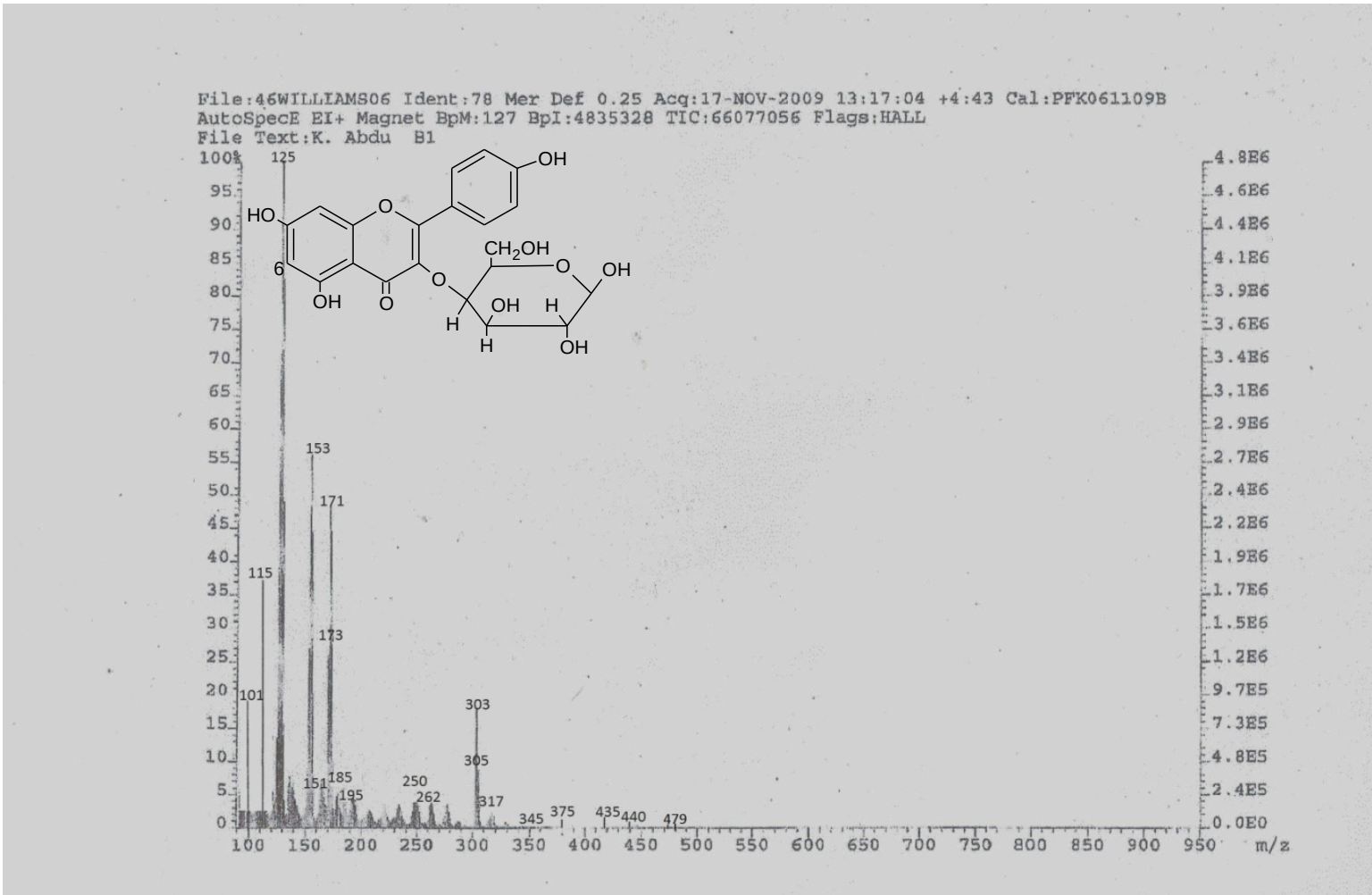


Fig 4.8: Mass spectra of compound B1

4.7 Spectral Analysis of Compound C1

4.7.1 ^{1}HNMR Spectral Analysis:

The ^{1}HNMR spectrum of C1 in DMSO-d6 400MHz shows the signals at δ 7.64 (1 H, dd, *J* = 8.8Hz, 2.0Hz) H -6′ indicate aromatic protons, and *J* = 8.8Hz refer to ortho coupling while 2.0Hz refer to para coupling. We have disubstituted Benzene ring (Ring A) of flavonoid nucleus.

