PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF, STEM BARK AND ROOT BARK EXTRACTS OF *STACHYTARPHETA ANGUSTIFOLIA* (MILL) VAHL.

(*VERBENACEAE*)

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JUNE, 2014

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(*VERBENACEAE*)

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Ph.D/Pharm Sci/00118/2006-07

BEING

A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL AHMADU BELLO UNIVERSITY ZARIA, NIGERA

IN PARTIAL FULFILMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY.

DEPARTMENTOF PHARMACEUTICAL AND MEDICINAL CHEMISTRY, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

JUNE, 2014

DECLARATION

I do solemnly declare that, this dissertation entitled **PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF, STEM BARK AND ROOT BARK EXTRACTS OF *STACHYTARPHETA ANGUSTIFOLIA* (MILL) VAHL. (*VERBENACEAE*)** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry under the Supervision of Dr. U. U. Pateh, Prof. M. Ilyas and Dr. A. M. Musa.

This dissertation has not been accepted in any previous application for higher degree elsewhere. The work of other investigators are acknowledged and referred to accordingly.

____________________ __________________ ______________

Name of Student Signature Date

CERTIFICATION

This Dissertation entitled the **PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES OF THE LEAF, STEM BARK AND ROOT BARK EXTRACTS OF *Stachytarpheta angustifolia* (MILL) VAHL. FAMILY (*VERBENACEAE*) by Musa Mohammed** meets the regulations governing the award of Doctor of Philosophy in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University Zaria, and it is approved for its contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENT

I do not have adequate word or phrase to express my profound gratitude to ALLAH (S.W.T) for granting me the opportunity throughout my endeavour including this research work. May His blessing be upon the last prophet (P.B.U.H).

Indeed, I wish to express my humble and sincere gratitude to Dr. U.U. Pateh, who designed and supervised this project. I am really grateful for all his contributions in the course of making this work a success. My co-supervisor, Prof. M. Ilyas is worth mentioning here. His contribution to the success of this work cannot be over emphasized. He epitomizes the masculine excellence of an intellectual coupled with patience in the course of scrutinizing this work. Oh! I want to say thank you sir.

I must say this with the greatest reverence to Dr. A. M. Musa who has been my immediate mentor; he has been an indispensable radar throughout every bit of this work. He has not only supervised the work thoroughly but critically, painstaking and constructively exposed me to the knowledge beyond this level of my academic pursuit. Oh! Thank you.

Mall. Ibrahim Adamu, Assistant chief technologist with Department of Pharmacology is worth mentioning here. I am highly indebted to Mall. Iliya Salisu , the chief technologist of the Department. This is for the provision of a conducive laboratory space and facilities without which the phytochemical aspect of this work would have not been a reality.

A big kudos to my colossal friends in persons of Dr. Adeiza, A. A (C.A.A.S) A.B.U Kaduna, Dr. Musa Y. Maikafi, of (C.A.S.) Lafiya Nasarawa state , Dr. Awwal Tijjani of Uni. Maiduguri and Dr. Musa G. Abdullahi of school of Health Tech. Kaduna state. So also to my well wishers and rest of my friends who have contributed in one way or the other to make this piece a bold reality.

I would like to acknowledge the good gesture of Ahmadu Bello University Zaria for giving me the opportunity to carry out this work. My regard also to Prof . Simon Gibbons of Centre for Pharmacognosy and Phytotheraphy, university of London for his assistance in the structural elucidation of these spectra.

In fact, to my parents, I have every cause to say “Rabbi irhamhuma kama rabbayani sagiran” (Oh Allah have mercy on them as they bring me up when I was an infant)

Lastly, to my humble wife, I say thank you.

“AL-HAMDULILLAH”

DEDICATION

This research work is dedicated to my beloved children.

Fatima Z. Musa

Hawwa'u Musa

Abdul Aziz Musa

Muhammad S. Musa

Abubakar S. Musa

Ibrahim Musa.

ABSTRACT

The extracts of the leaf, stem bark and root bark of *Stachytarpheta angustifolia* (Mill) Vahl. (Verbenaceae), were subjected to preliminary phytochemical screening and the result revealed the presence of carbohydrates, steroids, cardiac glycosides, terpenoids, flavonoids, tannins and Saponins. Silica gel column chromatography of n- butanol soluble fractions of the leaf, stem bark and root bark extract followed by gel filtration using sephadex LH_{20} and preparative thin layer chromatography resulted in the isolation of two iridoid glycosides 1-[3,4-dihydroxy-5-(3-hydroxy-cycloprop-1-enyloxy)-6-hydroxymethyl-tetrahydro- pyran- 2-yloxy]- 7-methyl-1, 4a, 5, 6, 7, 7a hexahydrocyclopenta [c] pyran -4-carboxylic methyl ester and 4a, 7 – dihydroxy – 7 – methyl – 1 – (3, 4, 5 – trihydroxy – 6–hydro methyl – tetrahydropyran – 2 – yloxy) – 1 -, 4a, 5, 6, 7, 7a, hexahydro – cyclopentano [C] pyran – 4 – carboxylic acid methyl ester from the leaf and root bark respectively, while -β – (3', 4' – dihydroxy phenyl) – ethyl – O - α - L – rhamnopyranosyl (1 → 3) – β – D – (4 – O - Caffeoyl) – glucopyranoside was isolated from the stem bark. The structures of these compounds were elucidated using chemical tests, spectroscopic techniques (FTIR, ID and 2D NMR) and by comparison with reference data. The ethanol leaf extract and its n-butanol and residual aqueous fractions exhibited concentration dependent contraction of the rabbit jejunum which was blocked by atropine, suggesting that the observed pharmacological effect were mediated through muscarinic receptors. In contrast, chloroform and Ethylacetate fraction of the ethanolic root bark extract exhibit concentration dependent relaxation of the rabbit jejunum. The ethanol leaf extract *was* screened for antimicrobial activity using disc diffusion and agar well dilution methods. The organisms tested were clinical isolates of *S. aureus*, S. pyogenes, *E. coli* and *S. typhi*. The ethanol extract inhibited the growth of all the microbes. The zone of inhibition ranging from 10-30mm). Conclusively, the use of *S. angustifolia* as a remedy for venerable infection by the folkloric healers has scientific basis .

Keywords: *Stachytarpheta angustifolia, Antimicrobial activity, Gastrointestinal activity.*

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LIST OF ABBREVIATIONS

Ach.	Acetylcholine
Atr.	Atropine
α	Alpha
Aq	Aqueous
β	Beta
^{13}C	Carbon-13
$CHCl_3$	Chloroform
Conc.	Concentration
C.N.S	Central nervous system
^{0}C	Degree Centigrade (Celsius)
$CDCl_3$	Deutrated chloroform
DEPT	Distortion less Enhancement by Polarization transfer
$FeCl_3$	Ferric chloride
g	Gramme
^{1}H	Hydrogen proton
HCl	Hydrochloric acid
HMBC	Homonucleir Multiple Bond Correlation
HSQC	Heternucleir Spin Quantum Correlation
I_2	Iodine
LD	Lethal dose
LBR	Liebermann Buchard Reagent
MH_Z	Megahertz

CD_3OD	Deutrated methanol
CH_3OH	Methanol
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
ml	Millilitre
NMR	Nuclear magnetic resonance
%	Percentage
cm^{-1}	per centimetre
KCl	Potassium chloride
KOH	Potassium hydroxide
H_2SO_4	Tetraoxosulphate (VI) acid
TLC	Thin Layer chromatography

CHAPTER ONE:

INTRODUCTION

1.0 General Introduction

The use of plant derived products by man as medicine dates back to pre-historic times. It was believed that at the beginning of creation, God made plants and herbs before creating man. This was done with the injunction that man should eat from the plants and herbs for his good health. Henceforth, herbs have been in use from antiquity and were the first medicine used by man (Kloss, 1992). From time immemorial man and animals have had to distinguish between those plants which are poisonous and those that are not, and they gradually developed the knowledge of the naturally occurring drugs which was transmitted at one time orally, later on as baked clay tablets, parchments, man scripted herbals, printed herbals, pharmacopoeias and most recently by computerized information – retrieved system (Trease and Evans, 2002). About half of the world's medicinal compounds are derived or obtained from plant (Geoffrey *et al.*, 2001). Nature has been a source of medicinal agents since times immemorial. The medicinal products from plants and other biota are in general more important to developing countries than industrialized nation, where the focus is very much on chemical discovery and synthesis of pharmaceutical drug products from biota as major contribution to human health service (Akerele, 1991).

Despite the immense technological advancement in modern medicine, a lot of African, approximately 80% of the population still relies on traditional healing practices and medicinal plants for their daily health care needs (Sofowora, 2008). The floral biodiversity of Africa provides the African traditional medical practitioner with an impressive 'natural pharmacy' from which plants are selected as remedies or as

ingredients to prepare herbal medicine (phytomedicines) for various human ailments (WHO, 2005). The Ayurvedic medicine preparations comprise of medicinal plants, mineral and organic matter. These are essentially primitive but are also preventive in therapeutic approach. Medicinal herbs constitute indispensable components of traditional medicine practiced worldwide due to low cost, easy access and ancestral experience (Marini-Bettolo, 1980).

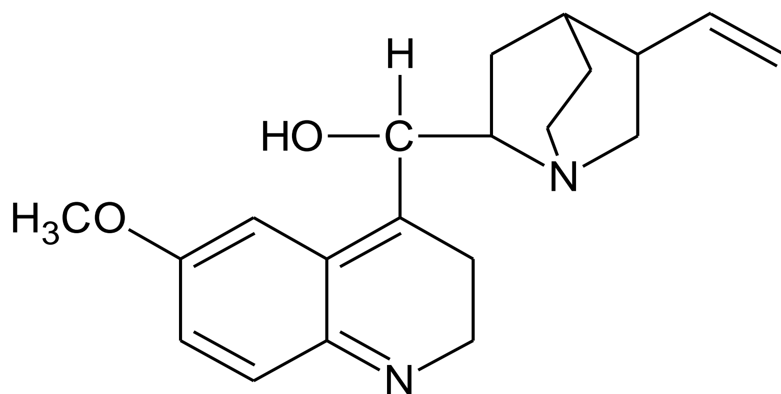
The use of traditional herbs in Nigeria for the treatment of various ailment has been fully accepted, this is because it serves as an alternative means of curing different diseases and as such its importance cannot be over emphasized. The historic period has it that, traditional medicine in most countries involves the use of medicinal herbs in their crude forms. The herbs are prepared in form of decoctions, infusion or powder to be taken and which has been in practice to date (Sofowora, 2008 ; Adjanohoun, 1991). Traditional medicines are an inspirational part of our health because it is the total sum of knowledge, skills and practice based on the theory, belief and experience used in maintaining good health as well as curing diseases using medicinal plants (Sofowora, 2008 ; Verporte *et al.,* 1987). Medicinal plants are define as those in which one or more of their parts contain metabolites that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. The medicinal properties of drug plants are dependent on the presence of these chemical substances known as the secondary metabolites. Secondary metabolites are constituents synthesized by the plant in addition to its basic metabolites, which may be concentrated in different part of the plant. Some of these compounds include saponins, glycosides, flavonoids, anthraquinones, alkaloids, steroids and terpenoids. Others include coumarins, tannins, gum and mucilage, volatile oil and organic acids. These chemical constituents are dominant in different drug plants given it a unique chemistry which differs from one plant to another at varying concentrations.

The phytochemical examination of all these constituents has been made possible and easier by the improved method of extraction, separation, isolation and characterization (Farnsworth and Soejarto 1991).

It is evident, that the plant kingdom still holds many species of plants containing substances of great medicinal value which have just been discovered or yet to be discovered (Farnsworth, 1996). Large numbers of plants are constantly being investigated for their possible pharmacological values (Trease and Evans, 2002).

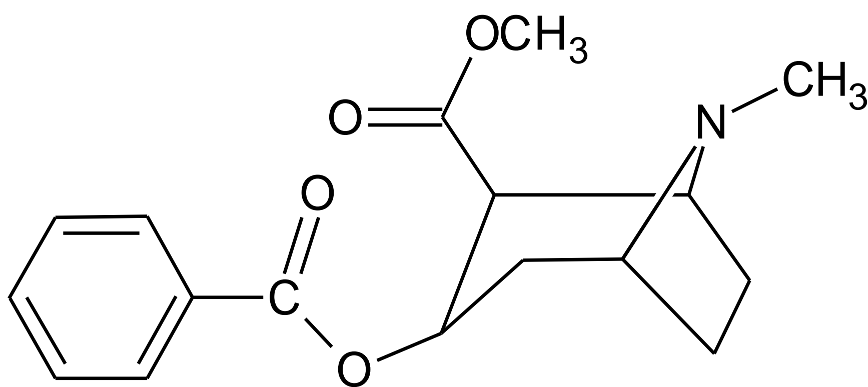
Plants may be regarded as a biosynthetic laboratory that produces or synthesizes a large variety of chemical compounds during its metabolic activities. These include compounds that are utilized by man and other animals to exert physiological effects on them (Richard, 1998). Some of the plants studied were found to possess antimicrobial activity. These include *Plumbago zeylanica* L., *Mitracarpus scaber* Zucc, *Euphorbia hirta* L, *Alstonia boonei* De Wild and *Eupatorium odoratunm* L. (Ekpendu *et al.,* 1989). Some plants are also known to combat and expel or destroy intestinal worms e.g. *Carica papaya* (Sofowora, 2008).

World Health Organization (WHO) defined Traditional herbs as finished, labeled medicinal products that contain as active ingredient from the aerial or underground part of a plant or other plant material or combination thereof whether in crude state or as plant preparation. Drugs from natural sources may fall into one of these categories of compounds: those that are isolated from natural source, those that are modified version of the natural products and those that are completely synthetic yet based on models of natural origin (Akerele, 1991). A recent survey revealed that 61% of 877 drugs introduced worldwide can be traced to or were inspired by natural products (Rouhi, 2003). Plants in the tropics are often used as direct source of drugs, for example, the anti malarial quinine (I) from *Cinchona species*.



I

Plants from tropics have produced compounds that served as lead for the synthesis of new drugs e.g cocaine (II) from *Erythroxylum coca* has served as lead compound for the synthesis of a number of local anesthetics such as procaine (III) and lidocaine (IV) (Cragg., *et al.*, 1997).



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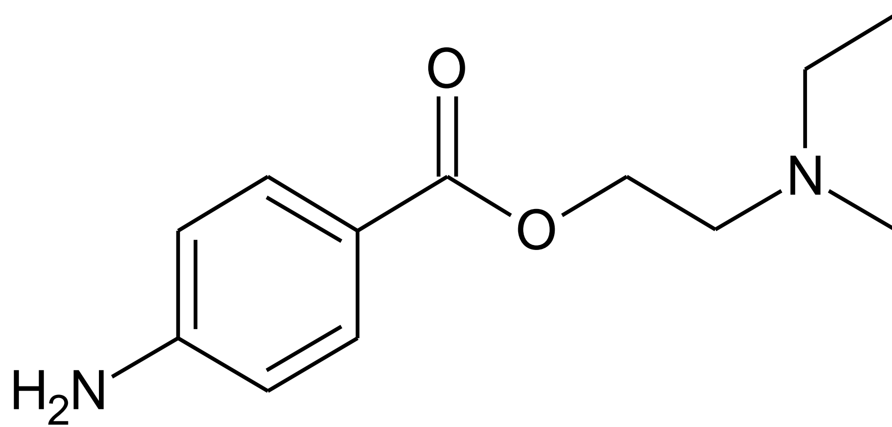

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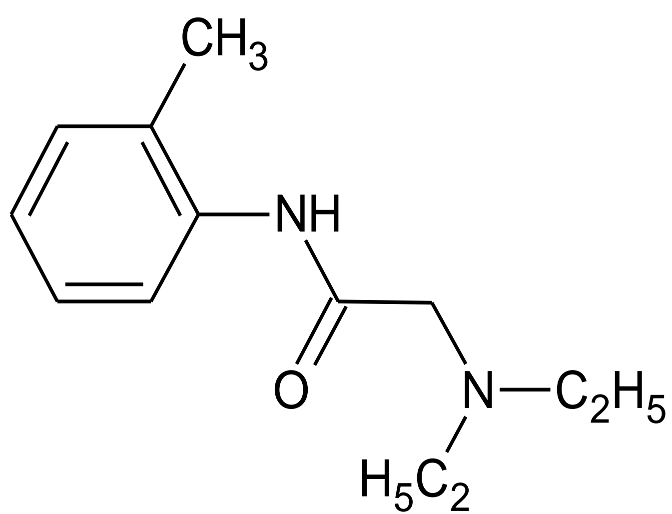


IV

An alkaloid, echitamine obtained from *Alstonia boonei* De wild and Scopolamine, isolated from the fruit of *Tetraptera Taub* have been reported to possess antispasmodic activity (Nwodo and Botting 1983).

1.1 BACTERIAL INFECTIONS AND LAXATIVE PROPERTIES

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

Laxative agents have smooth muscles relaxation properties and are used to decrease gastrointestinal motility, inhibit acid secretion and to relieve pain associated with diarrhea and other gastrointestinal disorders (Chris, 2006). The present study was prompted by the claim of Hausa traditional healers in Nigeria that aqueous portion of the plant has some laxative properties; this is with a view to validate the folkloric claim.

1.2 STATEMENT OF RESEARCH PROBLEMS

Despite the tremendous progress in drug development, disease caused by bacteria, virus and parasite are still major threat to the public health sector. Antimicrobial resistance and gastrointestinal problems are some of the biggest challenges facing global health sector, hence there is need to develop new antimicrobial and laxative agents that are highly effective, affordable with less toxicity. This has craved the need to search for biologically active compounds from plants that have been reported for use in condition related to bacterial infections as well as laxative agents, one of such plant is *Stachytarpheta angustifolia* .

1.3 AIM OF THE STUDY

The aim of this research work is to isolate some of the bioactive principles present in the leaf, stem bark and root bark extract of *Stachytapheta angustifolia* and to structurally elucidate these compounds using some phytochemical and spectroscopic techniques.

1.4 GENERAL OBJECTIVES OF THE STUDY

The primary objectives of this study were to identify, isolate and structurally elucidate bioactive compounds from the leaf, stem bark and root bark of *Stachytarpheta angustifolia* using standard phytochemical protocols. Secondly, to investigate the Laxative properties using pharmacological models and thirdly, using clinical isolates to determined the antimicrobial properties to validate the ethno medicinal uses of the plant.

1.5 SPECIFIC OBJECTIVES OF THE STUDY

Specific objectives 1:

- Collection and identification of the plant.
- Extraction of the plant material with organic solvents
- Preliminary phytochemical screening of the crude petroleum ether and ethanol extract.
- Isolates bioactive compounds from the selected fractions
- Identify and determine the chemical structures of the isolated compounds using spectral analysis.

Specific objectives 2:

- Determine acute toxicity of the crude ethanol extract
- Determine the gastrointestinal properties of the crude ethanol extract

Specific objectives 3:

- Determine the antimicrobial activity of the crude ethanol extract and other fraction using clinical isolates.

1.6 STATEMENT OF RESEARCH HYPOTHESIS

Stachytarpheta angustifolia contains compounds with laxative and antimicrobial activity.

1.7 JUSTIFICATION

Medicinal plants are believed to be important sources of new chemical substances with potential therapeutic effect (Farnsworth *et al.,* 1996). The research into plants with alleged various folkloric uses in the cure of various ailments should therefore be viewed as logical in the search for the treatment of various ailments (Elisabethsky *et al.,* 1995).

The World Health Organization recognized many herbal remedies and hence encouraged the developing countries to incorporate the use of folkloric plants due to their availability and affordability apart from being effective alternative to allopathic practice (WHO, 2005 ; WHO,1996). In Nigeria today, ethno medical treatment using herbal remedies has succeeded in the treatment of diseases like diabetics, mental disorder, breast cancer, sickle cell anemia and other forms of viral infections (Sofowora 2008). The synthetic laxative and antimicrobial drugs are associated with serious side effects, so the use of naturally occurring compounds may assist in reducing some of the undesirable effects. So also, most of the synthetic laxative and antimicrobial drugs are very expensive and unaffordable to majority of the populace especially in Africa. Therefore, the abundance of this plant within the African region will go a long way in providing cheaper reliable and affordable substitute.

Stachytarpheta angustifolia has been used in the management of some diseases related to bacterial infections and conditions related to gastrointestinal problems by the folkloric healers. Literature search shows that no much work has been reported on this plant. This prompted the screening of this plant to validate the folkloric claim.

CHAPTER TWO

2.0 LITERATURE REVIEW

The family Verbenaceae is generally known as the Teak family. It is a family of about 100 genera and 3000 species. In Nigeria, there are ten genera and two species which include *S. angustifolia* (Trease and Evans, 2002).

2.1 *Description of the family*

There are about 10 genera of this family found in Nigeria among which are *Stachytarpheta* e.g. *Stachytarpheta angustifolia.* Members of this family are composed of herbs, shrubs and trees while many are lianas found mostly in tropical, sub-tropical regions of Africa and other parts of the world (Keay, 1989).

2.2 Morphology of *Stachytarpheta*

The genus *Stachytarpheta* (Vahl) consist of about 100 species, most of which are shrubs or herbs (Dalziel, 1999). They have a long and cylindrical calyx, 5 – ribbed, 5 – toothed often variously slit in a later stage. They have a slender corolla tube, cylindrical limb patent with 5 – orbicular equal or unequal lobes. They have perfect stamen, 2 (anticuous pair) included in the corolla – tube.The cells are divaricates; ovules solitary attached lately near the base of the cells, style long filiform; stigma capitates, fruit cylindrical, separating into two long and a narrow truncate Pyrenees. The seeds are linear and exalbiminous. The leaves are usually opposite and petiole toothed. Inflorescence with a long spike and rachis usually thickened and hollowed out so as to form a cavity in which the flowers are immersed, while the bracts lanceolate are rigid and persistent. Corollas are blue, red or woody stem at least below or herbaceous with inflorescence up to 40cm (Dalziel, 1999 ; Burkill, 1995).

Plate 2.1 *Stachytarpheta angustifolia* (Mill) Vahl; Verbenaceae

2.3 TAXONOMY OF *Stachytarpheta angustifolia*

KINGDOM	=	Plantae
DIVISION	=	Angiosperm Dicotyledonous
SUB – CLASS	=	Sympetalae
ORDER	=	Tub florae
FAMILY	=	Verbenaceae
GENUS	=	*Stachytarpheta*
SPECIES	=	*Stachytarpheta angustifolia*
AUTHOR	=	(Mill) Vahl

Common Names: The plant is known as devils coach whip, verbena or Bastard Vervain (Trease and Evans, 2002). In Nigeria, the Hausas called it Tsarkiyar kusu or Wutsiyar Kadangare, while the Yorubas called it Iru – Alangba or Iru – Amure (Jinju, 1990).