The signal at δ 7.72 (1H, d, J = 1.6 Hz) H – 2′ indicate aromatic protons *J* = 1.6 Hz is meta coupling.

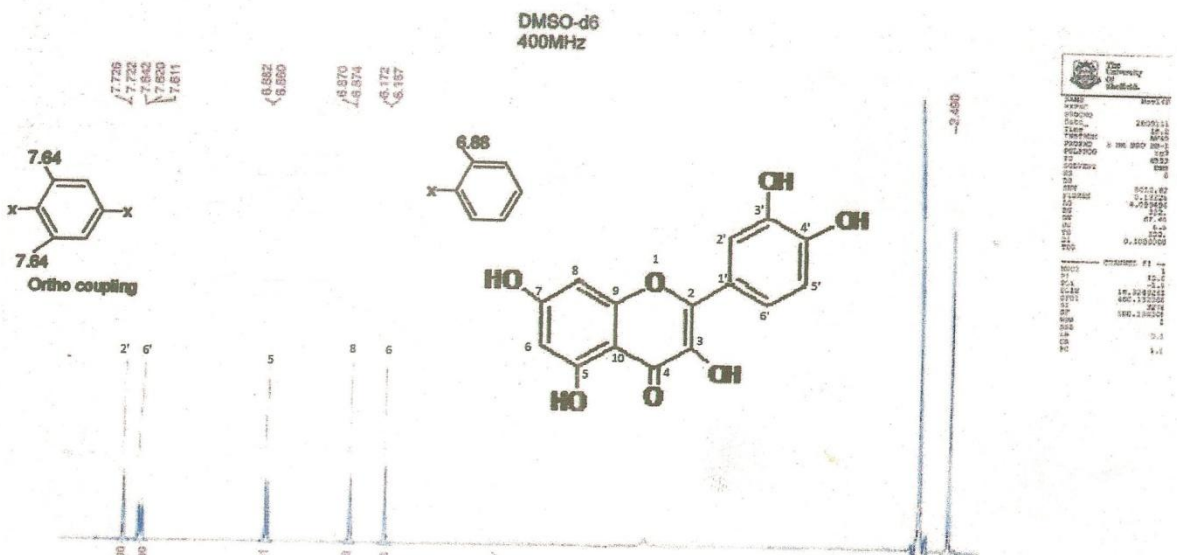


Fig 4.9: Proton NMR Spectra of C1

4.7.2 Mass spectrum Analysis of C1 :

The Ms spectrum of C1 revealed molecular ion peak at m/z 302(M^+,4%) while base peak at m/z 106 (100%), other prominent include m/z151(35%) , 127(6%), 153(5%), 271(5%),