Stachytarpheta angustifolia flowers around July to November. Each flower has a calyx – tube, fused to the ovary forming a simple style. Fruits start to emerge around September to December. This plant is a common weed of farm land (Burkill, 1995).

2.4 Distribution

Stachytarpheta angustifolia is widely distributed in the Northern and southern part of Nigeria. It is also found in Gambia (Hayes), Mali (Kabarah), Guinea Bissau (Kouroussa), Ghana (Achimota, Accra plains) and in Senegal (Pevrottet) and other parts of the world (Dalziel, 1999).

2.5 Ethno Medicinal Uses of *Stachytarpheta angustifolia* and other Species in the Genus *Stachytarpheta*

The decoction of the whole shrub mixed with trona is taken as a remedy for dysentery and also for similar condition in horses (Jinju, 1990). The cold infusion of the plant when mixed with trona is taken as a remedy for, gonorrhea and other forms of venereal diseases. It is also taken as a vermifuge or a purging vehicle for other vermifuge. The boiled leaf extract of the plant is taken as a purgative agent while the root is taken as a remedy for diabetes (Dalziel, 1999). In Asia and America the aerial parts of *Stachytarpheta angustifolia* is boiled and taken traditionally as a remedy for diarrhea, intestinal parasites, skin ulcer and as an abortificient (Eldridge *et al.,* 1975).

In Brazil, the triturated fresh leaf is applied locally for the treatment of ulcer and also as a good remedy for rheumatism (Dalziel, 1999). The leaf have also been used for the relief of sprain. The plant has been reported to contain a glucosidal substance called stachytarphine which has been reported to have abortificient property (Dalziel, 1999). In Ghana, the juice from the leaf is used as a remedy for eye trouble such as cataracts and also applied to sores in children's ear. The aqueous leaf extract of *Stachytarpheta angustifolia* is also used to cure heart problems. In papua – New – Guinea, oral infusion of the whole plant is used as an anti-fertility agent (Watt and Breyer Brandwijk 1963). In western part of India, the juice obtained from the fresh leaf is used as an emetic and also as a purgative agent, while the decoction of the whole plant is taken as an antihelmintic agent and as a remedy for gonorrhea (Dalziel, 1999).

The decoction of the aerial parts of *Stachytarpheta jamaicensis* (L) Vahl is used for the treatment of many ailments such as diarrhea, intestinal parasite, skin ulcer and as an Abortificient (Alberto, *et al.,* 2000). The Iridoid glycosides isolated from *Stachytarpheta indica* have been found to posses antimicrobial, antitumor, laxative,

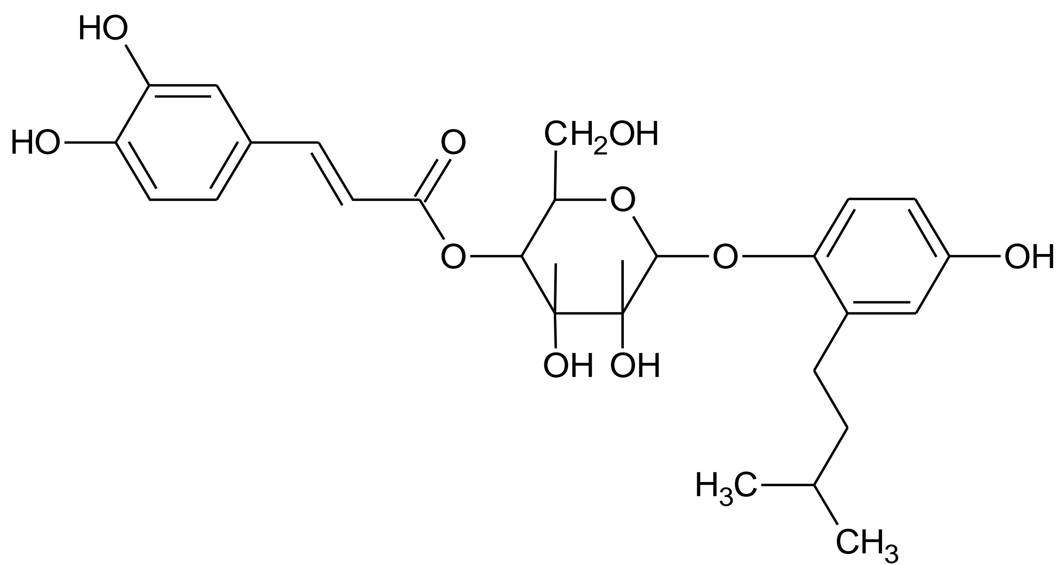
hemodynamic, choleretic, hepatoprotective and anti inflammatory activity (Sophon *et al.,* 2002).

Stachytarpheta cayennensis has been used by the Brazilian folk medicine as an anti-inflammatory, antipyretic, hepatoprotective, laxative and to treat gastric disturbances (Mesia – Vela *et al.*, 2004).

The infusions of the root and stem of *Javanica spreny* is used for the treatment of cough, colds and other related bronchial problem while the leaves are used as tea substitute (Watt and Breyer Brandwijk, 1963) .

2.6 Phytochemistryof *S.angustifolia* and other species inthegenus*Stachytarpheta*

Prenyl hydroquinone glycoside was reported as 1 – O – (4" – O – caffeoyl) – β – glucopyranosyl – 1 – 4 – dihydroxy – 2 – (3; 3 – dimethyl allyl) Benzene(V) and Acteoside (VI) were isolated from the leaf and stem bark of *Stachytarpheta cayemensis* (Cordell, 2000). Lamiide (VII), Ipolamiide (VIII) and Samangaoside (IX) were reported to be isolated from the leaf extract of *Stachytarpheta indica* (Sophon, 2002). While Lucidemic acid (X), korolkoside, a Bis – iridoid glycoside (XI), Citrifolinoside(XII), Isorhamnetin as 3-O- β-D- apio -D- furanosyl (1- 2)-β-D-galactopyranoside (XIII) and Serratoside (XIV) were isolated from *Stachytarpheta* species (Ganapaty *et al,* 1998 ; Farnsworth, 1996 ; Subramanan *et al.,* 1974).



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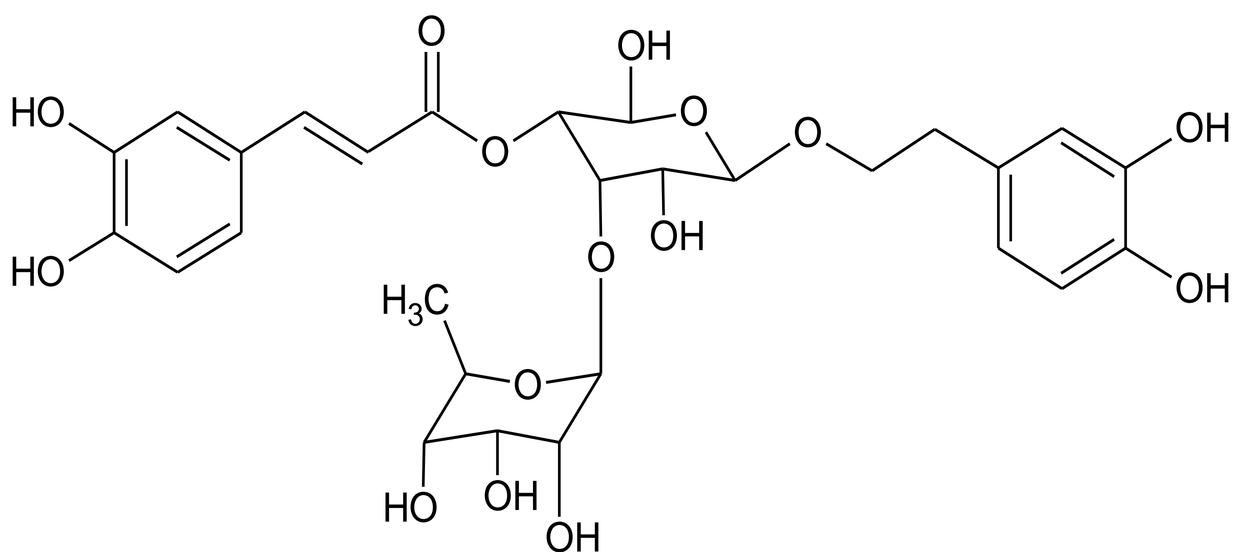

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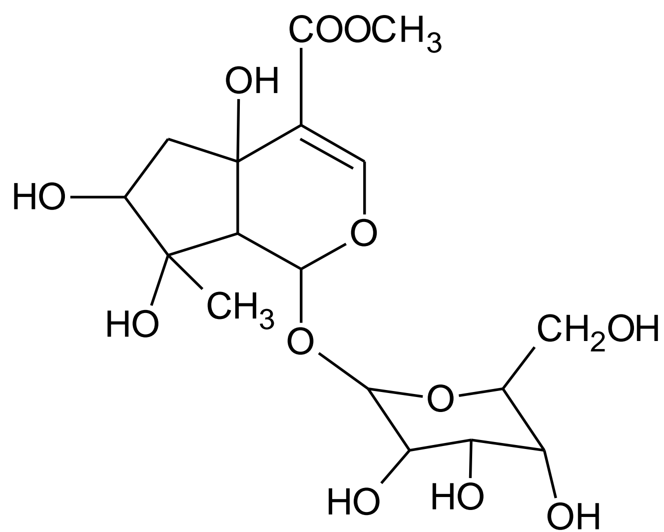

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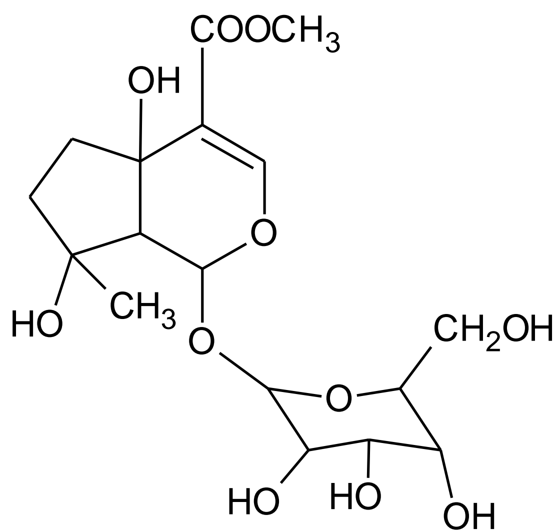

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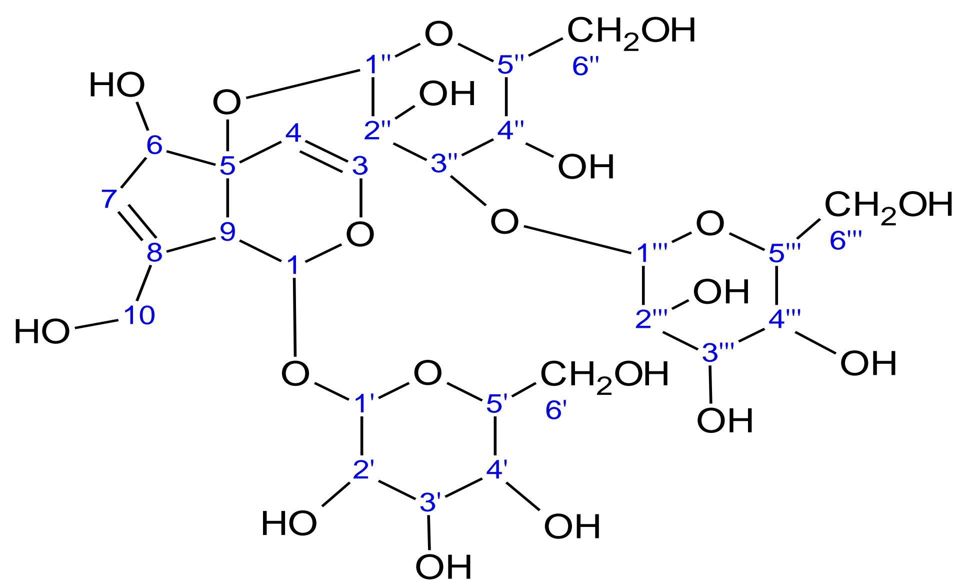

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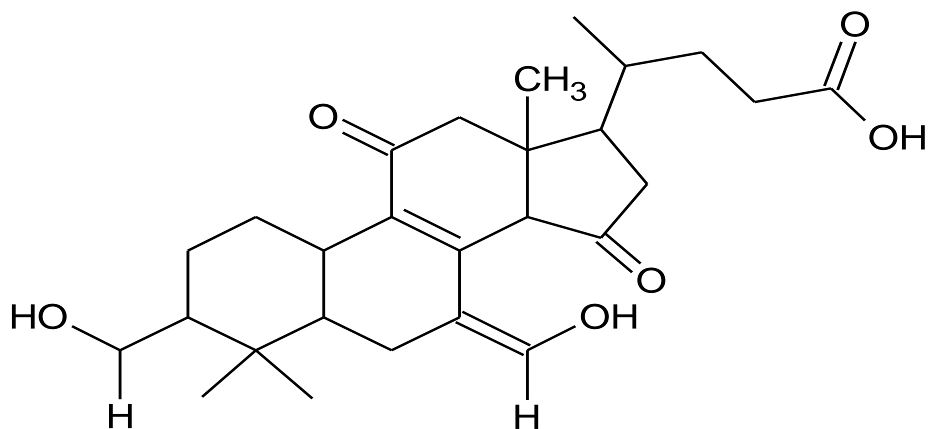
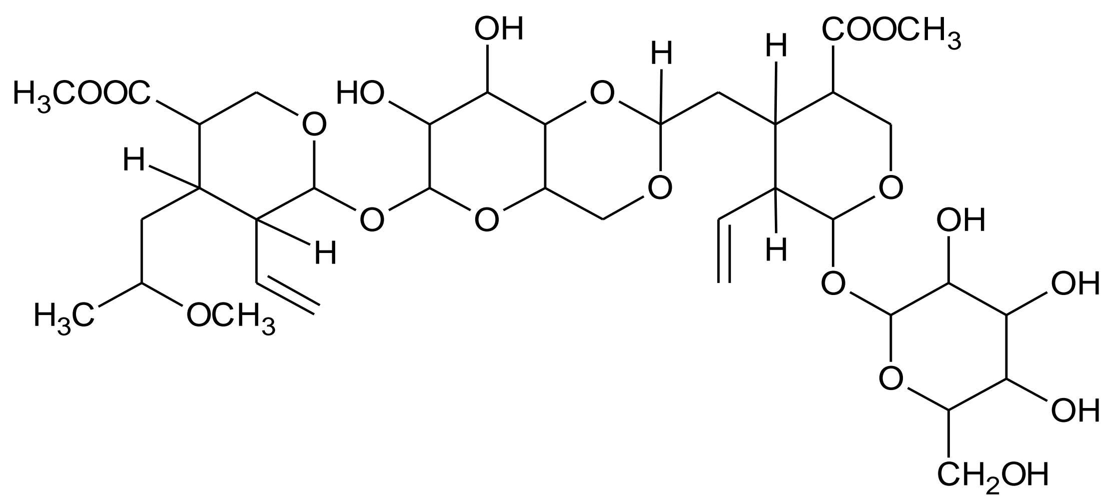


X



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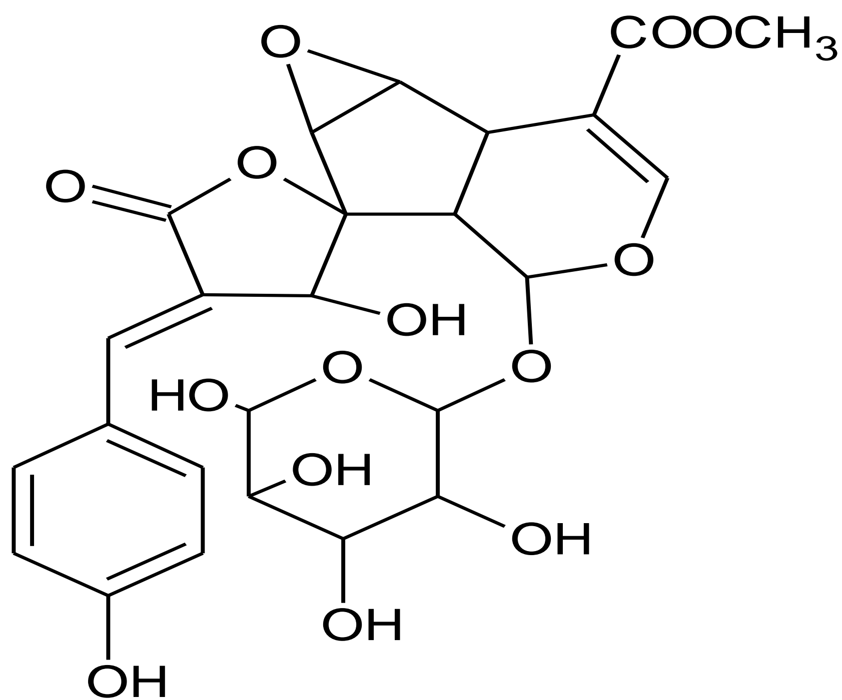

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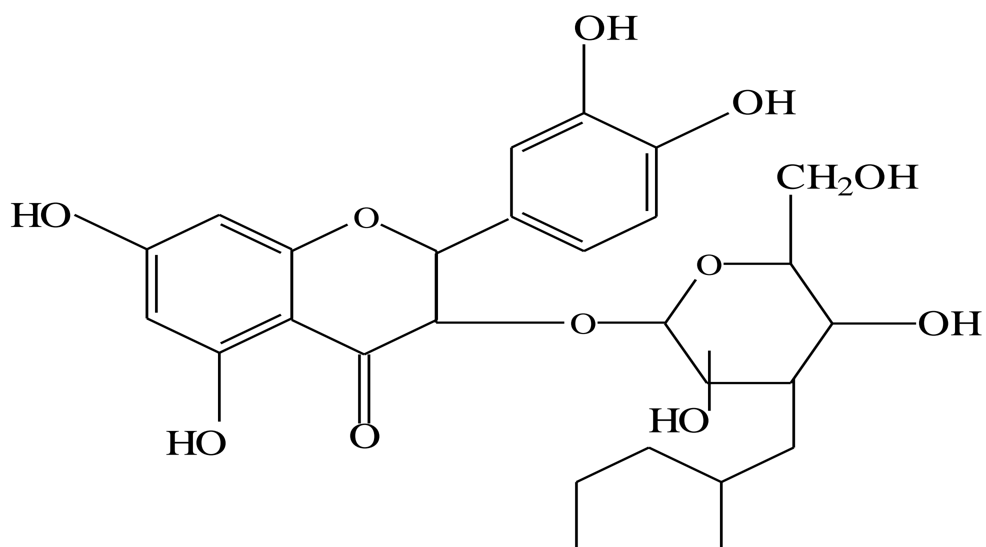

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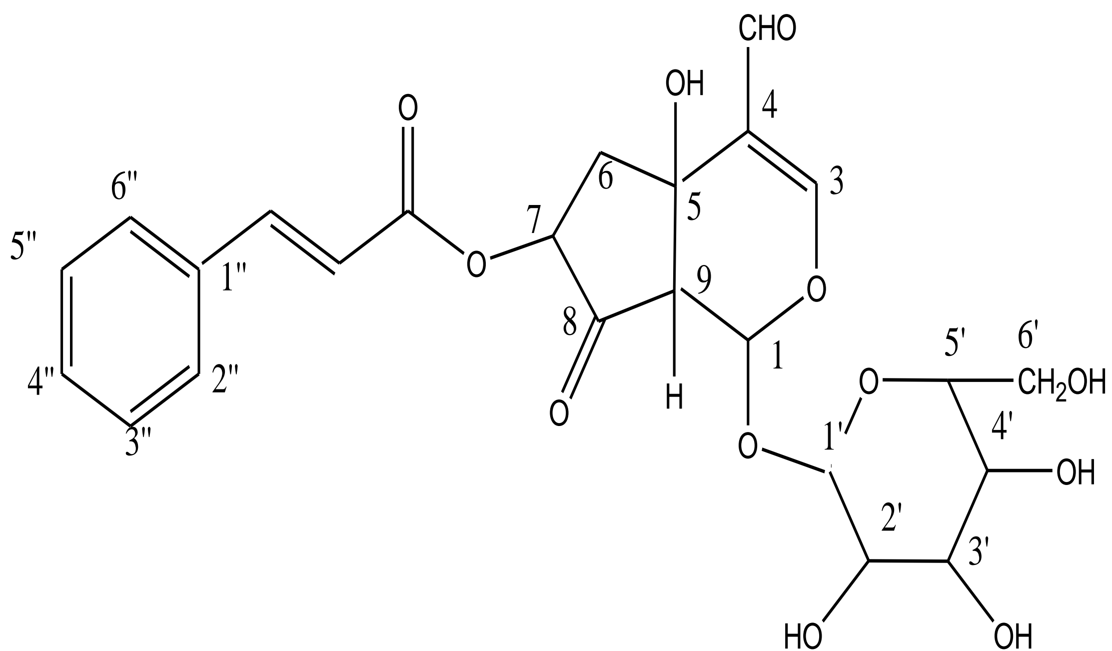

XIV

2.7 ^{13}C-NMR

This is a recently developed techniques for the elucidation of the structures of plant metabolites especially glycoside without chemical degradation, provided the spectral data of the aglycone and sugar moieties are available and also the carbon chemical shift of a number steroidal compounds and terpenes. For the assignment of the carbon signals of both the sugar and aglycone moieties of glycosides, the glycosidation shift rules (Carbon resonance displacement for both the sugar and aglycone moieties on glycoside formation) derived from ^{13}CNMR studies of a variety of prepared α - and β – anomeric part of D-glucopyranosides, D – mannopyranosides, L – arabinopyranosides has also been studies (Bernstein, 1994). The glycosylation shift of a number of natural steroid, steroidal alkaloid and triterpeniod oligoglycosides have also been reported. The anomeric configuration of different sugar moieties in glycosides can also be determined by ^{13}C NMR spectroscopy. Apart from the differences in chemical shift of α - and β – anomeric carbons the direct bonded C – H coupling constant of the C – 1 signal (Jc – 1, H – 1) of hexapyranose and pentopyranoses are characteristics of the anomeric configuration (Jc – 1, H – 1) in 155 H_Z. When H – 1 is axil where as it is C – 165 H_Z when H – 1 is equatorial (Robert, 2005).

Chemical shift

The processional frequency of all the protons in the same external field is not the same but depends on number of factors. The measurement of processional frequency of a group of nuclei in absolute frequency unit is extremely difficult. Therefore, the differences are measured with respect to some reference group of nuclei. Example of such nuclei is tetra methyl silane (TMS).

Thus, it is evident that ^{13}C- NMR spectroscopy has immensely help in the elucidation of the structures of plant metabolites such as the prenyl hydroquinone glycoside as 1 – O – (4" – O – caffeoyl) – β – glucopyranosyl – 1 – 4 – dihydroxy – 2 – (3; 3 – dimethyl allyl) Benzene, Myricetin 3 – (3, 4 – dimethyl -α - L – rhamnoside and Orobanchoside as – β – hydroxyl – β – (3, 4 – dihydroxyphenyl) – ethyl – O - α - L – rhamnopyranoside (1 – 2) – β – D – (- O – caffeoyl – glucopyranoside (Robert, 2005).

2.8 Infrared Absorption Spectroscopy

The infra red absorption spectroscopy is concerned with the measurement of absorption of electromagnetic radiation by molecules due to vibrational energy inherent in them. Thus, when infra red radiation of an appropriate frequency interacts with a molecule, the energy is absorbed leading to an increase in vibration energy of that bond. There two types of vibration in organic molecules which includes the following (Robert, 2005; Olaniyi, 2000)

- Stretching (Symmetrical and Asymmetrical)
- Bending (Scissoring, Rocking, Wagging and Twisting).

Advantages of infrared spectroscopy

- Identification

The IR spectra especially in the finger print region (1500-650 cm^{-1}) offers considerable detail information and direct comparism of two spectra which have been under same sampling conditions offers one of the most reliable ways of examining the identity or unidentity of two samples.

- Detection of impurities

  Impurities absorb strongly within a region where main component is reasonably transparent are easily detected e.g adrenalone in adrenaline.

- Recognition of functional groups.

  The IR spectrum is capable of yielding considerable information about the bonds present in a molecule. The functional group frequency concept makes it possible to identify particular functional group within a molecule and hence make IR the simplest ,and most reliable method of assigning a compound to its class, thus contributing to structural elucidation of organic molecules (Olaniyi, 2000; Kemp, 1991)

2.9 Pharmacological actions of *Stachytarpheta angustifolia* and other Species in the genus.

A survey on the biological studies of *Stachytarpheta* species revealed its various activities such as abortificient, anti-microbial, anti-ulcerogenic, laxative, anti-hypertensive, analgesic, anti- pyretic hepatoprotective, anti-rheumatism, anti- helminthes and anti- inflammatory properties (Mesia-Vela *et al.,* 2004).

The alcoholic leaf extract of *Stachytarpheta angustifolia* has been reported to show some antimicrobial activities against *Mycobacterium tuberculosis, Staphylococcus aureus* and *Escherichia coli (*Dalziel, 1999*)*. The whole plant extract of *Stachytarpheta angustifolia* was also reported to have abortificient property (Watt and Breyer Brandwijk, 1963). The aqueous leaf portion of *S. angustifolia* has been reported also to have laxative and anti-diabetic property (Naomesi, 1977; Jinju, 1990), anti-pyretic, hepatoprotective, anti-helminthes and anti- diarrhea properties (Eldridge, 1975). A similar member of the genus (*Stachytarpheta cayennensis*) has been reported to have anti-inflammatory property (Alberto, *et al.,* 2000), while *Stachyterpheta jamaisensis* has been reported to have anti-diarrhea properties (Chanch *et al.,* 1988).

CHAPTER THREE

3.0 METHODOLOGY

3.1 MATERIALS / REAGENTS / EQUIPMENTS AND ANALYTICAL PROCEDURE

3.1.1 Apparatus and Equipment

i. Soxhlet Extractor
ii. Evaporating Dish.
ii. Column (Sintered glass) of various sizes.
iii. TLC tank of various sizes.
iv. Oven
v. Distillation Apparatus
vi. Gallenkemp (Melting point Apparatus)
vii. U.V light (254nm)
viii. Iodine Tank
ix. Test tubes, Beaker's Spatula, Retort stand, Maceration Bottle, Funnel, Cotton, Reagent bottle, Micro slides, small bottles and capillary tubes.
x. Water bath and Rotary Evaporator
xi. I. R. and G. C Mass Spectrometer
xii. ^{1}H NMR and ^{13}C NMR

3.1.2 The Chemicals use in this Study

These include petroleum ether, Ethanol, Chloroform Ethyl acetate, n-butanol, Acetone and formic acid. The chemicals were obtained from BDH chemical Ltd and sigma Aldrich. All the solvents were distilled before use.

3.1.3 Analytical Procedure

Collection, Identification and Preparation of Plant

(Maceration) Petroleum ether (60 – 80^{0}C)/ Ethanol (95%)

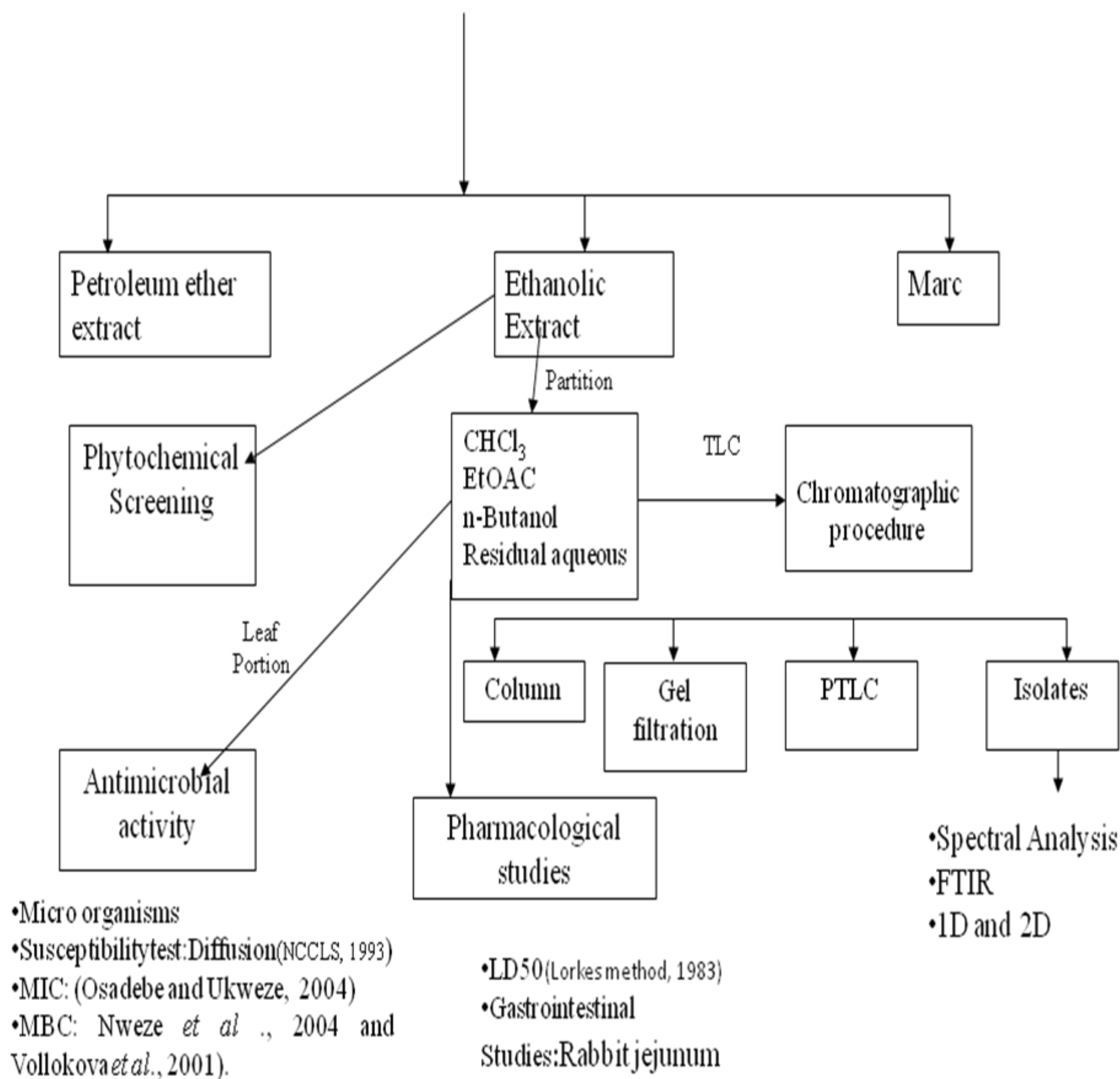

Fig.:3.1Chromatographic and Pharmacological studies of *Stachytarpheta angustifolia*

3.1.4 Collection, Identification and Preparation of Plant Materials

The plant *Stachytarpheta angustifolia* (MILL) Vahl. Verbenaceae was collected from a farm land in Basawa, a village located on the outskirt of Zaria, Kaduna State, Nigeria in the month of October, 2007. The plant was authenticated by comparing with the existing species reserved at the herbarium section of the Department of Biological Science, Ahmadu Bello University Zaria, Nigeria, and voucher No. 900188 was obtained. The fresh plant material was carefully separated into different parts, the leaf, stem bark and root bark. They were cut, air-dried and made into powder using pestle and mortar and subsequently referred to as powdered plant material of leaf, stem bark and root bark.

3.1.5 Extraction

The powdered materials of the leaf (1000 g), stem bark (940 g) and root bark (840 g) of *Stachytarpheta angustifolia* were separately extracted with petroleum ether 60 – 80^{0}C (5 x 600 ml) to exhaustion using cold maceration to obtain pet- ether extracts. In each case, the defatted marc was air dried at room temperature and exhaustively extracted with 95% ethanol (7 x 500 ml) using the same procedure to obtain ethanol extracts. The solvents were removed *in-vacuo* to afford an oily, light green and a dark brown gummy mass referred to as petroleum ether extract coded "L_{ps}" "S_{ps}" "R_{ps}" for the leaf, stem bark and root while the ethanol extracts were coded as "L_{ES}" "S_{ES}" "R_{ES}" for the leaf, stem bark and root extract respectively.

The ethanol extract of the leaf (25 g), stem bark (30 g) and root bark (30 g) were each suspended in water (500 ml) and sequentially partitioned with chloroform (3 x 500 ml), ethyl acetate (4 x 500 ml), and n-butanol (5x 500 ml) to afford chloroform, ethyl acetate and n-butanol soluble portions respectively (Yaching, *et al.,* 2004; Shengmin *et al.,*

2001). The residual aqueous portions were also concentrated separately using rotary evaporator.

3.2 PRELIMINARY PHYTOCHEMICAL SCREENING

The leaf, stem bark and root bark crude extracts of *S. angustifolia* were subjected to phytochemical screening using standard protocols (Sofowora, 2008 ; Trease and Evans, 2002). The spray of each chromatogram was done using 10% tetraoxosulphate (VI) acid solution to access the composition of the extract. (Richard 1998).

3.2.1 Test For Carbohydrates

(i) General Test (Molisch's)

A few drops of the molisch reagent were added to a clear solution of the extract dissolved in water. This was followed by the addition of 1ml of Conc. Sulphuric acid down the side of the test tube, the mixture was allowed to stand for 2 minutes and then dilute with 5ml of water. The appearance of a red colour at the interphase of the two layers, confirms the presence of carbohydrate (Brain and Tuner, 1975).

(ii) Test for sugar (Barfoed's)

To a clear solution of the extract dissolved in water in a test tube, 1ml of Barfoed's reagent was added and heated on the water bath for 2 minutes. A red precipitate of Cu_2O was taken as a positive test for the presence of sugars (Trease and Evans, 1983).

(iii) Test for reducing sugar

To a clear solution of the extract dissolved in water and heated with 5ml equal volume of fehlings solution A and B. The formation of a red precipitate of Cu_2O indicates the presence of reducing sugar (Brain and Tuner, 1975).

3.2.2 Test For Tannins

A small quantity of each extract was boiled with 10ml of water, cooled and filtered. The filtrate was used for the following test.

(A) A few drops of lead ethanoate was added to 1ml of the filtrate, the formation of a white precipitate indicate the presence of tannins.

(B) To another portion of the filtrate, a few drops of 1% iron (ii) chloride solution was added, the presence of blue–black, green or blue-green precipitate was an evidence for the presence of tannins (Harbone, 1973).

(C) About 2ml of 10% ethanolic acid and 10% lead ethanoate was added to 10ml of each filtrate. The formation of a white precipitate indicates the presence of tannins (Trease and Evans, 1983).

(D) The filtrate was boiled with 3 drops of 10% Hcl and 1 drop of methanol. The appearance of red precipitate was an evidence for the presence of tannins (Trease and Evans, 1997).

3.2.3. Test For Saponins

Little portion of the extract was shaken with water in test tube and then warm on water bath. Persistent frothing indicates the presence of Saponin (Sofowora, 1986).

3.2.4 Test For Flavonoids

(i) **Shinoda's Test**

A little portion of the extracts was dissolved in ethanol this was further warmed and filtered. Three to four pieces of magnesium chips was added to the filtrate, followed by the addition of few drops of Conc. Hydrochloric acid (Hcl). The

presence of pink colouration was an indication of the presence of Flavonoids (Trease and Evans, 1997).

(ii) **Ferric Chloride Test**

Little portion of the extract was dissolved in ethanol and boil with few drops of 10% $fecl_3$ solution. A violet colouration observed was an indication of the presence of a phenolics hydroxyl group (Trease and Evans, 1997).

3.2.5 Salkowskii Reaction

0.2g portion of each extract was dissolved in 2.5ml of chloroform. Sulphuric acid was carefully added down the test tube to form a lower layer. A reddish brown colour at the interphase indicates the presence of steroidal ring (i.e. aglycone portion of the cardiac glycoside (Sofowora, 1982).

(ii) **Liebermann Burchard Test**

To a small quantity of each extract portion, dissolved in chloroform (2ml) was filtered. The filtrate was mixed with (2ml) of acetic acid and cooled in iced. Concentrated tetraoxosulphate (VI) acid was added down the side of the test tube. A colour change from violet to blue or bluish green indicates the presence of steroidal nucleus (i.e. aglycone portion of the cardiac glycoside (Sofowora, 1982).

3.2.6 Test For Glycosides

(i) General Test

A small quantity of each extract was boiled with 25ml of 2.5m tetraoxosulphate (VI) acid. This was cooled and neutralized with 20% potassium hydroxide and then boil again with 5ml of a mixture of equal volume of fehlings solution A and

B. Formation of a brick red precipitate shows the presence of glycosides (Trease and Evans, 1997).

(ii) **Legal Test**

Little portion of each extract was dissolved in pyridine and a few drops of 2% sodium Nitroprusside together with few drops of 20% sodium Hydroxide (NaoH) was added. A deep red colour which faded to brownish yellow indicates the presence of Cardenolides (RiChard, 1998).

(ii) **Keller – Kiliani Test**

Little portion of the extract was dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under laid with 1ml of concentrated Sulphuric acid. A brown ring obtained at the interphase is the presence of sugar characteristic of Cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Richard, 1998).

3.2.7 Test For Alkaloids

Little portion of the extract was stirred with 5ml of 1% aqueous hydrochloric acid (HCl) on water bath and later filtered. 3ml of the filtrate was divided into 3 portion of 1ml each.

- The first 1ml was treated with few drops of Dragendoff's reagent to give an orange red precipitate as an evidence of Alkaloid.
- The second 1ml portion, Mayer's reagent was added to give a buff coloured precipitate as an evidence of Alkaloid.

- Lastly, the third portion was treated with Wagner's reagent to give a dark brown precipitate. All the three colours confirms the presence of an alkaloid (Brain and Tuner, 1975).

3.3 CHROMATOGRAPHIC PROCEDURE

3.3.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on silica gel precoated Aluminium plates Pf_{254} with layer thickness of 0.2 mm.

Technique: One way ascending.

Spotting and development: Spots were applied manually using capillary tube; plates were dried using air blower and developed at room temperature using Shandon chromatographic tank.

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

a. Petroleum ether: Chloroform (8:2)

b. Petroleum ether: Ethyl acetate (9:1)

c. Petroleum ether: Chloroform (7:3)

d. Ethyl acetate : Methanol (8:1)

e Chloroform : Methanol (7:2)

f. Chloroform: Methanol: water (3:3:1)

g. Ethyl acetate : Methanol: Water (100:16.5:13.5)

h. Ethyl acetate: Formic acid: Water (upper layer) (10:1:2)

i. Pet. ether: Ethyl acetate: Methanol: H_2O (3:7:5:5)

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

3.3.2. Column Chromatography

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

(a) Techniques – Gradient elution.

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

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

(d) Column packing – Wet slurry method.

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

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

I - Petroleum ether

II - Chloroform

III – Ethyl acetate

IV - Methanol

3.3.3. Gel Filtration Chromatographic Techniques

Gel filtration was performed using Sephadex LH-20 (sigma).

Packing method: The Sephadex $LH\text{-}_{20}$ was suspended in methanol 100% as the eluent. This was then allowed to swell for 24 hours prior to use. It was then poured in to the column and allowed to settle overnight.

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

Solvent: Methanol and Acetone.

3.3.4 Preparative Thin Layer Chromatography (PTLC)

PTLC was carried out using Fluka silica gel precoated glass plates 20×20 cm with layer thickness of 0.5-4mm sorbent thickness. A thin line of about 1.5 cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of solvent to give an approximate concentration of 20 mg/ml. It was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using an appropriate solvent system. The developed plate was air dried in a fume cupboard and the position of the band of interest was marked with pencil and scraped off the back of the plate on to a foil. The scraped sorbent was size reduced using pestle and mortar, transferred to a sintered glass funnel and washed repeatedly with Acetone 100% and the solution obtained was evaporated to give the isolated compound (Richard, 1998).

3.3.5 Melting Point (m.p.) Determination

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

3.3.6 Spectral Analysis

Infrared IR spectra were recorded on spectrophotometer shimadzu 8400s.

Melting points were determined on GallenKemp apparatus and results were uncorrected. ^{1}H NMR and ^{13}C NMR were performed on Bruker spectrometer 500 and 600 MHz for ^{1}H NMR and 100 and 124 MHz for ^{13}C NMR. Spectra's were referenced to the CD_3OD solvent signals at δ3.80 (1H) and 49.00 (^{13}C) with TMS as an internal standard. Chemical shift values (δ) were reported in parts per million (ppm) in relation to the appropriate internal solvent standard (TMS). The coupling constant (J-values) were given in Hertz. TLC was carried out on plates precoated with R-P18 gel (Merck) and silica gel 60-120 mesh were used for column chromatography. Spots on plates were visualized by spraying with 10% H_2SO_4 followed by treating in even. Column chromatography was performed on silica gel 60-120 mesh size.

Gel-filtration techniques were carried out on sephadex L_H20. TLC visualization was by u.v absorption at 245nm and deutrated methanol was used in all cases.

3.4 CHROMATOGRAPHIC SEPARATION OF MNF, B, and MND

3.4.1 Thin Layer Chromatography (TLC)

(i) Thin Layer Chromatography of the petroleum ether and the ethanol leaf, stem bark and root bark extract of *Stachytarpheta angustifolia* was conducted. The solvents used were Petroleum ether: Chloroform (8:2), Petroleum ether: Ethyl acetate (9:1) and Petroleum ether: Chloroform (7:3).

3.4.2 Thin layer chromatography of the n-butanol leaf extract L(n-bt) of *S. angustifolia*

The Leaf n-butanol extract L(n-bt) of *S. angustifolia* was subjected to thin layer chromatography using precoated TLC aluminium plate. The solvent system used was Chloroform: Methanol: Water (3:3:1).

3.4.3 Column Chromatography of the n-butanol leaf fraction L(n-bt) of *S. angustifolia*

L (n-bt) fraction (2.5g) was chromatographed over glass column (100 cm×4 cm) packed with silica gel (60-120 mesh). The column was eluted continuously using chloroform, chloroform/Ethyl acetate mixture, Ethyl acetate, Ethyl acetate/methanol mixture and finally with methanol by gradient elution technique. The progress of elution was monitored using thin layer chromatography to afford a total of 450 fractions of 10 ml aliquot. Fractions were pooled together base on their TLC profile to afford a total of 11 major fractions (F_I- F_{11}). Fraction F_4 consisting of 2 major spots was subjected to repeated gel filtration to afford 5 sub-fractions (A_1-A_5). Sub- fraction A_3 (6-18) gave a single spot when subjected to TLC using two solvent systems (Ethyl acetate: methanol (8:2) and Chloroform: Methanol (7:3) to afford MNF.

3.4.4 Thin layer chromatography of the n-butanol stem bark extract S (n-bt) of *S. angustifolia*

The n-butanol stem bark extract S(n-bt) of *S. angustifolia* was subjected to thin layer chromatography using precoated TLC aluminium plate. The solvent system used was Ethyl acetate: Methanol: Water (100:16.5:13.5).

3.4.5 Column Chromatography of the n-butanol stem bark fraction S(n-bt) of *S. angustifolia*

The n-butanol fraction (3.0 g) S (n-bt) was chromatographed over glass column (100 cm×4 cm) packed with silica gel (60-120 mesh).

The column was eluted continuously using chloroform, chloroform/ Ethyl acetate mixture, Ethyl acetate, Ethyl acetate/ Methanol mixture and finally with Methanol by gradient elution technique. The progress of elution was monitored using thin layer chromatography. A total of 465 fractions of 10 ml aliquot were obtained. Fractions were combined based on their TLC profile to afford 10 major fractions B_1- B_{10}. Fraction B_4 consisting of two major spots was subjected to repeated gel filtration using sephadex LH-$_{20}$, eluted with methanol and final purification with preparative thin layer chromatography (PTLC) to afford a single spot on TLC coded as B.

3.4.6 Thin layer chromatography of the n-butanol root bark R (n-bt) of *S. angustifolia*

The n-butanol root extract R(n-bt) of *S. angustifolia* was subjected to thin layer chromatography using precoated TLC aluminium plate. The solvent system used was Chloroform: Methanol: Water (3:3:1).

3.4.7 Column Chromatography of the n-butanol root bark fraction R (n-bt) of *S. angustifolia*

The n-butanol fraction (1.8 g) R (n-bt) was chromatographed over glass column (100 cm×4 cm) packed with silica gel (60-120 mesh). The column was eluted continuously using chloroform, chloroform/ethyl acetate mixture, ethyl acetate, ethyl acetate/ methanol mixture and finally with methanol by gradient elution technique. The progress of elution was monitored using thin layer chromatography. A total of 388 fractions of 10ml aliquot were obtained. Fractions were combined based on their TLC profile to afford 10 major fractions D_1- D_{10}. Fraction D_4 (81-242) consisting of two major spots was subjected to a repeated gel filtration technique using sephadex LH-20 and eluted with methanol to obtain 5 sub fractions (D_1-D_5).

Sub fractions D_2 (19-39) gave a single spot when subjected to TLC using two solvent systems (Ethyl acetate: methanol (8:2) and Chloroform: Methanol (7:4) to afford MND.