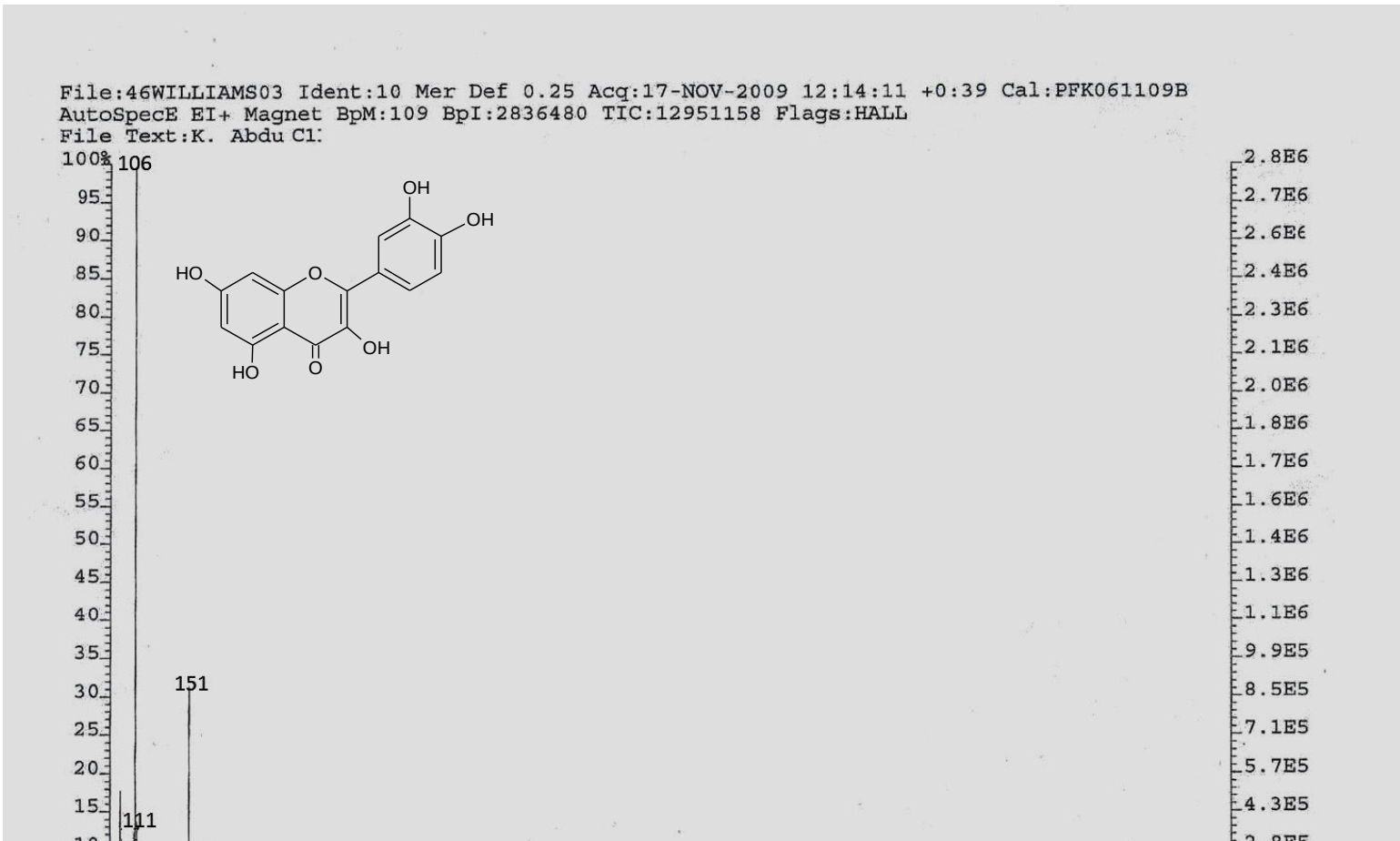


Fig 4.10: Mass spectra of compound C1

4.8 **Result of pharmacological studies**.

4.8.1 Acute toxicity studies.

The LD_{50} of the ethanol extract of *Chrozophora senegalensis* in mice (IP) was found to be 2154.1mg/kg

4.8.2 Effect of ethanol extract on acetic acid induced writhing in mice

The effect of ethanol extract of root of the plant on acetic acid induced whrithing in mice showed a dose- dependent inhibition and statistically significant at $p \leq 0.05$ as shown in Table 4.6

Table 4.6: Effect of Ethanol extract (EE) of root of *Chrozophora senegalensis* on acetic acid induced writhing test in mice.

Treatment	Conc(mg/kg)	Mean no. of writhing (± SEM)	% inhibition
Normal Saline	10	18.8 ± 5.1	0
EE	600	1.3 ± 1.9*	93
EE	300	1.7 ± 2.1*	91
EE	150	3.7 ± 4.1*	80
Ketoprofen	10	0.6 ± 0.8	97

The Result was expressed as mean ± standard error of the mean (SEM)

* P ≤ 0.05: Statistically significant from control (post Hoc Test)

4.8.3 Effect of the ethanol extract on carrageenan induced oedema in rats

The result of anti inflammatory studies is presented in Table 4.7. The aqueous solution of ethanol extract of *Chrozophora senegalensis* root administered at a dose of 600mg/kg induced a significant (p≤0.05) oedema inhibition (39.5%) at 135min, whereas 300 and 150mg/kg induced 30.2 and 27.9% inhibition of oedema respectively.

Table 4.7; **Effect of the ethanol extract on carrageenan induced oedema in rats**

TEST	**TIME INTERVALS IN MINUTES**					
GROUP	0 min	45 min	90 min	135 min	180 min	
Control	0.19±0.01	0.33±0.03	0.42±0.02	0.43±0.04	0.40±0.04	MV
(Normal saline)		42.4	57.8	58.8	60.5	%II
Ketoprofen	0.22±0.008	0.24±0.02	0.29±0.03	0.29±0.02	0.27±0.01	MV
10mg/kg		8.3	24	24	18.5	%II
		27.3	30.1	32.6	32.5	%I

EE 600mg/kg	0.21±0.01	0.26±0.02	0.26±0.01	0.26±0.01	0.25±0.01	MV
		19.2	19.2	19.2	15.3	%II
		21.2	38.1	39.5*	37.5	%I
EE 300mg/kg	0.22±0.008	0.27±0.03	0.32±0.03	0.30±0.05	0.27±0.03	MV
		18.5	31.3	26.7	31.0	%II
		18.8	23.8	30.2*	27.5	%I
EE 150mg/kg	0.21±0.01	0.25±0.02	0.33±0.03	0.31±0.03	0.29±0.02	MV
		20.0	39.4	33.3	31.0	%II
		24.3	21.4	27.9*	27.5	%I

Key: MV= Mean volume, %II = Percentage increase in inflammation

% I= Percentage inhibition of inflammation E.E = Ethanol extract of root of *Chrozophora senegalensis*

* $P \leq 0.05$: Statistically significant from control (post Hoc Test)

4.9 Result of Antimicrobial studies of Ethanol Extract, Ethyl acetate and n-butanol soluble fraction

The result of anti-microbial studies of the ethanol extract, ethyl acetate and n-butanol soluble fractions is shown in Table 4.8, expressing the zone of inhibition of bacterial and fungus growth. The extract and fractions showed considerable amount of inhibition against *E.coli, S. aureaus, Bsubtilis, P.auriginosa, C. albicans, T.mentagrophyte,* but ethanol extract and ethyl acetate has no activity on S. aureus at low concentration of 20mg/ml . The standard antibiotic disc used in this study inhibited the growth of test bacteria and *C. albicans* but inactive against *T. mentagrophyte.*

Table 4.8; Zone of inhibition (in mm) of Ethanol extract, ethyl acetate and n-butanol soluble fraction and streptomycin.

Organism	Ethanol		Ethyl acetate		n-Butanol		Streptomycin	
	I	II	I	II	I	II	III	IV
E.coli	19.0	24.0	19.0	24.0	15.0	16.0	30.0	32.0
S.aureus	0.0	19.0	0.0	19.0	17.0	19.0	32.0	33.0
B.subtilis	19.0	23.0	19.0	23.0	21.0	25.0	35.0	33.0
P aeruginosa	15.0	25.0	15.0	19.0	18.0	20.0	35.0	36.0
C.albicans	21.0	25.0	15.0	25.0	17.0	19.0	30.0	32.0
T. mentagrophyte	22.0	24.0	22.0	24.0	23.0	26.0	0.0	23.0

Key: I = 0.02 mg/kg, II = 0.05mg/kg, III = 0.02 mg/kg, IV = 0.05 mg/kg

Table 4.9 Minimum inhibitory concentration (MIC) in mg/ml of ethanol extract, ethyl acetate and n-Butanol fraction.

The test-organisms has MIC in mg/ml ranging from 0.06 to 0.25 using the extract and fractions

Test organism	**Ethanol Extract**	**Ethyl acetate fraction**	**N-butanol fraction**

E.coli	0.25	0.25	0.25
S. aureus	0.25	0.25	0.25
B. subtills	0.13	0.06	0.13
P. aeruginosa	0.13	0.13	0.25
C. albican	0.13	0.25	0.25
T. mentagrophyte	0.13	0.13	0.13

Table 4.10 Minimum Bactericidal / Fungicidal concentration (MBC)/(MFC) in mg/ml of Ethanol extract, Ethyl acetate and n-Butanol soluble fraction.