Compound MNF, B and MND were subjected to TLC using the following solvent system of different polarity as chloroform: methanol: water (3:3:1), Ethyl acetate : methanol: water (100:16.5:13.5) and Ethyl acetate: formic acid : water (upper) 10:1:2 to obtain a single spot.

3.4.8 Solubility Test

The solubility of each compound MNF, B and MND was determined in chloroform, Ethyl acetate, Acetone and Methanol at room temperature.

3.4.9 Melting Point Determination.

The melting point of compound MNF, B and MND were determined using Gallenkamp apparatus. Results were uncorrected.

3.4.10 Identification tests for compounds MNF, B and MND

(i) The developed chromatogram of MNF, B and MND were separately sprayed with dissolved Vanillin (4g) in concentrated Tetraoxosulphate (VI) acid in (100mL). This was then heat at 100^0C until coloration appears.

(ii) The chromatogram of MNF, B and MND were separately sprayed with 20% (w/v) aqueous perchloric acid solution. The plate was then heat at 100^0C until coloration appears.

(iii) The chromatogram of MNF, B and MND were separately sprayed with 5% ferric chloride in 0.5%N HCl. The plate was then heat gently in the oven (Richard, 1998, Trease And Evans, 2002, Manguro and Lemmen, 2007).

3.5 PHARMACOLOGICAL STUDIES

3.5.1 Acute Toxicity Study on the Leaf, Stem bark and Root bark extract of *Stachytarpheta angustifolia*.

LD_{50} determination was carried out using the method of Lorke (1983). Thirteen mice each were used to study the extracts from the leaf, stem bark and root bark. In the first phase, three doses of the leaf, stem bark and root bark ethanol extract each (10,100, 1000 mg/kg) were administered intraperitoneally (*i.p.*) to three groups each containing (n=3) mice. In the second phase, four doses of Leaf extract (600, 370, 225, 140 mg/kg), Stem bark extract (610, 370, 226, 142 mg/kg) root bark (620, 371, 222 and 138 mg/kg) each were administered through the same route to four groups each containing one mouse respectively. The median lethal dose (LD_{50}) value was calculated as the geometric mean of the highest non lethal dose and the lowest lethal dose for each of the extract.

3.5.2 Studies on isolated rabbit jejunum.

The rabbit was starved for 24 hours and sacrificed by a blow on the head and killed. The abdomen was dissected carefully to reveal its abdominal cavity. The jejunum was carefully removed and cleaned free of its content, and 2-3 cm were cut (Schlemper *et al.,* 1996 ; Akah *et al.,* 1996). The tissue was mounted into a 25 ml organ bath containing a standard physiological (Tyrode solution) of the following composition (mM): D-glucose 10.0 g, NaCl 90.0 g, KCl (10% solution) 20 ml, $NaH_2PO_4.2H_2O$ (10%) 5ml, $NaHCO_3$ 10 g, $MgCl_2.6H_2O$ 1.0 ml and $CaCl_2$ (10% solution) 20ml. This was prepared in 10 litre of de ionized water (Kitchen, 1994). The solution was aerated with air maintained at $37^0C \pm 1^0C$. The initial concentration of acetylcholine (0.05-0.8 ug/ml) and contractile responses were recorded isometrically through an isometric micro dynamometer 7004 on an Ugo Basile unirecorder 7050.

The tissue was equilibrated for 60 - 90 minutes during which the physiological solution was replaced every 15 minutes (Samuelson, *et al.,* 1991; Vongtau *et al.,* 2000). At the end of the equilibration period, the effect of the ethanol extracts of the leaves, stem bark and root bark at concentration of (0.4, 0.8, 0.16 and 0.32 mg/ml) each were investigated. The chloroform, ethyl acetate, n-butanol and aqueous fractions were also investigated on the isolated jejunum at concentration of (0.4, 0.8, 0.16 and 0.32 mg/ml). The effect of the leaf ethanol extract of *S. angustifolia* was also investigated in the presence of atropine on the isolated rabbit jejunum. This was repeated four times (Vongtau *et al.,* 2000; Michelson, 1984).

3.6 ANTIMICROBIAL ASSAY

3.6.1 Microorganism

The clinical isolates uses in this experiment were obtained from the Medical Microbiology Department of Ahmadu Bello University Teaching Hospital (ABUTH), Zaria. These isolates used were checked for purity and maintained in slants of nutrient agar. These include *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Salmonella typhi.*

3.6.2 Antimicrobial Susceptibility Testing

The disc diffusion method (Sardari *et al.,* 1998) was used for the test. Filter paper disc (6 mm in diameter) impregnated with sample solution were placed on nutrient agar plates which had been inoculated with the test microorganism according to standard protocol described by National Committee of Chemical laboratory Standard (1993). The leaf extract and the partitioned factions were dissolved in their solvent of extraction. This was tested at the initial concentration of 20 mg/ml. The inoculated plates were then incubated at 37^0C for 24 hrs, after which the diameter of the incubated zones were measured with a transparent ruler and the results recorded in millimeter.

Filter paper disc containing the solvent of extraction without the extract was used to serve as the control. (Cowan, 1999 ; Ntiejumokwu and Alemika 1991). The reference antibiotic Ampicillin-cloxacillin 5mg/ml was used as the positive control.

3.6.3 Minimum Inhibitory Concentration (MIC)

MC-Farland's turbidity standard scale number 0.5 (Manufacturers standard) was prepared to give a turbid solution. Nutrient broth was prepared ,10 ml was dispensed into test tube and was sterilized at 121^0 C for 15 minutes after which, the broth was allowed to cool. Normal saline was prepared, 10 ml was dispensed into sterile test tube and the test microorganisms were inoculated and incubated at 37^0C for 6 hrs. Dilution of the test microbes in the normal saline was done continuously until the turbidity marched that of the MC-Farland's scale by visual comparison; at this point the test microbes have a concentration of 1.5 x 10^8cfu/ml (Ogbulie *et al.,* 2007; Kunle *et al.,* 2003).

Two fold serial dilution of the extract in the sterile broth were made and the following concentrations were obtained 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml. About 0.1ml of the test microorganism in the normal saline was measured using pasture pipette and then inoculated in to the different concentrations of the extract in the sterilized broth. The incubation was made at 37^0C for 24hrs after which the test tubes were observed for turbidity (growth). The lowest concentration of the extract in the broth which shows no turbidity was recorded as the minimum inhibitory concentration (NCCLS, 1993).

3.6.4 Minimum Bactericidal Concentration (MBC).

Minimum bactericidal concentrations was to determine whether the test microbes were killed or their growth was only inhibited. Nutrient agar was prepared according to manufacturer's instruction.

This was sterilized at 121^{0}C for 15 minutes, poured into sterile petri dishes and the plates were allowed to cool and solidify (Nweze *et al.,* 2004 ;Vollokova *et al.,* 2001). The contents of the inhibitory concentration test tubes in the serial dilution were then sub-cultured into the prepared medium, the incubation was made at 37^{0}C for 24 hrs after which the plates were observed for colony growth. The MBC observed as the plate with the lowest concentration of the extract without colony growth (Meenakshi *et al.,* 2001; Sidney *et al .,*1978).

CHAPTER FOUR

RESULTS

4.1 Result obtained from leaf, stem bark and root bark extraction.

The result of petroleum ether leaf extract gave (yellow oily color), stem bark extract (Orange color) and root bark extract gave (lemon green). The ethanol leaf extract gave (light brown color), stem bark extract gave (Dark brown color) while the root bark extract gave (Greenish color) respectively (Table 4.1).

4 .2 Phytochemical Screening

The results of preliminary photochemical screening of the petroleum ether and ethanol extract of the leaf, stem bark and root bark extracts of the plant were summarized in Tables below. The petroleum ether extract revealed the presence of sterols and terpenoids (Table 4.2) while the ethanol extract revealed the presence of flavonoids, steroid, terpenoids, tannins, Cardiac glycoside and saponins (Table 4.3).

Table 4.1 Result obtained from the extraction and partition of the leaf, stem bark and root bark extract of *S. angustifolia*

Extracts	Yield/ weight(g)	% Yield	Colour
LEAF Extract			
Petroleum ether	35.60	3.7	Light Yellow oily
Ethanol extract	60.40	6.0	Light brown
Chloroform fraction	2.30	0.20	"
Ethyl acetate fraction	1.86	0.90	"
n-Butanol fraction	5.43	0.50	"
Residual aqueous	8.60	0.95	"
STEM BARK			
Petroleum ether	32.00	3.4	Light Orange
Ethanol extract	54.00	5.7	Dark brown
Chloroform fraction	4.5	0.50	"
Ethyl acetate fraction	2.3	0.25	"
n-Butanol fraction	5.00	0.45	"
Residual aqueous	6.20	0.67	"
ROOT BARK			
Petroleum ether	28.00	3.3	Lemon green
Ethanol extract	49.00	5.80	Green
Chloroform fraction	3.80	0.47	"
Ethyl acetate fraction	1.90	0.23	"
n-Butanol fraction	5.00	0.50	Bluish green
Residual aqueous	4.72	0.62	"

Table. 4:2 Phytochemical screening of the leaf, stem bark and root bark Petroleum ether extract of *Stachytarpheta angustifolia.*

CONSTITUENTS	TEST		LEAF	STEM	ROOT
		Observation	Inference	Inference	Inference
Carbohydrate					
General test	Molisch	Red colour	-	-	-
Monosaccharide	Barfoed's	Red. ppt	-	-	-
Reducing sugar	Fehling's	Red. ppt	-	-	-
Tannins	Lead ethanoate	White ppt	-	-	-
	Iron (III) chloride	Blue-black	-	-	-
Saponins	Frothing	Persist frothing	-	-	-
Sterols	Liebermann, Burchard	Blue-green	+	+	+
	Salkowski	Red ring at interphase	+	+	+
Terpenoids	Liebermann, Burchard	Brown ring with brown interphase	+	+	+
Alkaloids	Dragendoff's	-	-	-	-
	Mayer's	-	-	-	-
	Wagner's	-	-	-	-
Flavonoids	Shinoda	Pink coloration	-	-	-
	Ferric chloride	Blue-green coloration	-	-	-
Cardiac glycoside	Keller-Kilanis	Reddish brown	-	-	-
	Legal's	Deep red colour	-	-	-

Key: + = Present, - = Absent

Table. 4:3 Phytochemical screening of the leaf, stem bark and root bark ethanol extract of *Stachytarpheta angustifolia.*

CONSTITUENTS	TEST		LEAF	STEM	ROOT
		Observation	Inference	Inference	Inference
Carbohydrate					
General test	Molisch	Red colour	+	+	+
Monosaccharide	Barfoed's	Red. ppt	+	+	+
Reducing sugar	Fehling's	Red. ppt	+	+	+
Tannins	Lead ethanoate	White ppt	+	+	+
	Iron (III) chloride	Blue-black	+	+	+
Saponins	Frothing	Persist frothing	+	+	+
Sterols	Liebermann, Burchard	Blue-green	+	+	+
	Salkowski	Red ring at interphase	+	+	+
Terpenoids	Liebermann, Burchard	Brown ring with brown interphase	+	+	+
Alkaloids	Dragendoff's	-	-	-	-
	Mayer's	-	-	-	-
	Wagner's	-	-	-	-
Flavonoids	Shinoda	Pink coloration	+	+	+
	Ferric chloride	Blue-green coloration	+	+	+
Cardiac glycoside	Keller-Kilanis	Reddish brown	+	+	+
	Legal's	Deep red colour	+	+	+

Key: + = Present, - = Absent

4.3. Result of Thin Layer Chromatography of the ethanol leaf extract.

Thin layer chromatography of the crude ethanol leaf extract using chloroform: MeOH (7:3) and n-butanol fraction of the plant using chloroform: methanol: water (3:3:1) as the solvent system revealed several spots. The chromatograms were sprayed with 10% H_2SO_4 and heated in the oven at 110^0 C for 5min (Table 4.4).

Table. 4:4. TLC Analysis of crude ethanol leaf extract using Chloroform: MeOH(7:3).

Spots	Colours with . 10% H_2SO_4	R_f
1	Light –blue	0.09
2	Blue green	0.18
3	Purple	0.33
4	Purple	0.47
5	Brown	0.51
6	Brown	0.56
7	Greenish	Solvent front

Plate: 4.1 The TLC profile of n-butanol leaf fraction using chloroform: methanol: water (3:3:1) and 10% H_2SO_4 spray reagent.

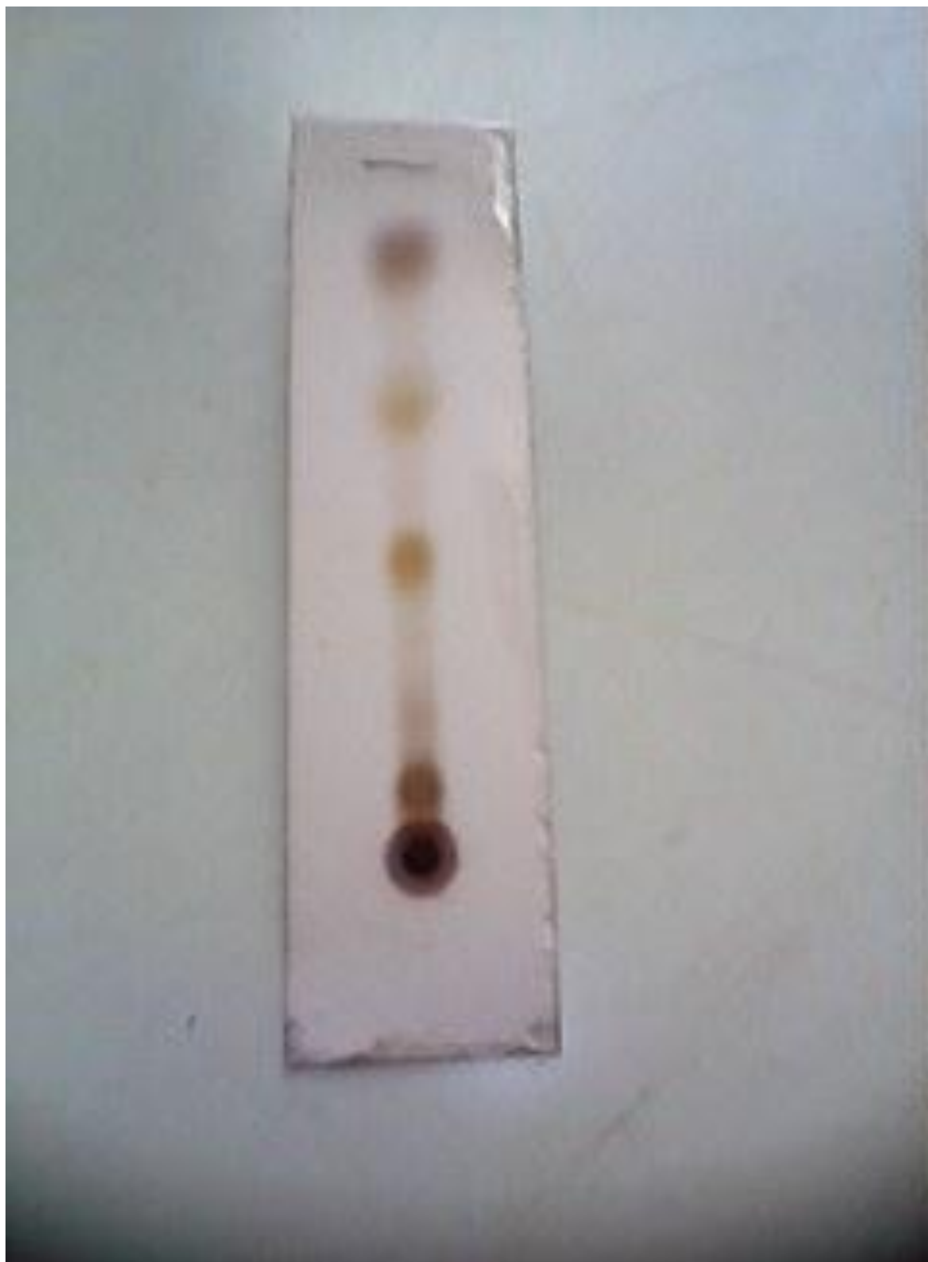

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Table. 4: 5 The TLC of n-butanol leaf fraction using chloroform: methanol: water (3:3:1) with 10% H_2SO_4 spray reagent.

Spots	Colours with 10% H_2SO_4	R_f
1	Deep-green	0.12
2	Blue-green	0.31
3	Brownish	0.48
4	Brown	0.71
5	Brown	Solvent-front

Table 4:6. TLC of crude ethanolic stem bark extract using ethyl acetate: methanol (8:2). The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110 ^{0}C for 5min.

Spots	Colours with 10% H_2SO_4	R_f
1	Deep-blue	0.12
2	Blue-green	0.21
3	Blue-green	0.31
4	Brown	0.41
5	Dark brown	0.55
6	Light purple	Solvent front

Table 4:7 TLC of n-butanol fraction of the stem bark extract using Ethyl acetate : Methanol (8:2). The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110 ^{0}C for 5min.

Spots	Colours with 10% H_2SO_4	R_f
1	Deep blue	0.13
2	Blue-Green	0.33
3	Dark brown	0 .53
4	Purple	1.00

Table 4:8 TLC of n-butanol fraction of the root bark extract using chloroform: methanol: Water (3:3:1) The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110 ^{0}C for 5min.

Spots	Colours with 10% H_2SO_4.	R_f
1	Orange	0.12
2	Purple	0.21
3	Bluish-green	0.40
4	Bluish-green	0.70

4. 4 Results of Column Chromatographic Separation

4.4.1 Isolation of compound MNF

The fractionation of the n-butanol fraction of the leaf extract using column chromatography yielded 11 pooled fractions (Table 4.9). Repeated gel chromatographic separation of fraction F_4 yielded a light brown amorphous solid (47 mg) coded as compound MNF.

Table: 4.9. Fractions obtained from the n-butanol column chromatographic separation of the leaf extract to yield 11 pooled fractions (F_1- F_{11}).

S/no	Fraction	Eluting solvent	Spots	Weight (mg)	Code
1	5-35	EtOAc 100%	3	0.01	F_1
2	36-37	EtOAc: MeOH (90:10)	4	-	F_2
3	73-102	EtOAc: MeOH(80:20)	3	22	F_3
4	**103-184**	**EtOAc: MeOH(70:30)**	**2**	**95**	**F_4**
5	185-196	EtOAc: MeOH(60:40)	2	10	F_5
6	179-220	EtOAc: MeOH(50:50)	3	50	F_6
7	221-295	EtOAc: MeOH(40:60)	4	97	F_7
8	296-340	EtOAc: MeOH(30:70)	3	67	F_8
9	341-382	EtOAc: MeOH(20:80)	3	51	F_9
10	383-421	EtOAc: MeOH(10:90)	2	20	F_{10}
11	422-450	MeOH (100%)	4	12	F_{11}

4.4.2 Melting Point of compound MNF

The melting point of MNF was found to be 218-220 ^{0}C.

4.4.3 Solubility of compound MNF

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

4.4.4 Result of Identification test

Compound MNF produced a positive result with Vanillin/ H_2SO_4 test but gave a negative result with perchloric acid and ferric chloride test.

Plate 4.2 TLC profile of compound MNF using EtOAc : MeOH : Water (100:16.5:13.5) and $CHCl_3$: MeOH : Water (3:3:1) as solvent system. The chromatogram was sprayed with 10% H_2SO_4 and heated in the oven at 110 ^{0}C for 5min.

A **B**

I II

Key:

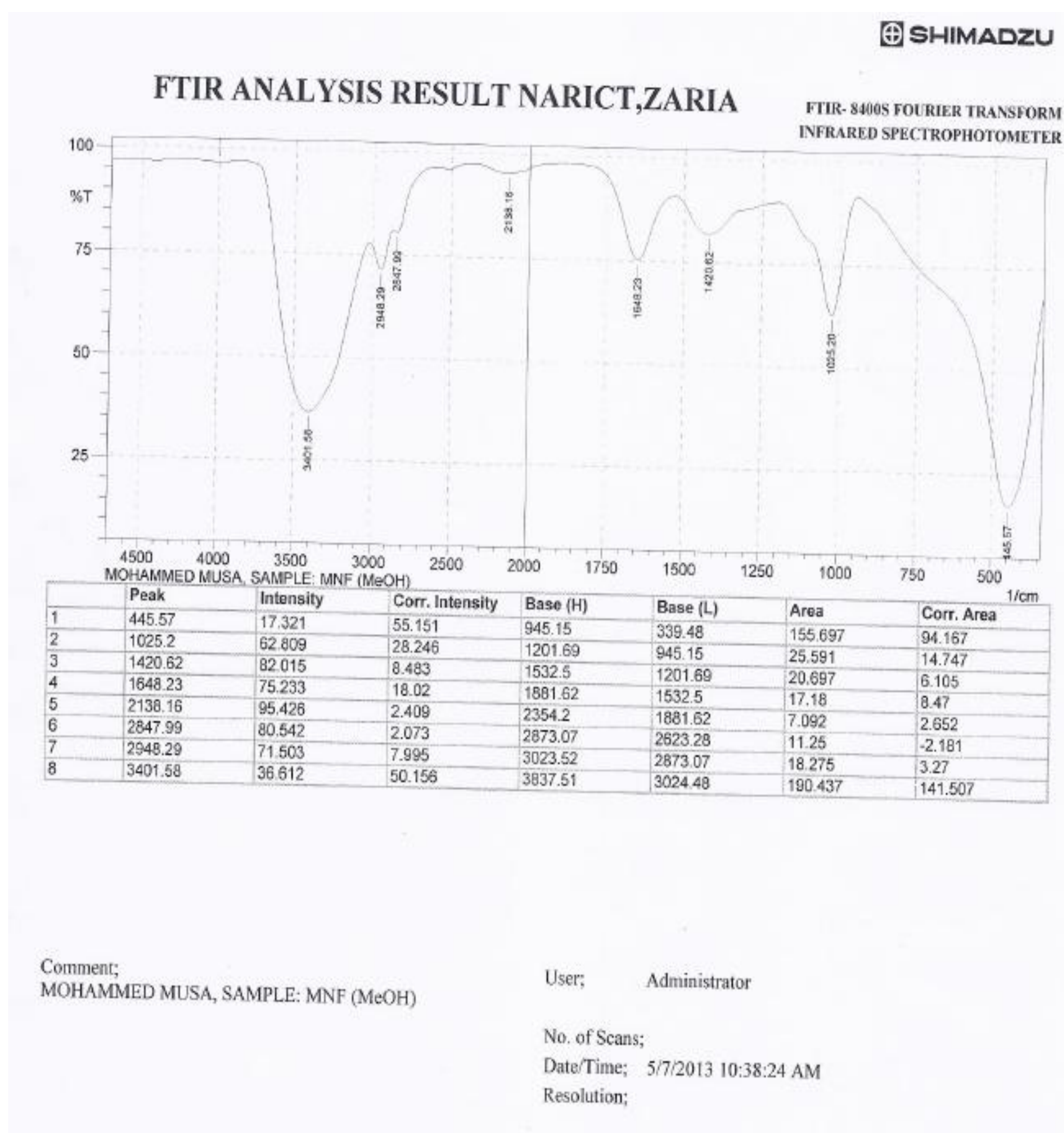
I = EtOAC : MeOH : Water (100:16.5:13.5)

II = $CHCl_3$: MeOH : Water (3:3:1)

4.4.4.1 Spectral Analysis of Compound MNF

Infrared spectroscopic analysis of compound MNF

The IR spectrum of MNF revealed the presence of the following frequency 3419 cm^{-1}, 2848 cm^{-1}, 1648 cm^{-1}, 1486 cm^{-1} and 1021 cm^{-1}(Fig. .4.1).