The test organisms has MBC/MFC in mg/ml ranging fom 0.25 to 1.0 using the extract and fractions

Test organism	Ethanol Extract	Ethylacetate fraction	N-butanol fraction
E.coli	0.50	0.5	0.50
S. aureus	0.50	1.0	0.50
B. subtills	0.50	0.13	0.25
P aeruginosa	0.25	0.50	0.50
C. albicans	0.25	0.50	0.50
T. mentagrophyte	0.25	0.25	0.25

CHAPTER FIVE

5.0 DISCUSSION

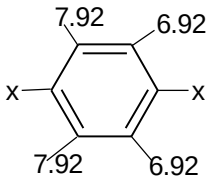
The preliminary phytochemical screening carried out on the root extracts of *Chrozophora senegalensis* revealed the presence of flavonoid, saponin, Tannins Alkaloids, phlobatannins Cardiac glycosides. These secondary metabolites have been shown to be responsible for the therapeutic activity of plants (Trease and Evans, 1996).

Also plants containing these metabolites usually demonstrate antimicrobial properties (Hung *et al.,* 2006) Tannins have been reported to inhibit growth of microorganism by precipitating microbial protein and making nutritional proteins unavailable to them (Ogunleye and Ibitoye, 2003). Saponins are special class of glycosides that have been shown to have antifungal potential (Sadipo *et al.,* 1991).

Column chromatographic separation of ethylacetate soluble fraction over silica gel led to the isolation of three compounds, coded A4, B1 and C1.

Chemical tests of isolated compounds using Shinoda and Ferric chloride gave positive test for flavonoids (Harbon,*et al*., 1975).

The ^{1}H-NMR spectrum of A4 in DMSO-d6 showed the following characteristics proton signals at; δ 7.92 (2H, d, *J*= 8.4 Hz) and δ 6.97 (2H, d, *J* = 8.0 Hz) which were assined to aromatic protons. The *J* value of 8.0 and 8.4 Hz indicate an ortho coupling for both protons and disubstituted benzene ring i.e.



Singlet at δ 6.11, 6.47 were assigned to H – 3, H – 6 respectively which are ring A – protons characteristics of a flavone. Protons at C-2′, 3′ ,5′ ,6′ due to free rotation of B-ring , appeared as two pairs of ortho coupled doublets. The H-3′, 5′ doublets occur up field from the H-2′,6′ doublet due to the shielding effect of the oxygen substituent and to the deshielding influence of C-ring functions on H-2′ and 6′.

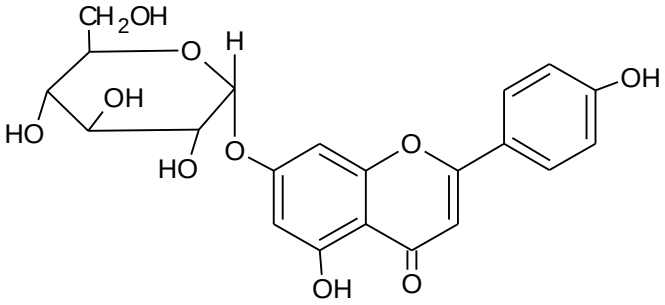
In ^{13}CNMR , a total of 21 Carbon atoms were observed which agree favorably with aglycon moiety of apigenin and glucose. 15 for apigenin while 6 for glucose.

The deshielded signal at 181.54 ppm was assign to C – 4, other down field signals were assigned similarly 160.9 (C – 5), 161.0 (C – 4′), 163.6 (C – 7). The signals at 78.0 for C-3′′ and 77.5 for C-5′′ indicate a glucose moiety.

The fragmentation pattern obsreved in the mass spectrum showed moeity m/z at 271 which corresponds Apigenin-7-0-glycoside (m/z 270) , 256 $[270+CH_2]^+$, 153$[A1+H]^+$, 121$[B2]^+$, It is reported in the literature Apigenin-7-glucoside has the following fragments;

M/z ; A^+(m/z 153), B^+(m/z 119) (Victoria, *et al..,* 2009)

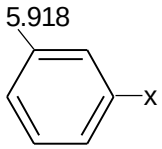
M/z 270 [aglycon fragment], 256 $[270-CH_2]^+$, 153[A1+H] ,121[B2]+ , 94$[B2+H+CO]^+$ (Yassan, *et al..,*2007



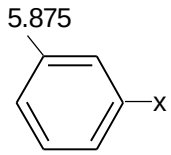
Compound A4 – Apigenin glycoside

The ^{1}HNMR spectrum of B1 in DMSO-d6 (indicates the aglycon moiety of Aromadendrin and glucose) showed the following characteristic proton:

Signals at δ 5.918 (IH, d, *J*= 1.6 Hz) H – 8 assigned to aromatic protons, *J* – value = 1.6 Hz show meta coupling.

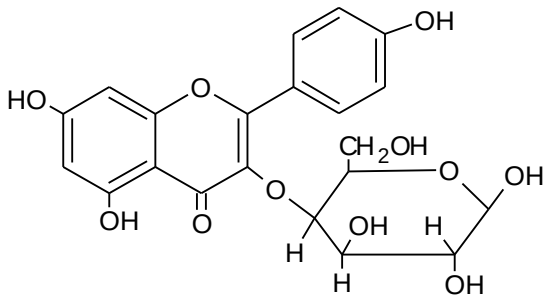


Also signal at δ 5.87 (IH, d, *J* = 2.0Hz) H – 6 shows aromatic proton. *J* = value = 2.0Hz indicate meta coupling.



Both the protons H-8 and H-6 are aromatic protons of ring A system of flavonoid nucleusIn the ^{13}C NMR, there are total of 21 carbon atoms, 15 for Aromadendrin while 6 for glucose. In the spectrum we have signals at 130.2 (C-3), 164.4(C-7), 119.4(C-1'), 136.7(C-4'), 100.5(C-1''), 73.7(C-2'') for Aromadendrin aglycon while at 76.6 for C-3'' and 77.2 for C-5'' showing glucose moiety. In the mass spectrum of the compound (B_1) there is peak at m/z 151(5%),153(55%), 195(4%),125(100%) The comparison of this peak with the one reported in the literature indicate Aromadendrin (Tsukasa *et al.,* 2007). Based on spectroscopic data analyzed the compound is identified as; 5,7-dihydroxy-2-(4-hydroxyl phenyl)-4-chromenone-3-glucose.

Structure of Aromadendrin glucoside is shown below

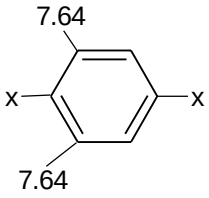


5,7-Dihyroxy-2-(4-hydroxyphenyl)chromen-4-one-3-glucose

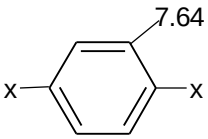
(Aromadendrin glucoside)

Compound C_1 was isolated as a brownish yellow amorphous solid (10mg), has melting point 317^0C, but from the literature it has melting point of 316^0C (Aarti *et al*., 2012) and Rf value of 0.6. The ^{1}HNMR spectrum in DMSO-d6 400MHz shows the following resonance δ value

The signals at δ 7.64 (1 H, dd*, J* = 8.8Hz, 2.0Hz) H -6' indicate aromatic protons, and *J* = 8.8Hz refer to ortho coupling while 2.0Hz refer to para coupling.



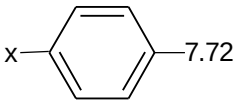
Ortho coupling



Meta coupling

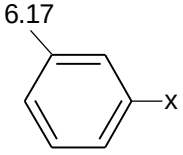
We have disubstituted Benzene ring (Ring A) of flavonoid nucleus.

The signal at δ 7.72 (1H, d, J = 1.6 Hz) H – 2′ indicate aromatic protons *J* = 1.6 Hz is meta coupling.

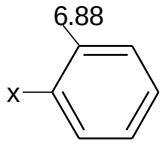


1H, d, *J* = 1.6 Hz refer to H-2' protons of Ring B.

The signals at δ 6.17 (IH, d*, J* = 2.0Hz), H- 6 indicate an aromatic proton and *J*-value of 2.0Hz indicate meta coupling.

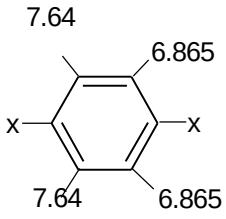


The signal at 6.88 (IH, d, J = 8.8 Hz) H- 5 indicate aromatic proton, *J*- value of 8.8Hz an ortho coupling



The signals at δ 7.64 (IH, dd, J= 8.8, 2.0 Hz), 7.72 (IH, d, J = 1.6Hz) are characteristics of a 1,2,4 trisubstituted benzene ring (The ABC system with ortho and meta coupling, the B-ring of the flavonol) and two doubles at δ 6.17 (IH, d, *J* = 2.0Hz) δ 6.37(1H,d,*J*= 2.0) and δ 6.88 (1H , d, *J* = 8.8Hz) characteristics of a 1,2,3,5 tetra-substituted benzene ring (the AB system with meta coupling, the A – ring of the flavonol) . The combination of the substitution pattern of the A and B rings suggested that the compound is 3', 4', 5, 7 Tetrahydroxy flavonol (quecetin).