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	445.57	17.321	55.151	945.15	339.48	155.697	94.167
2	1025.2	62.809	28.246	1201.69	945.15	25.591	14.747
3	1420.62	82.015	8.483	1532.5	1201.69	20.697	6.105
4	1648.23	75.233	18.02	1881.62	1532.5	17.18	8.47
5	2138.16	95.426	2.409	2354.2	1881.62	7.092	2.652
6	2847.99	80.542	2.073	2873.07	2623.28	11.25	-2.181
7	2948.29	71.503	7.995	3023.52	2873.07	18.275	3.27
8	3401.58	36.612	50.156	3837.51	3024.48	190.437	141.507

Fig. 4.1. Infra-red band spectrum of Compound MNF

Table. 4.10 FT I R spectrum data of Compound MNF.

Bands (cm^{-1})	Intensity	Vibrations
3419	Broad	OH Stretch
2848	M	C-H
1648	S	C=O
1486	M	-C=C Stretch
1021	S	C-O or OH(def)

Keys.

M= Medium

S= strong

4.4.4.2 Proton Nuclear Magnetic Resonance of Compound MNF

Fig.4.2 Below shows the ^{1}H NMR spectrum of compound MNF with the following resonance at δ_H 5.6 ppm (1H, J=1.1H_Z, H-1), δ_H 7.3 ppm (1H, s, H-3), δ_H 1.9 ppm (1H, m, H-6), δ_H 1.7 ppm (IH, m, H-7), δ_H 3.7 ppm (1H,H- 9), δ_H 0.96 ppm (3H, s, H-10), δ_H 3.65 ppm (3H, s, OCH_3), δ_H 4.40 ppm (J=9.85/H-1'), δ_H 3.5 ppm (H- 2'), δ_H 3.1 ppm (H-3'), δ_H 3.2 ppm (IH, H-4'), δ_H 3.4 ppm (1H,m, H-5'), δ_H 3.6 ppm (2H,m, H-6), δ_H 3.6 ppm (1H,m, H-2"), δ_H 3.5 ppm (1H,m, H-3").

4.4.4. 3. Carbon-13 Nuclear Magnetic Resonance of Compound MNF

Fig. 4.3 Below shows the ^{13}C-NMR spectrum of compound MNF showing the presence 19carbon signals. The following signals were attributed to various carbons that is, δc 93.4 ppm, (C-1), δc 151.6 ppm (C-3), δc 114.6 ppm (C-4), δc 106.3 ppm (C-5), δc 36.0 ppm (C-6), δc 38.0 ppm (C-7), δc 104.5 ppm (C-8), δc 61.1 ppm (C-9), δc 23.5 ppm (C-10), δc 167.1 ppm (C-11), δc 52.0 ppm (OCH_3), δc 98.6 ppm (C-1'), δc 73.5 ppm (C-2') , δc 70.8 ppm (C-3'). δc 77.7 ppm (C-4'), δc 76.4 ppm (C-5'), δc 61.8 ppm (C-6'), δc 152.0 ppm (C-1") , δc 82.8 ppm (C-2") and δc 74.8 ppm (C-3").

4.4.4.4 Dept Spectrum of Compound MNF

Fig. 4.4 Below shows the DEPT spectrum of compound MNF showing various Methine, methylene and Methane signals. DEPT spectrum of MNF experiments below also indicated the presence of 19 carbon atoms. The spectrum exhibited 1 methyl carbon atom, 3 methylene carbon atoms, 10 methine carbon atoms and a single signal indicative of a methoxy carbon.

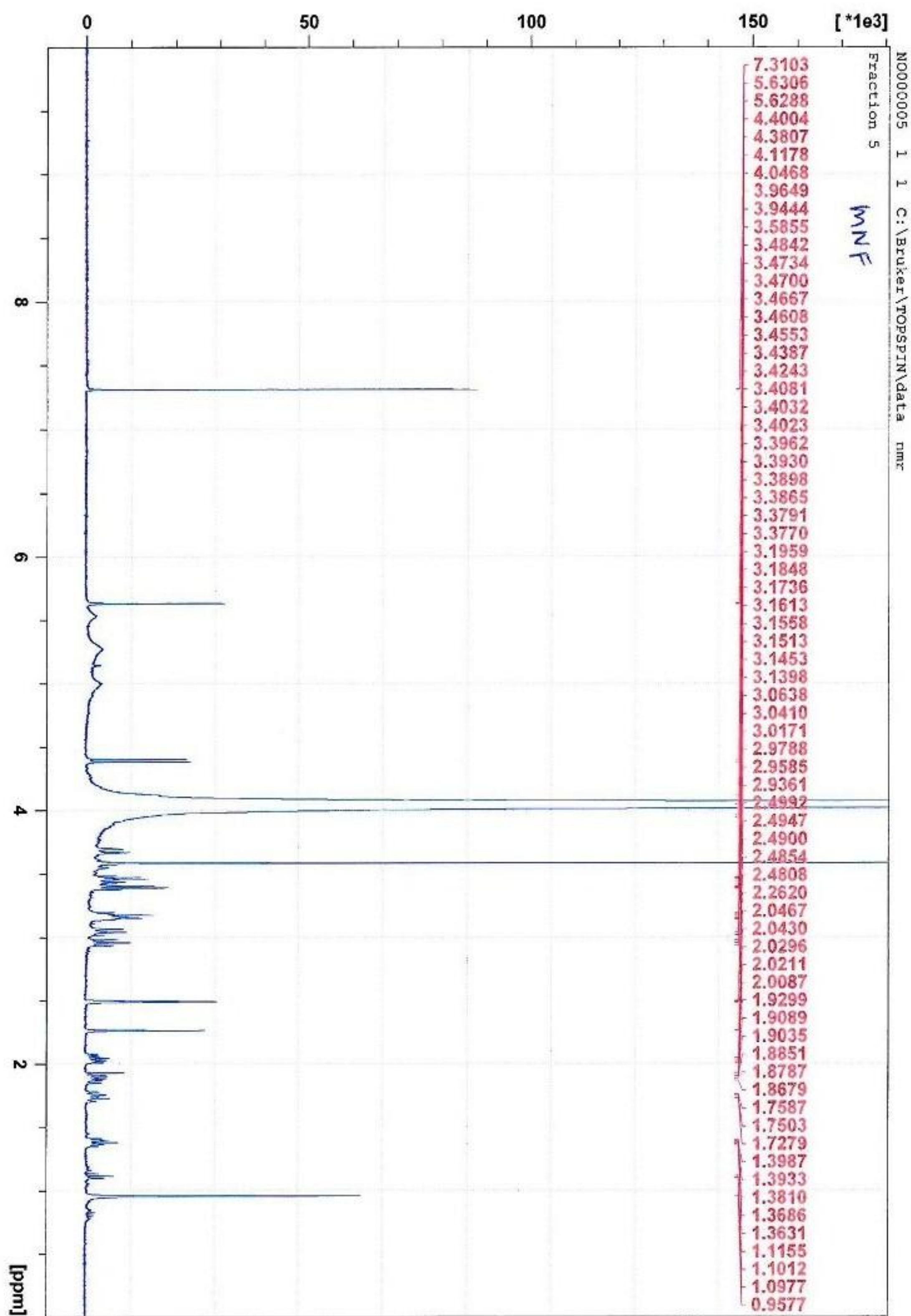


Fig. 4. 2 Proton Nuclear Magnetic Resonance (Nmr) spectrum of Compound MNF

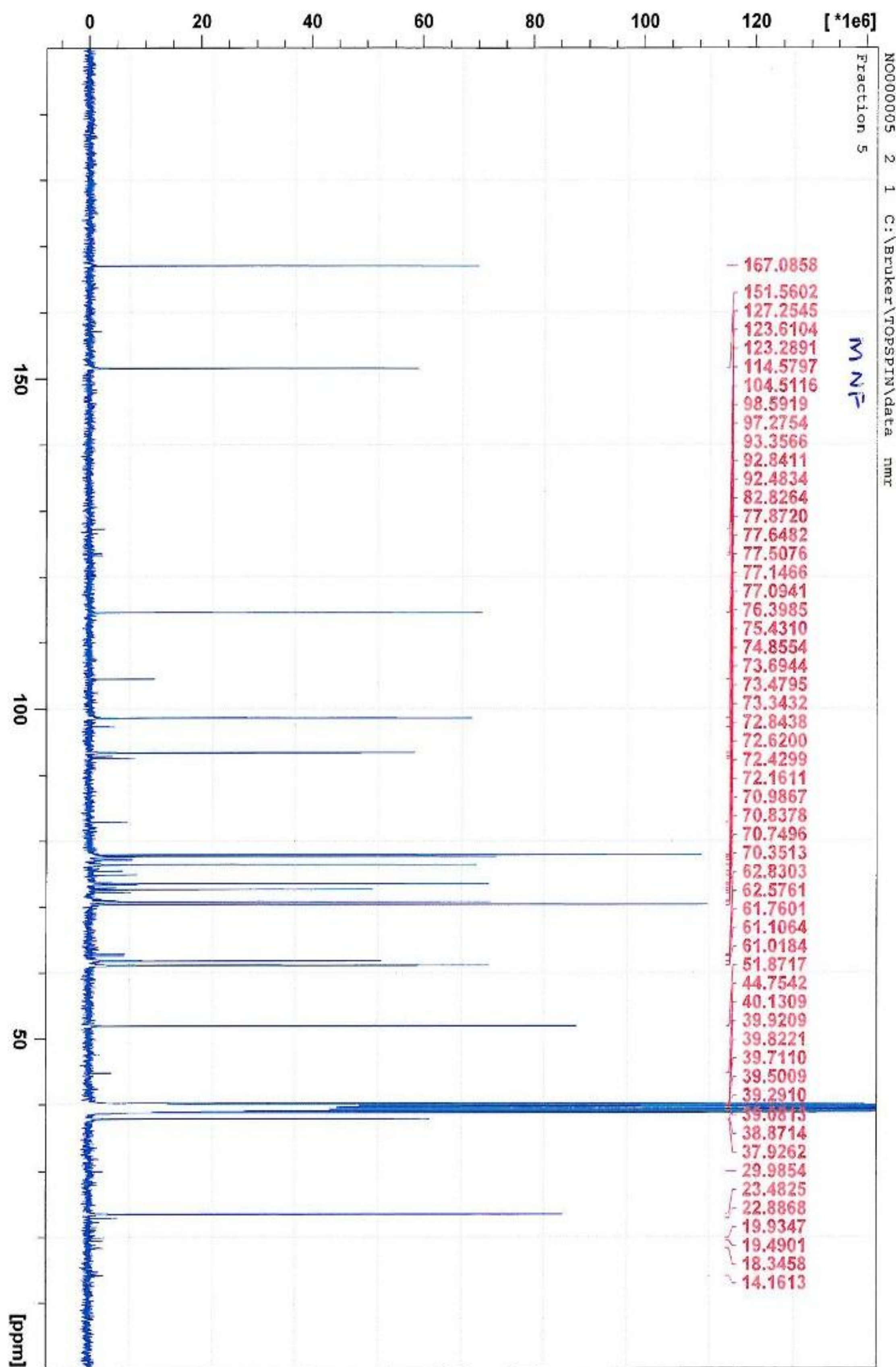


Fig. 4.3 ^{13}C NMR Spectrum of Compound MNF

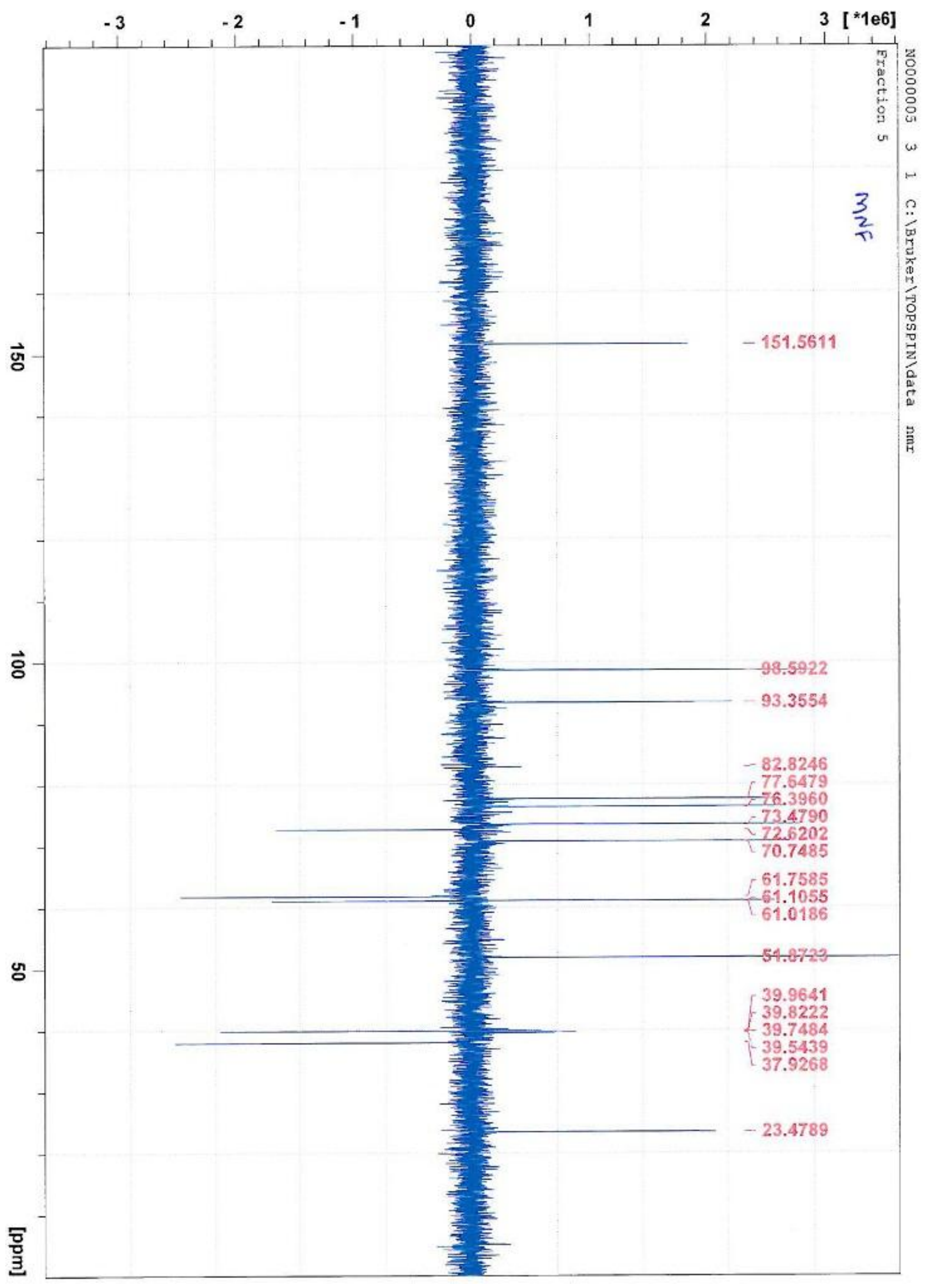


Fig. 4.4 DEPT Spectrum of Compound MNF in CD_3OD

4.4.4.5 HSQC Spectrum of Compound MNF

Fig.4.5 Below shows the HSQC spectrum of compound MNF showing the assignment of protons to their respective carbon atoms. The following correlations were observed, δ_c 98.4 ppm →of δ_H 5.60ppm, 7.3ppm → δ_c 151.6ppm, δ_H 1.9ppm→δ_c 36.0ppm, δ_H 1.7ppm→ δ_c 38.0 ppm, δ_H 3.7ppm→ δ_c 61.1ppm, δ_H 0.96ppm → δ_c 23.5ppm, δ_H 3.65ppm → δ_c 51.9ppm, δ_H 4.4ppm → δ_c 98.6ppm, δ_H 3.5ppm → δ_c 73.5ppm, δ_H 3.1ppm → δ_c 70.8ppm, δ_H 3.2ppm → δ_c 77.7ppm, δ_H 3.4ppm → δ_c 76.4ppm, δ_H 3.6ppm→ δ_c 61.8ppm, δ_H 3.6ppm → δ_c 82.8ppm and δ_H 3.1ppm → δ_c 74.8ppm.

4.4.4.6 COSY Spectrum of Compound MNF

Fig. 4.6 Below shows the COSY spectrum of compound MNF showing the (^1H-^1H COSY) correlation of protons situated in the same environment also exhibiting the same nature of substitution on the aglycone and the sugar moiety. Signal at δ_H 3.7ppm correlated to δ_H 4.4ppm and δ_H 0.96ppm, signal at δ_H 3.5ppm was also found to correlates with δ_H 4.4ppm and δ_H 3.1ppm, while δ_H 3.2ppm was found to correlates with δ_H 3.4ppm of the cyclo propyl ring respectively.

4.4.4.7 NOESY Spectrum of Compound MNF

Fig. 4.7 and 4.8 Below shows the NOESY spectrum of compound MNF showing the showing correlations of protons situated in the same environment also exhibiting the same nature of substitution on the aglycone and the sugar moiety.