The signals at δ 7.64 (2H, d, *J* = 6.4 Hz) H – 2', 6' indicate aromatic protons, *J* –value = 6.4Hz shows ortho coupling. The signals at δ 6.86(2H, d, *J* = 6.8 Hz) H – 3', 5' are also aromatic protons, *J* – value of 6.8 Hz) also shows ortho coupling. i.e. The H-2',6',3',5' are protons of ring B of flavonoids nucleus.



Based on the above spectroscopic data the compound is quercetin

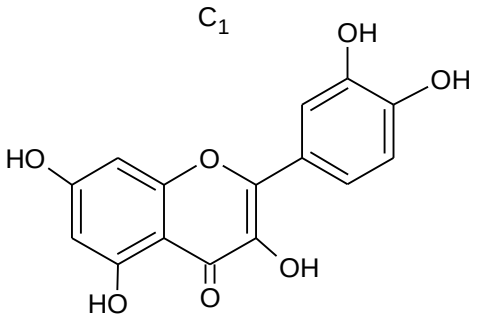
The mass spectra shows molecular ion peak at m/z 302 which represents quercetin (302+H)

The result of mass spectroscopy is shown below;

m/z 302(M^+,4%) 106 (100%), 151(35%) , 127(6%), 153(5%), 271(5%),

It is reported in the literature that quercetin has the followings fragments;

m/z: 302(M^+, 100%), 301(46%),285(4%), 284(3%), 274(6%), 273(9%), 153(5%), 152(10%), 150(4%), 137(6%), 136(17%) (Bahman *et al*., 2003)



Compound C $_1$- Quercetin

The UV Spectra analysis of compounds A4 reveals that the compound (A4) was maximally absorb at λmax 268, 333nm. This seem to be in agreement with values reported by Hashem *et al.,* 2007 for apigenin 7-O- glycoside (λmax:268,332) , also reported in the literature that Apigenin-7-O-glucoside has λmax(MeOH) = 270,335 (Yassan *et al.*., 2007)

Compound B1 showed a λmax of 268, 353 comparable to the values recorded for Aromadendrin- 3- glucoside, λmax(nm) mthanol 290, 330(Tsukasa *et al*.,2007)

In the first phase of LD_{50}, none of the animals die while in the second phase the dose of 1600mg/kg did not kill the animals but the doses of 2900 and 5000mg/kg kill the animals.

Therefore preliminary acute toxicity studies suggest that, intraperitonially, the crude extract (LD_{50} 2154.1) are moderately toxic according to classification of toxicity (Loomis and Hayes, 1996) it is therefore recommended that these drugs should be used with caution.

The acetic acid induced writhing in mice; shows that 600mg/kg of ethanol root extract of *Chrozophora senegalensis* is 93% (% inhibition) while 300mg/kg was 91%, the percentage of inhibition falls to 80% with 150mg/kg. Using a standard drug which is ketoprofen (10mg/kg) the Percentage inhibition was 97%. While no inhibition was observed with normal saline which is negative control. The analgesic effect of the extract may be either due to its action on visceral receptor sensitive to acetic acid, to the inhibition of the algogenic substances or the inhibition of the algogenic substances or the inhibition of transmission of painful message at the central level. (Hosseizadch *et al*., 2002).

The acute anti-inflammatory effect was evaluated by carrageenan-induced hind paw edeme.The result shows that after 3hrs, ketoprofen (10 mg/kg) has 32.5% inhibition of inflammation ,while 600 mg/kg of the extract has 37.5%, 300 mg/kg has 27.5% and 150 mg/kg has 27.5%.

The ability of the extract on carrageenan induced inflammation is as a result of inhibition of the enzyme cyclo-oxygenase and subsequent inhibition of prostaglandin synthesis. Significant inhibitions of paw oedema in the early hours of study could be attributed to the inhibition of histamine and/or serotonin (Nemat *et al.,* 2013).