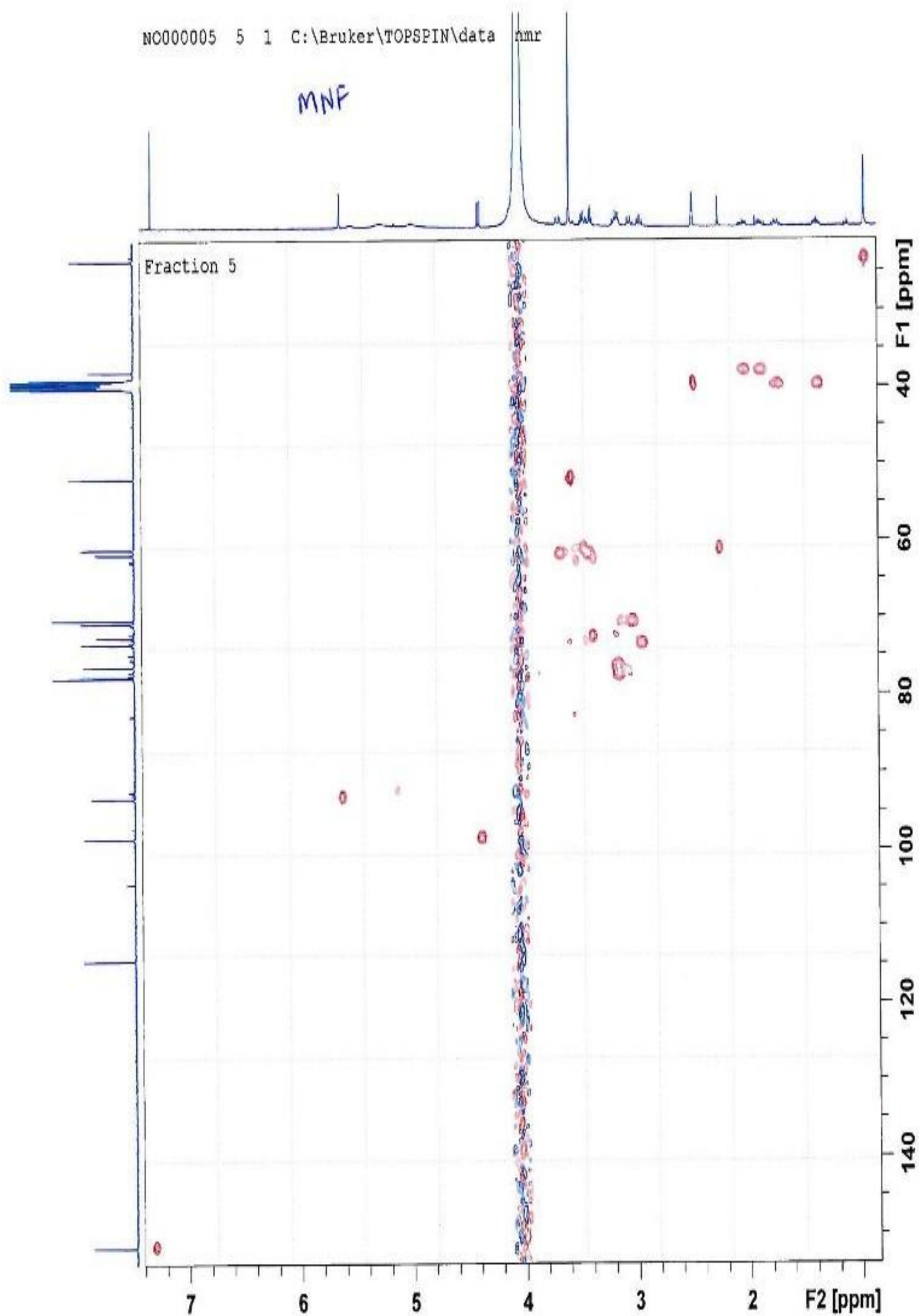


Fig. 4.5 HSQC Spectrum of Compound MNF in CD_3OD

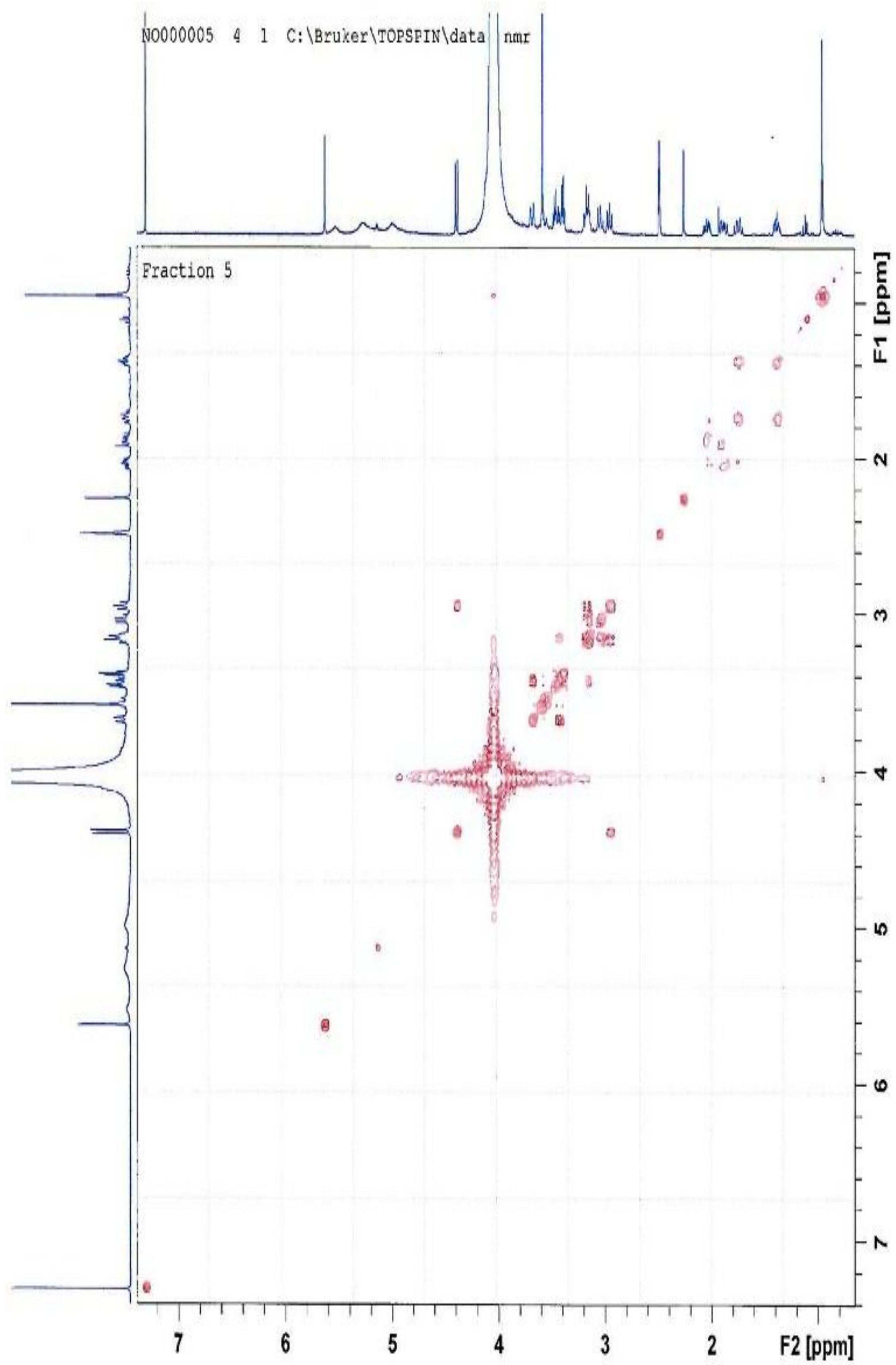


Fig. 4.6 COSY spectrum of compound MNF in CD_3OD

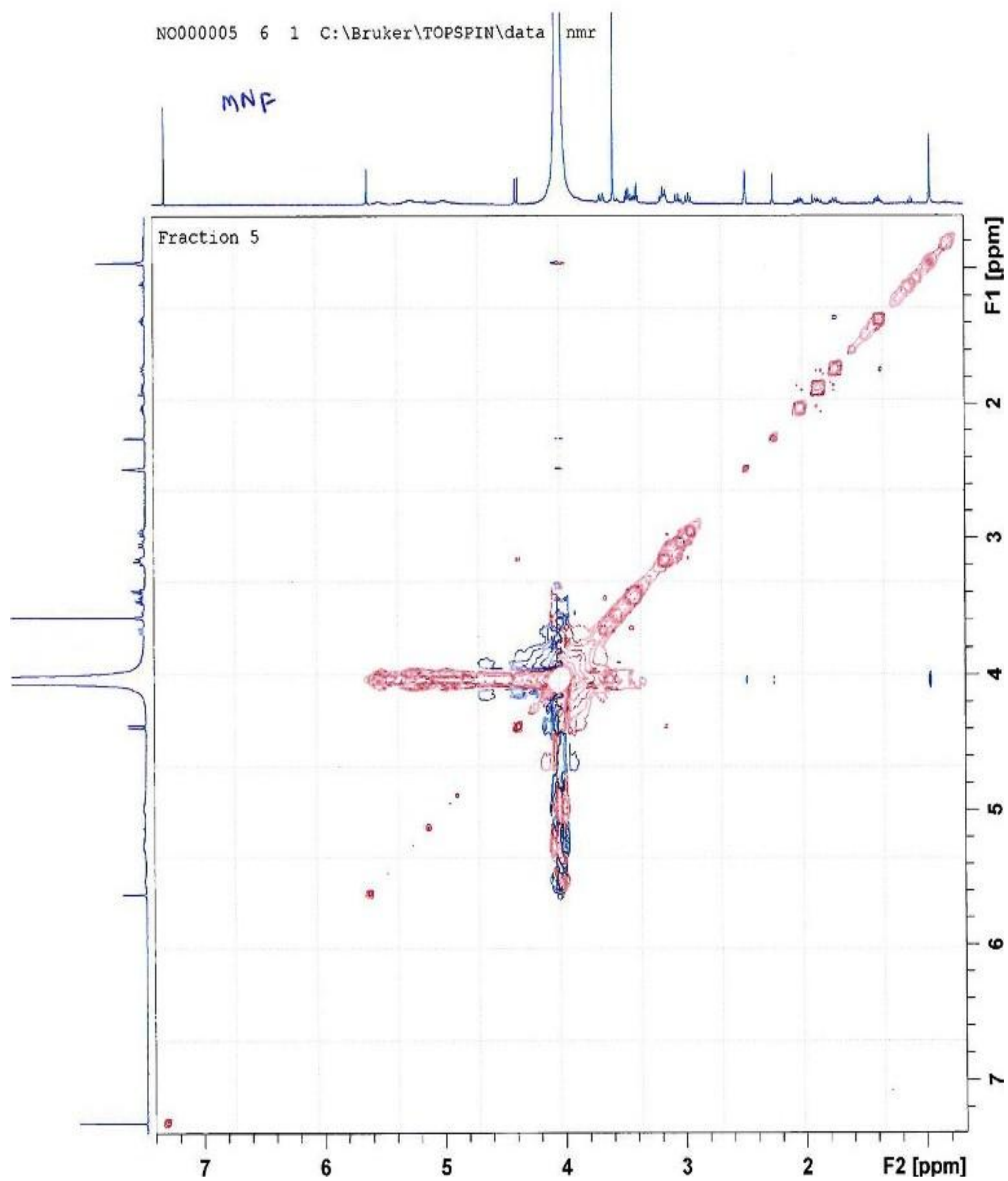


Fig. 4.7 NOESY Spectrum of Compound MNF in CD_3OD

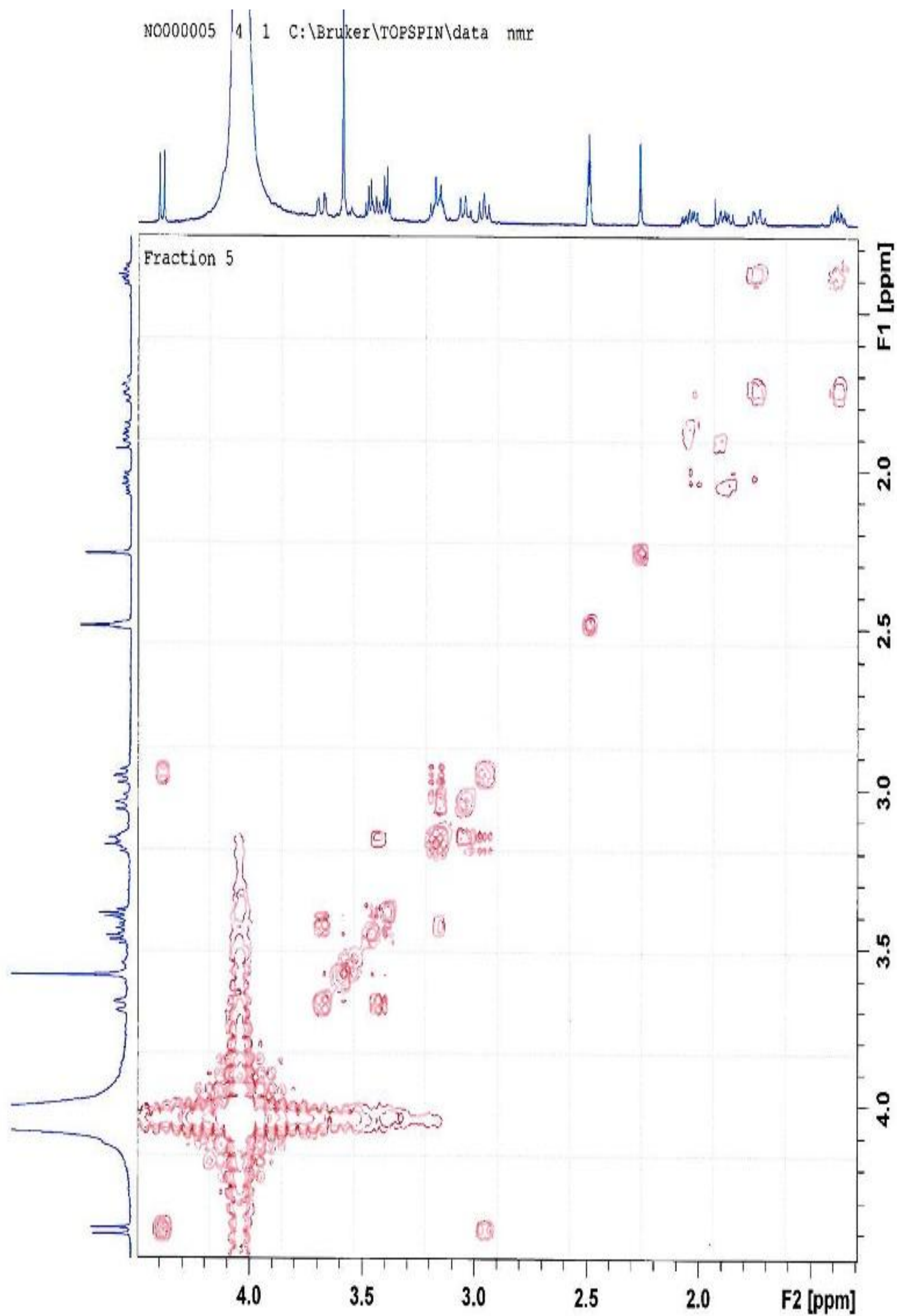


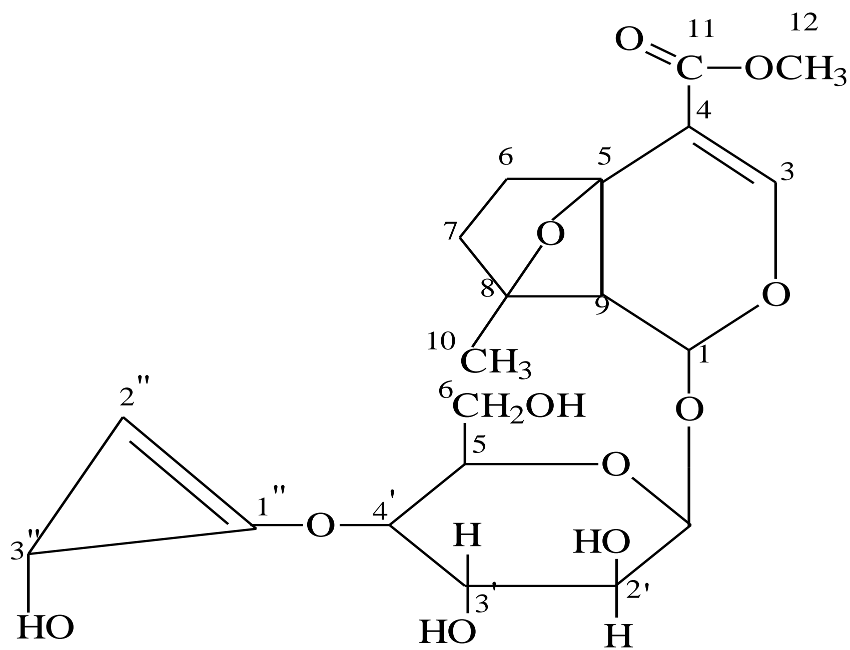
Fig 4.8 NOESY (Expanded) Spectrum of Compound MNF in CD_3OD

Table 4.11 ^{13}C (150 MHz) and ^{1}H (500 MHz) NMR spectra data of Compound MNF in CD_3OD, (δ ppm, J in H_Z).

POSITION	**δc (PPM)**	**δ_H (ppm)**	$J=H_Z$
1	93.4	5.6	
3	152.0	7.3(s)	
4	114.0	-	
5	106.3	-	
6	36.0	1.90(d)	
7	38.0	1.70,1.4	
8	104.5	-	
9	61.10	3.7	
10	23.5	0.96 (s)	
11	167.1	-	
12	51.9	OCH_3	
1'	98.6	4.4	9.85
2'	73.5	3.5	
3'	70.8	3.1	
4'	77.7	3.2	
5'	76.4	3.4	
6'	61.8	3.6	9.12
1''	152.0	-	
2''	82.8	3.7	
3''	74.8	3.4	

Table: 4.12 ID and 2D NMR Spectral Summary for compound MNF in CD_3OD (500MH_Z, 125MH_Z).

Position	$\delta_{H\ ppm}$ (J= H_Z)	$\delta_{C(ppm)}$	DEPT	HSQC	NOESY
1	5.60	93.4	CH	H-1	H-1'
3	7.30	151.6	CH	H-3	
4	-	114.6	C	-	
5	-	106.3	C	-	
6	1.90(d)	36.0	CH_2	H-6	
7	1.70,1.4	38.0	CH_2	H-7	
8	-	104.5	C	-	
9	3.4	61.1	CH	H-9	
10	0.96	23.5	CH_3	H-10	
11	-	167.1	C	-	
12	3.65(S)	51.9	OCH_3	12	
1'	4.40 (9.85Hz)	98.6	CH	H-1'	H-2'
2'	3.50	73.0	CH	H-2'	
3'	3.10	70.8	CH	H-3'	H-3''
4'	3.20	77.7	CH	H-4'	
5'	3.40	76.4	CH	H-5'	
6'	3.70	61.8	CH_2	H-6'	
1''	-	152.0	C	-	
2''	3.6	82.8	CH	H- 2''	
3''	3.5	74.8	CH	H-3''	



XV

4.5 Isolation of compound B

Fractionation of the n-butanol fraction of the stem bark extract using silica gel column chromatography yielded 10 pooled fractions B_1 - B_{10}. Repeated gel chromatographic separation of fraction B_3 (58 – 179) and subsequently PTLC afforded compound B (Table 4.13).

Table 4:13 Fractions obtained from the column chromatographic separation of n-butanol fraction of the ethanolic stem bark extract..

S/No	Fraction	Eluting Solvent	No of spots	Weight(mg)	Code
1	6-25	EtOAc 100%	3	0.1	B_1
2	26-57	EtOAc: MeOH (90:10)	4	15	B_2
3	**58-179**	**EtOAc: MeOH (80-20)**	**2**	**86**	**B_3**
4	180-188	EtOAc: MeOH (70-30)	3	61	B_4
5	189-244	EtOAc: MeOH (60:40)	2	10	B_5
6	245-258	EtOAc: MeOH (50:50)	3	50	B_6
7	259-315	EtOAc: MeOH (40:60)	2	55	B_7
8	316-343	EtOAc: MeOH (30:70)	3	87	B_8
9	344-368	EtOAc: MeOH (20:80)	3	51	B_9
10	383-465	EtOAc: MeOH (10:90)	2	20	B_{10}

4.5.1 Melting Point of compound B

The melting point of compound B was determined to be 222-224^{0}C.

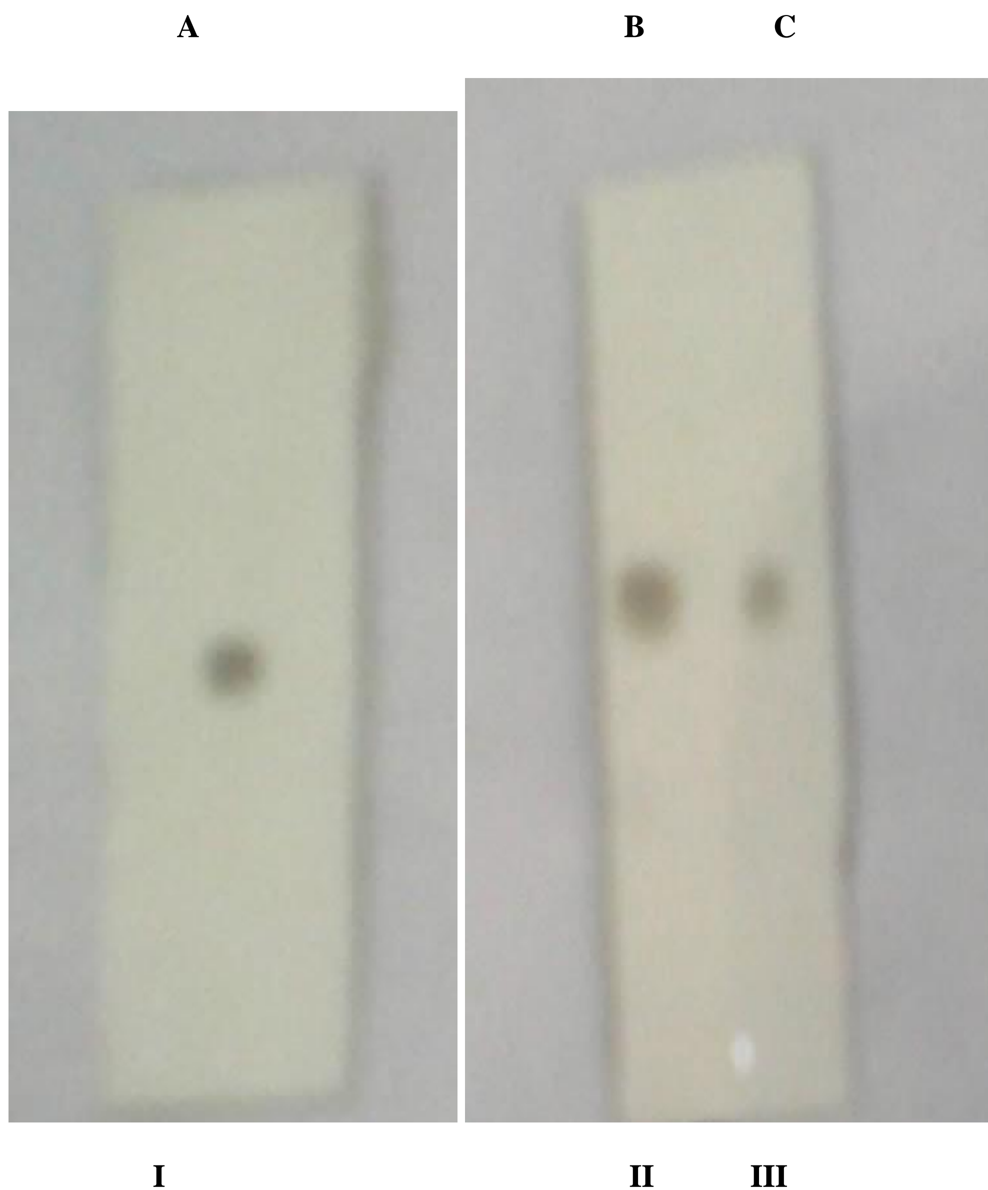
4.5.2 Solubility of compound B

Compound B was found to be soluble in Acetone and Methanol.

4.5.3 Identification test for compound B

The chromatogram of compound B gave a bluish – green coloration showing the presence of phenolics, while the Vanillin/ H_2SO_4 and perchloric acid test gave negative result.

Plate 4. 3 TLC profile of compound B using EtOAc : MeOH : Water (100:16.5:13.5) , $CHCl_3$: MeOH : Water (3:3:1) and EtOAc : formic acid : Water (10:1:2) as solvent system and the chromatogram was sprayed with 10% H_2SO_4.. This revealed a single spot With R_f value of 0.54 , 0.62 and 0.65 for A , B and C respectively**.**



Key

I = EtOAc : MeOH : Water (100:16.5:13.5)

II = $CHCl_3$: MeoH : Water (3:3:1)

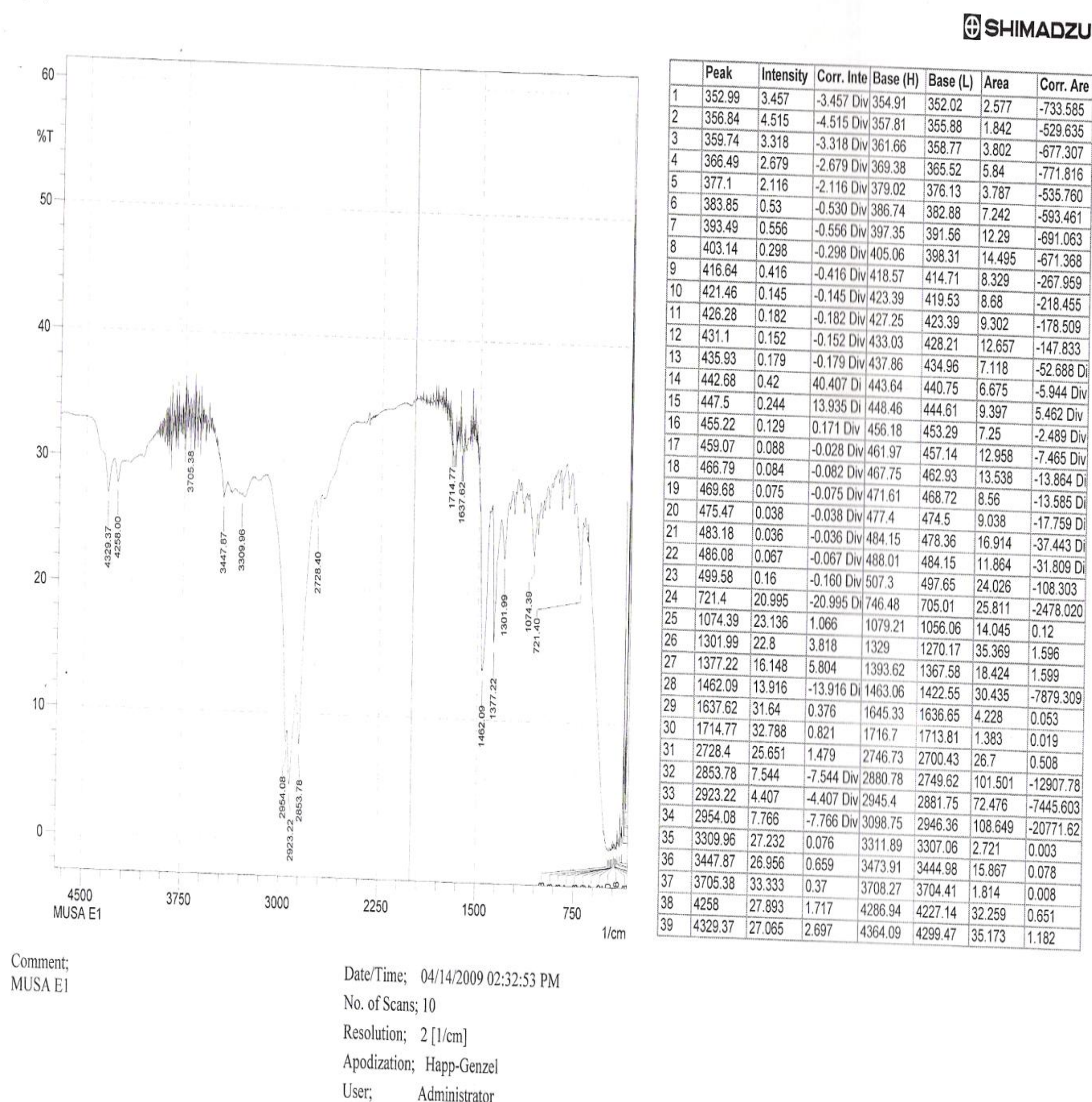
III= EtOAc : formic acid : Water (10:1:2)

4.5.4 Spectral Analysis of Compound B

Infrared spectrum absorption of compound B

The Infra-red spectroscopic analysis of compound B shows the absorptions at 3487cm^{-1}, 2954 cm^{-1} , 2923 cm^{-1}, 1462 cm^{-1}, 1201 cm^{-1} and 721 cm^{-1}.