Both the result of analgesic and Anti inflammatory studies were analysed using ANNOVA and are statistically significant at P less than 0.05

The extract possesses both anti-inflammatory and analgesic activities probably mediated through a common mechanism. Anti-inflammatory and Analgesic activities are commonly possessed by the non-steroidal anti-inflammatory drugs (NSAIDs).These NSA-IDs exert anti-inflammatory effect principally by inhibiting the synthesis of prostaglandins (Vane, 1971) an eicosanoid mediator of the inflammatory response (Foegh and Ramwell,2001)

The study shows that the extract of the plant has antimicrobial property which is due to the phytochemical constituents which include flavonoids, saponins, tannins etc. present in the plant extract.

The antimicrobial screening are recorded in Table 4.11 expressing the zone of inhibition of bacterial and fungus growth. The extract and fractions showed considerable amount of inhibition against *E coli, S. aureus, B subtilis, P. auriginosa, C albicans, T. mentagrophyte* but ethanol extract and ethyl acetate has no activity on *S. aureus* at low concentration of 20 mg/ml. The standard antibiotic disc used in this study inhibited the growth of the test bacteria and *C. albicans* but inactive against *T. mentagrophyte*.

Ethanol extract has MIC 0.13mg/ml on all the organisms tested except on *E.coli* and *S.aureus* which has MIC of 0.25mg/ml each . Ethyl acetate has MIC of 0.25mg/ml on *E.coli, S.aureus and C. albicans* each while 0.065mg/ml on *B. subtilis* and 0.13mg/ml on *P.aureginosa* and *T. mentagrophyte*

The minimum bacteria / fungicidal concentration of ethanol extract, ethyl acetate and n-butanol fraction was found to be 0.50mg/ml against *E. coli*, also 0.50mg/ml against *S . aurues* for ethanol extract and n-butanol fraction but 1.0mg/ml for ethyl acetate fraction. The MBC of 0.50mg/ml was also obserbed on *P.aeruginosa* using ethyl acetate and n-

butanol fraction but 0.25mg/ml using ethanol extract. The MFC of 0.25mg/ml was obserbed on *T. mentagrophyte* using the extract and fraction so also *C. albicans* but 0.50mg/ml using ethyl acetate and n-butanol

CHAPTER SIX

6.0 SUMMARY

The preliminary phytochemical screening revealed the presence of carbohydrates, flavonoids, saponin and tannin. Chromatographic purification led to the isolation of three compounds whose structures were confirmed by the use of ^{1}HNMR, ^{13}C NMR , mass spectra and UV spectroscopy with comparison with literature value, as Apigenin Glycoside, Myricetin Glycoside Quercetin.The acute toxicity studies for ethanol extract in mice (IP) were found to be 2154.1 mg/kg. The ethylacetate, ethanol and n-butanol extract shows a remarkable antimicrobial activity producing zones of inhibition ranging from 15.0 to25.0 mm. The effect of concentration 600 mg/kg extract (ethanol) of *Chrozophora senegalensis* root on acetic acid induced writhing in mice were found to be 93 % , compared to ketoproten (10mg/kg), standard drug used as reference which was 97%. Also the effect of this plant extract (600mg/kg) on carrageenan-induced rat hing paw oedema were found to be 37.5% while ketoprofen (10mg/kg) was found to be 32.5%.

6.1 CONCLUSION

It can be concluded that the effect of the extract of *Chrozophora senegalensis* root to inhibit the growth of micro-organism and on acetic acid induced writhing in mice and on carrageenan-induced rat hing paw oedema test justify the traditional use of the plant. This research work has led to the isolation of three flavonoids for the first time from root of this plant which includes apigenin glycoside that has anti-inflammatory properties, myricetin glycoside and quercetin. To the best of our knowledge, this is the first time in which root extract of *Chrozophora senegalensis* is reported to have anti microbial, analgesic and anti inflammatory activities.

6.2 RECOMMMENDATION

Further research need to be conducted on this plant in order to identify more compounds that can be used in treating ringworm or rheumatism. Antidiarrheal studies of leaves

extracts of the plant be studied since it is used in treating green diarrhea in children. The isolation of other metabolites apart from flavonoids is recommended.

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