Fig.4.9 FTIR result of compound B with all the spectral bands using spectrophotometer shimadzu 8400 model..



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	352.99	3.457	-3.457 Div	354.91	352.02	2.577	-733.585
2	356.84	4.515	-4.515 Div	357.81	355.88	1.842	-529.635
3	359.74	3.318	-3.318 Div	361.66	358.77	3.802	-677.307
4	366.49	2.679	-2.679 Div	369.38	365.52	5.84	-771.816
5	377.1	2.116	-2.116 Div	379.02	376.13	3.787	-535.760
6	383.85	0.53	-0.530 Div	386.74	382.88	7.242	-593.461
7	393.49	0.556	-0.556 Div	397.35	391.56	12.29	-691.063
8	403.14	0.298	-0.298 Div	405.06	398.31	14.495	-671.368
9	416.64	0.416	-0.416 Div	418.57	414.71	8.329	-267.959
10	421.46	0.145	-0.145 Div	423.39	419.53	8.68	-218.455
11	426.28	0.182	-0.182 Div	427.25	423.39	9.302	-178.509
12	431.1	0.152	-0.152 Div	433.03	428.21	12.657	-147.833
13	435.93	0.179	-0.179 Div	437.86	434.96	7.118	-52.688 Di
14	442.68	0.42	40.407 Di	443.64	440.75	6.675	-5.944 Div
15	447.5	0.244	13.935 Di	448.46	444.61	9.397	5.462 Div
16	455.22	0.129	0.171 Div	456.18	453.29	7.25	-2.489 Div
17	459.07	0.088	-0.028 Div	461.97	457.14	12.958	-7.465 Div
18	466.79	0.084	-0.082 Div	467.75	462.93	13.538	-13.864 Di
19	469.68	0.075	-0.075 Div	471.61	468.72	8.56	-13.585 Di
20	475.47	0.038	-0.038 Div	477.4	474.5	9.038	-17.759 Di
21	483.18	0.036	-0.036 Div	484.15	478.36	16.914	-37.443 Di
22	486.08	0.067	-0.067 Div	488.01	484.15	11.864	-31.809 Di
23	499.58	0.16	-0.160 Div	507.3	497.65	24.026	-108.303
24	721.4	20.995	-20.995 Di	746.48	705.01	25.811	-2478.020
25	1074.39	23.136	1.066	1079.21	1056.06	14.045	0.12
26	1301.99	22.8	3.818	1329	1270.17	35.369	1.596
27	1377.22	16.148	5.804	1393.62	1367.58	18.424	1.599
28	1462.09	13.916	-13.916 Di	1463.06	1422.55	30.435	-7879.309
29	1637.62	31.64	0.376	1645.33	1636.65	4.228	0.053
30	1714.77	32.788	0.821	1716.7	1713.81	1.383	0.019
31	2728.4	25.651	1.479	2746.73	2700.43	26.7	0.508
32	2853.78	7.544	-7.544 Div	2880.78	2749.62	101.501	-12907.78
33	2923.22	4.407	-4.407 Div	2945.4	2881.75	72.476	-7445.603
34	2954.08	7.766	-7.766 Div	3098.75	2946.36	108.649	-20771.62
35	3309.96	27.232	0.076	3311.89	3307.06	2.721	0.003
36	3447.87	26.956	0.659	3473.91	3444.98	15.867	0.078
37	3705.38	33.333	0.37	3708.27	3704.41	1.814	0.008
38	4258	27.893	1.717	4286.94	4227.14	32.259	0.651
39	4329.37	27.065	2.697	4364.09	4299.47	35.173	1.182

Fig. 4.9 Infrared spectrum absorption bands of compound B

Table.4.14 FT I R spectrum data of Compound B using spectrophotometer shimadzu 8400 model.

Bands (cm^{-1})	Intensity	Vibrations
3487	Broad	OH Stretch
2954	M	CH(stretch)
2923	B	COOH
1462	S	-C=C Stretch
1201	M	C=O (Stretch)
721	B	Weak bending vibration of –-- (CH2)-

S= Strong, M= Medium

4.5.5 Proton Nuclear Magnetic Resonance of Compound B

Fig. 4.10 Below shows the ^{1}H NMR spectrum of compound B with the following resonance at δ_H 7.1 (H-2), 6.5 (H-5), 6.8 (H-6), 7.5 (H-7), 6.3 (H-8), 6.7 (H-2$'$, 6.5 (H-5$'$), 6.7 (H-6$'$), 2.8 (H-7$'$), 3.8 (H-8$'$), 4.5 (H-I$''$), 3.6 (H-2$''$), 3.9 (H-3$''$), 4.8 (H-4$''$), 3.4 (H-5$''$) , 3.6(H-6$''$), 5.3(H-1$'''$), 3.7(H-2$'''$), 3.3(H-3$'''$), 3.1(H-4$'''$), 3.4(H-5$'''$) and 1.1(H-6$'''$).

The Proton Nuclear Magnetic Resonance of compound B shows signals at δ_H 6.80 ppm, δ_H 6.25 ppm and δ_H 6.65 ppm are indicative of aromatic protons. The signals at δ_H 5.30 ppm and δ_H 4.80 ppm are characteristic of anomeric sugar protons attributed to glucose and rhamnose moiety respectively. Signal observes at δ_H 1.1ppm could be due to proton on the methyl group of rhamnose and δ_H 3.6 ppm is for oxymethylene proton of the glucose moiety.

4.5.6 Carbon – 13 Nuclear Magnetic Resonance of Compound B

Fig.4..11 Below shows the ^{13}C-NMR spectrum of compound B showing the presence 29carbon signals. The ^{13}CNMR spectrum of compound B revealed resonance at δ_C 127.7(C-1), 115(C-2), 147(C-3), 149.4(C-4), 115.0(C-5), 123.2(C-6), 148.0(C-7), 117.1(C-8), 168.3(C-9), 131.5(C1$^{1'}$), 116.5(C-2$'$), 146.2(C-3$'$), 144.7(C-4$'$) , 116.3(C-5$'$), 121.3(C-6$'$), 36.6(C-7$'$), 72.1(C-8$'$), 103.1(C-1$''$), 78.2(C-2$''$), 81.6(C-3$''$), 76.1(C-4$''$), 70.4(C-5$''$), 62.4(C-6$''$), 104.2(C-1$'''$), 72.4(C-2$'''$), 72.3(C-3$'''$), 73.8(C-4$'''$), 70.6(C-5$'''$) and 18.5(C-6$'''$).

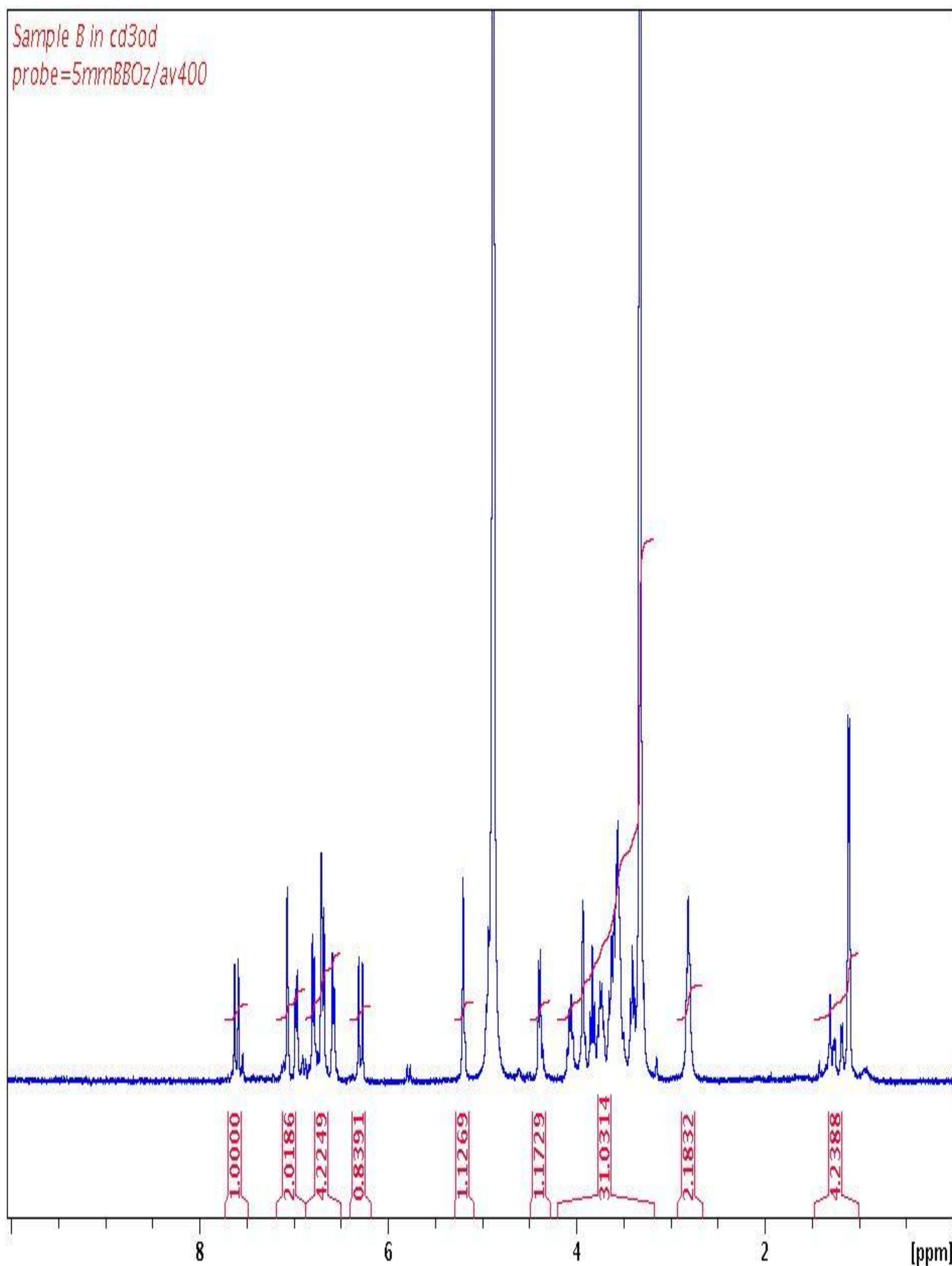


Fig.4..10 Proton Nuclear Magnetic Resonance (NMR) Spectrum of Compound B in CD_3OD.

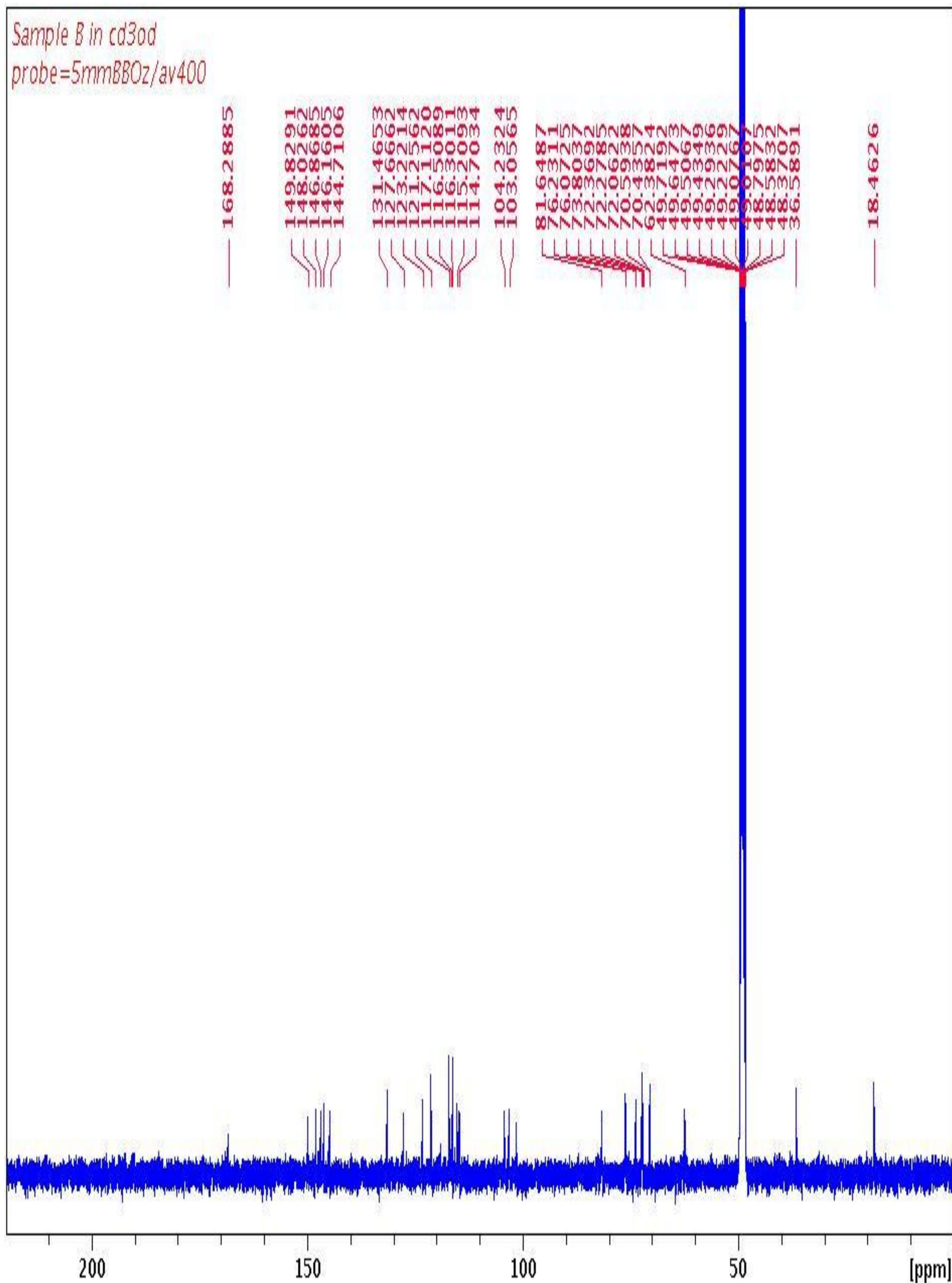
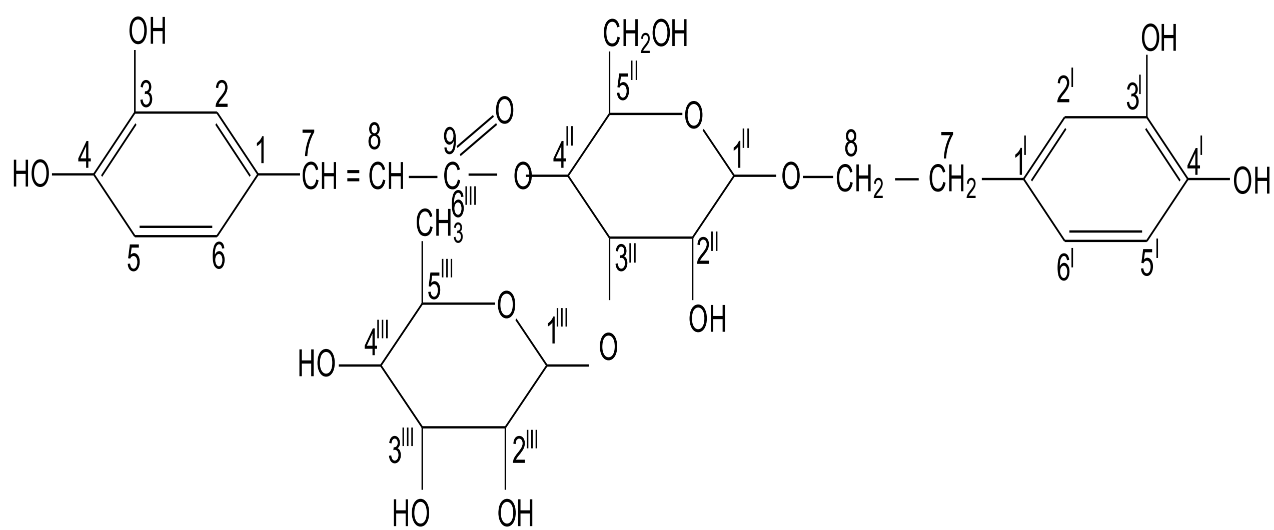


Fig. 4.11 Carbon-13 magnetic resonance of compound B in CD_3OD

Table: 4.15. H NMR and ^{13}C NMR spectrum data (δ in ppm) of Compound B in CD_3OD. Chemical shift were reported in part per million in relation to Internal standard**.**

Position	δ_C	δ_H value (ppm)
1	128.0	-
2	115.2	7.1
3	146.5	-
4	150.0	-
5	115.0	6.5
6	123.2	6.8
7	148.0	7.5
8	117.0	6.3
9	168.3	-
1'	132.0	-
2'	117.0	6.7
3'	146.2	-
4'	145.0	-
5'	116.3	6.5
6'	121.3	6.7
7'	37.0	2.8
8'	72.1	3.8
1"	103.1	4.5
2"	76.2	3.3
3"	82.0	4.8
4"	70.4	3.4
5"	76.1	3.6
6"	62.4	3.8
1"'	104.2	5.3
2"'	72.4	3.7
3"'	72.3	3.3
4"'	74.0	3.1
5"'	71.0	3.4
6"'	18.4	1.1



XVI

4.6 Isolation of compound MND

Fractionation of the n-butanol fraction of the root bark extract using silica gel column chromatography yielded 10 pooled fractions (Table 4.16). Repeated gel chromatographic separation of fraction D_4 yielded compound MND.

Table 4.16. Fractions obtained from the column chromatographic separation of n- butanol fraction of the ethanolic root bark extract of *S. angustifolia*.

No	Fraction	Eluting solvent	No of spots	Weight (mg)	Code
1	4-26	EtOAc 100%	3	0.1	D_I
2	27-60	EtOAc: MeOH (90:10)	4	15	D_2
3	61-80	EtOAc: MeOH (80-20)	2	19	D_3
4	**81-242**	**EtOAc: MeOH (70-30)**	**3**	**136**	$\mathbf{D_4}$
5	241-252	EtOAc: MeOH (60:40)	2	10	D_5
6	253-258	EtOAc: MeOH (50:50)	3	50	D_6
7	259-315	EtOAc: MeOH (40:60)	2	55	D_7
8	316-343	EtOAc: MeOH (30:70)	3	87	D_8
9	344-368	EtOAc: MeOH (20:80)	3	51	D_9
10	369-388	EtOAc: MeOH (10:90)	2	20	D_{10}

4.6.1 Melting Point of MND

The melting point of compound MND was determined to be 216-218^{0}C.

4.6.2 Solubility of MND

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

4.6.3 Identification test for MND

Compound MND produced a positive result with Vanillin/ H_2SO_4 test but gave a negative result with perchloric acid and ferric chloride test.

Plate 4.4. TLC profile of compound MND using EtOAc : MeOH : Water (100:16.5:13.5) , $CHCl_3$: MeOH : Water (3:3:1) and EtOAc : formic acid : Water (10:1:2) as solvent system. This revealed a single spot With R_f value of 0.48, 0.63 and 0.56 for A , B and C respectively (Fig. 4.11).

A **B** **C**

I II III.

Key

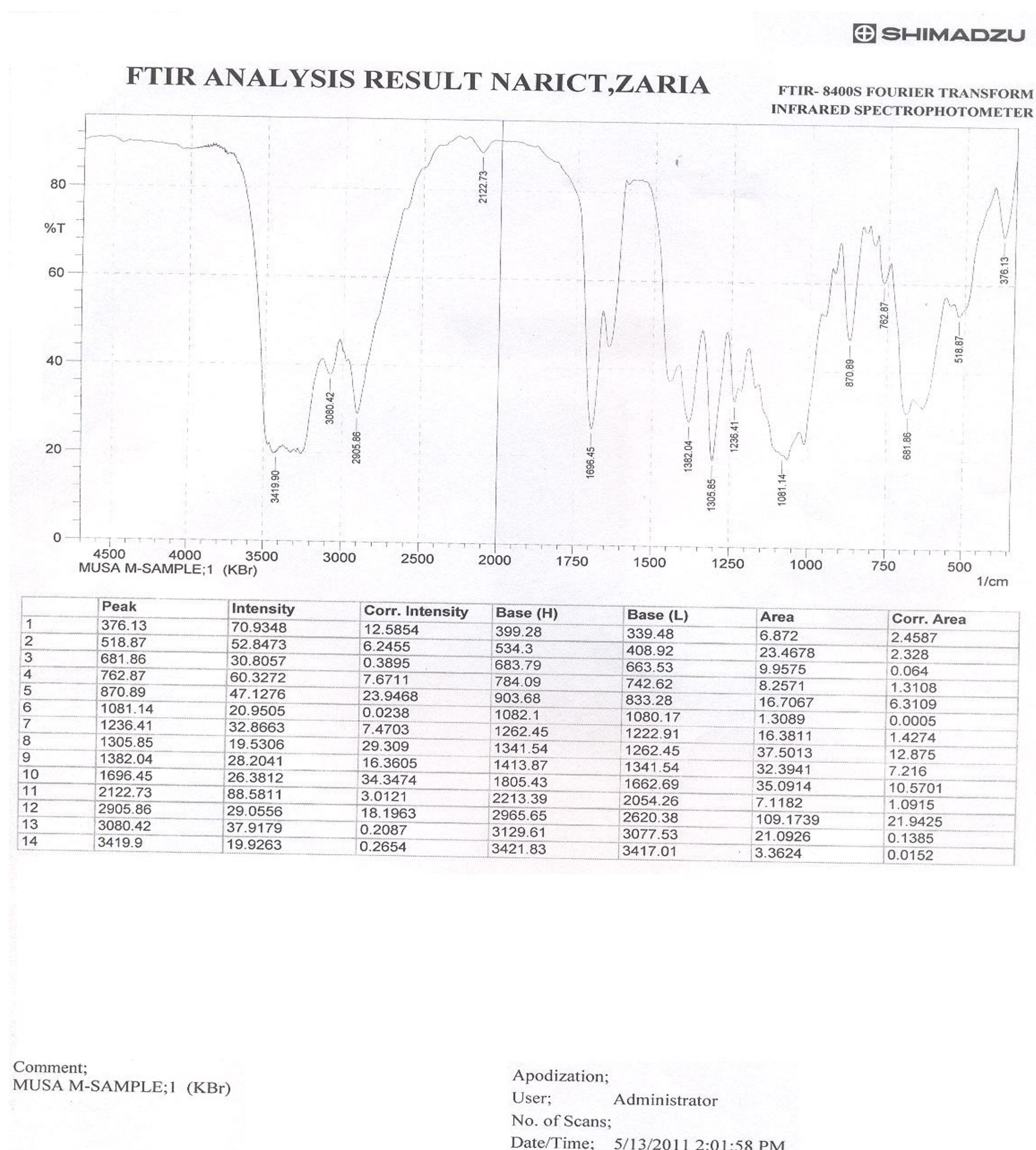
I = EtOAc : MeOH : Water (100:16.5:13.5)

II = $CHCl_3$: MeOH : Water (3:3:1)

III= EtOAc : formic acid : Water (10:1:2)

4.6.3.1 Infrared Spectral Analysis of MND

The Infra-red spectroscopic analysis of MND shows the absorption bands at 3419 cm^{-1} , 2848 cm^{-1} ,1486 cm^{-1} and 1021 cm^{-1}(Fig. 4.12.) .



SHIMADZU

FTIR ANALYSIS RESULT NARICT,ZARIA

FTIR- 8400S FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER

MUSA M-SAMPLE;1 (KBr)

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	376.13	70.9348	12.5854	399.28	339.48	6.872	2.4587
2	518.87	52.8473	6.2455	534.3	408.92	23.4678	2.328
3	681.86	30.8057	0.3895	683.79	663.53	9.9575	0.064
4	762.87	60.3272	7.6711	784.09	742.62	8.2571	1.3108
5	870.89	47.1276	23.9468	903.68	833.28	16.7067	6.3109
6	1081.14	20.9505	0.0238	1082.1	1080.17	1.3089	0.0005
7	1236.41	32.8663	7.4703	1262.45	1222.91	16.3811	1.4274
8	1305.85	19.5306	29.309	1341.54	1262.45	37.5013	12.875
9	1382.04	28.2041	16.3605	1413.87	1341.54	32.3941	7.216
10	1696.45	26.3812	34.3474	1805.43	1662.69	35.0914	10.5701
11	2122.73	88.5811	3.0121	2213.39	2054.26	7.1182	1.0915
12	2905.86	29.0556	18.1963	2965.65	2620.38	109.1739	21.9425
13	3080.42	37.9179	0.2087	3129.61	3077.53	21.0926	0.1385
14	3419.9	19.9263	0.2654	3421.83	3417.01	3.3624	0.0152

Comment;
MUSA M-SAMPLE;1 (KBr)

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Fig. 4. 12 **FTIR spectral bands of compound MND.**.

Table 4. 17 FTIR Data of Compound MND

Bands (cm^{-1})	Intensity	Vibrations
3419	Broad	OH Stretch
2848	M	C-H stretch
1648	S	C=O
1486	M	-C=C Stretch
1021	S	C-O or OH (def)

Key: S= Strong, M= Medium

4.6.3.2 Proton Nuclear Magnetic Resonance of MND

Fig.4.13 Below shows the ^{1}H NMR spectrum of compound MND with the following resonance at δ_H 5.8(1H, d, J=1.1H_Z, H-1), 7.4 (1H, s, H-3), 2.2 (H-5), 1.9 (1H, m, H-6), 1.6 (1H,m, H-7), 2.5 (1H, brs, H-9), 1.2 (3H, s, H-10), 3.7 (3H, s), 4.6 (1H, d, J= 7.8H_Z H-1'), 3.2 (3H,m, H-2'), 3.3 (3H,m, H-3'), 3.4 (3H,m, H-4'), 3.4 (3H,m, H-5'), (1H,dd, H-6').

4.6.3.3 Carbon-13 Nuclear Magnetic Resonance of MND

Fig.4.14 Below shows the ^{13}CNMR spectrum of compound MND with the following resonance at δ_c 93.0ppm(C-1), 151.2 (C-3), 114.0 (C-4), 70.3(C-5), 38.0 (C-6), 39.0 (C-7), 78.0(C-8), 60.5 (C-9), 22.0 (C-10), 167.0(C-11), 50.3 (OCH_3), 98.2 (C-1'), 73.0 (C-2'), 76 (C-3'), 70.3 (C-4'), 77.0 (C-5') and 61.4 (C-6').

4.6.3.4 DEPT Spectroscopic Analysis of MND

Fig.4.15 Below shows the DEPT spectrum of compound MND. The DEPT spectrum analysis exhibited 17 carbon atoms. This shows the presence of a single methyl carbon atom , two methylene carbon atoms, ten methine and a methoxy group.

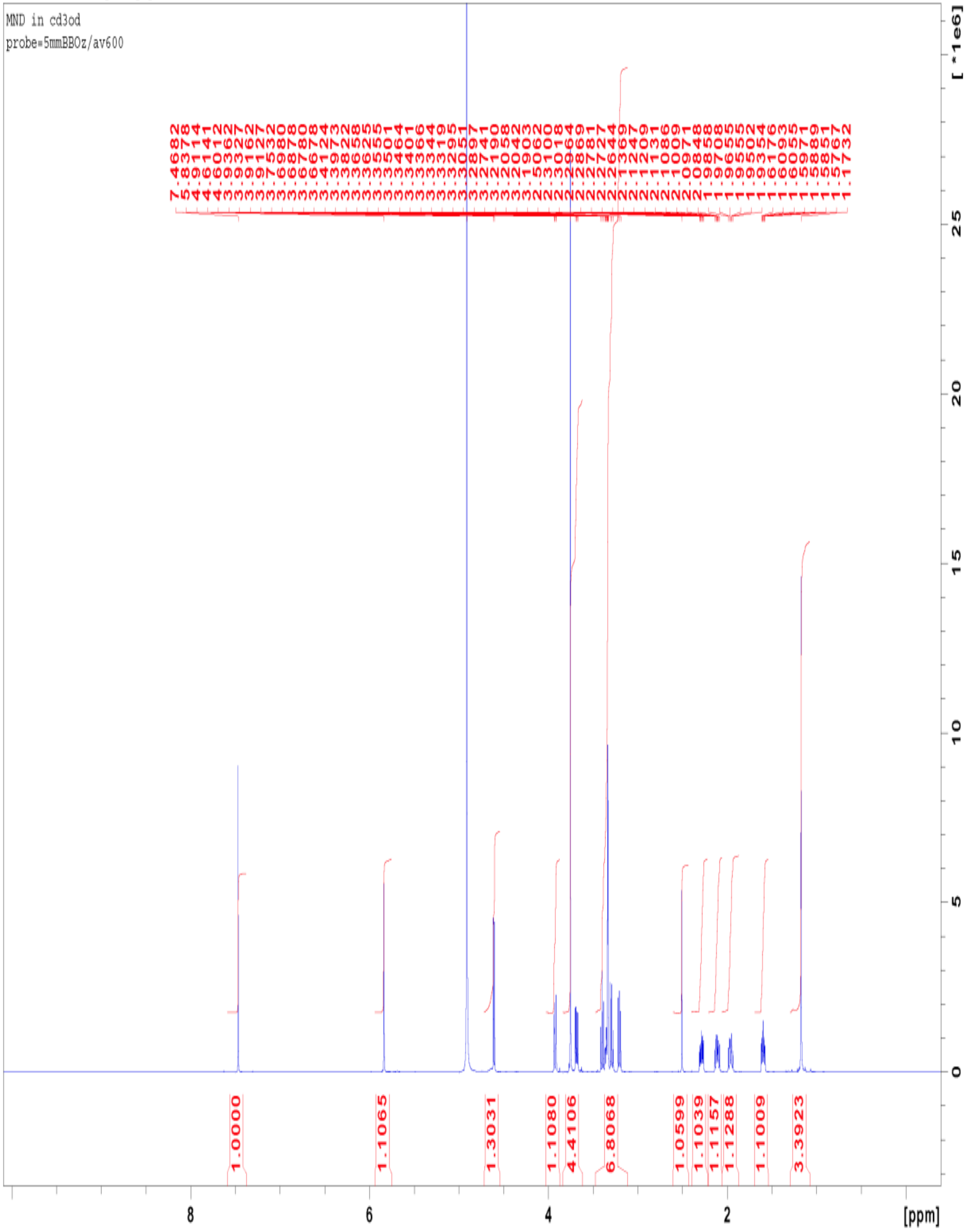


Fig. 4.13 Proton Nuclear Magnetic Resonance Spectrum of MND in CD_3OD.

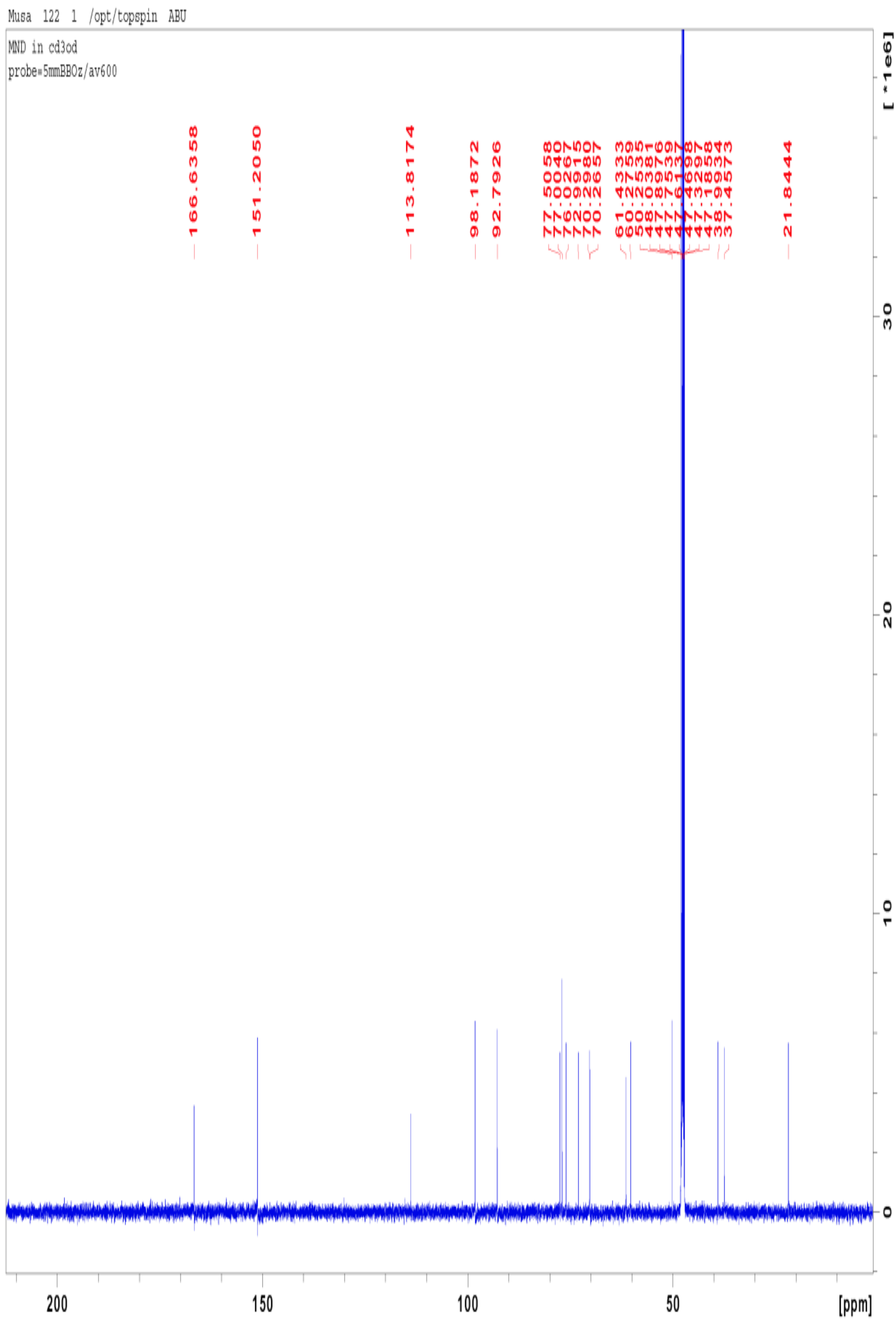


Fig. 4.14 Carbon -13 Nuclear magnetic resonance Spectrum of MND in CD_3OD

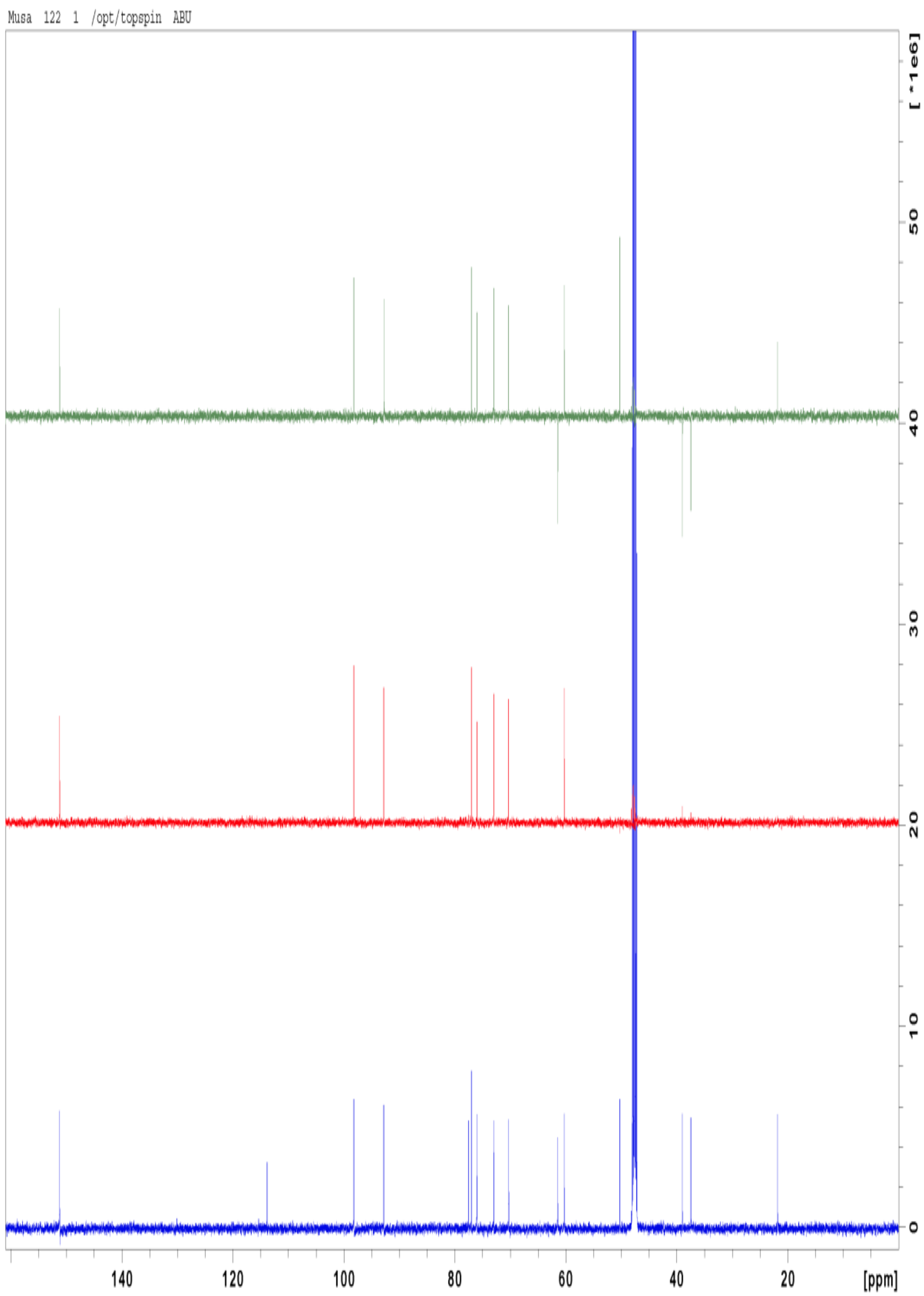


Fig. 4.15. C-13 (DEPT) Spectrum of MND in CD_3OD

4.6.3.5 HSQC OF MND

Fig.4.16 Below shows the HSQC spectrum of compound MND. The HSQC spectrum of MND was used in the assignment of proton to their respective carbons. The correlation of δ_H 5.80ppm to δ_c 92.80ppm was observed and δ_H 4.50ppm to δ_c 98.20ppm was also established. Other correlations include δ_H 7.4ppm (1H, s) to δ_c 151.2ppm, δ_H 1.9ppm(1H, m) to δ_c 38.0ppm, δ_H 1.6(1H,m), δ_H 2.05ppm to δ_c 39.0ppm, 2.5ppm (1H,brs) to δ_c 60.5ppm, δ_H 1.2ppm (3H, s) to δ_c 22.00ppm and δ_H 3.7 ppm(3H,s) to δ_c 50.3ppm for the aglycone. The signal observed at δ_H 4.6 (1H,d,j=7.8Hz) which is correlated to δ_c 98.2ppm could be attributed to the anomeric proton, signal at δ_H 3.6ppm (1H, dd, j=6.0Hz) correlated to δ_c 61.4ppm could be assigned to the oxymethylene proton of the sugar moiety. While signals observed at δ_H 3.2-3.4ppm correlated to δ_c 73.0, 76.0,70.3 and77.0ppm could be attributed to the overlapping signals of the sugar nucleus .

4.6.3.6 ^1H-^1H COSY spectroscopic analysis of MND

Fig. 4. 17 Below shows the COSY spectrum of compound MND showing the (^1H-^1H COSY) correlation of protons situated in the same environment. The 2D ^1H-^1H COSY Correlation of MND also exhibited the same nature of substitution on the aglycone and the sugar moiety.

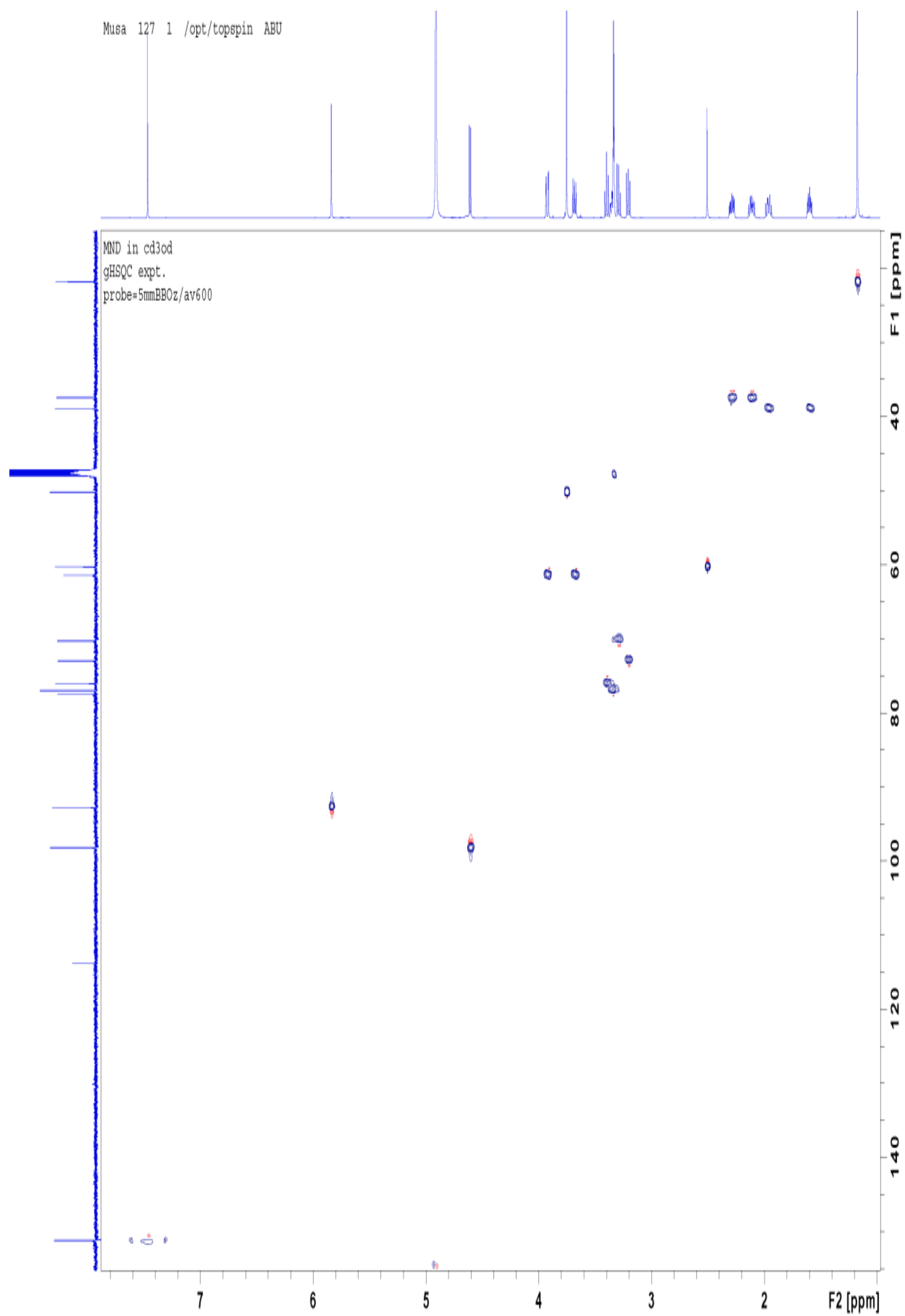


Fig: 4. 16 HSQC Spectrum of MND in CD_3OD.

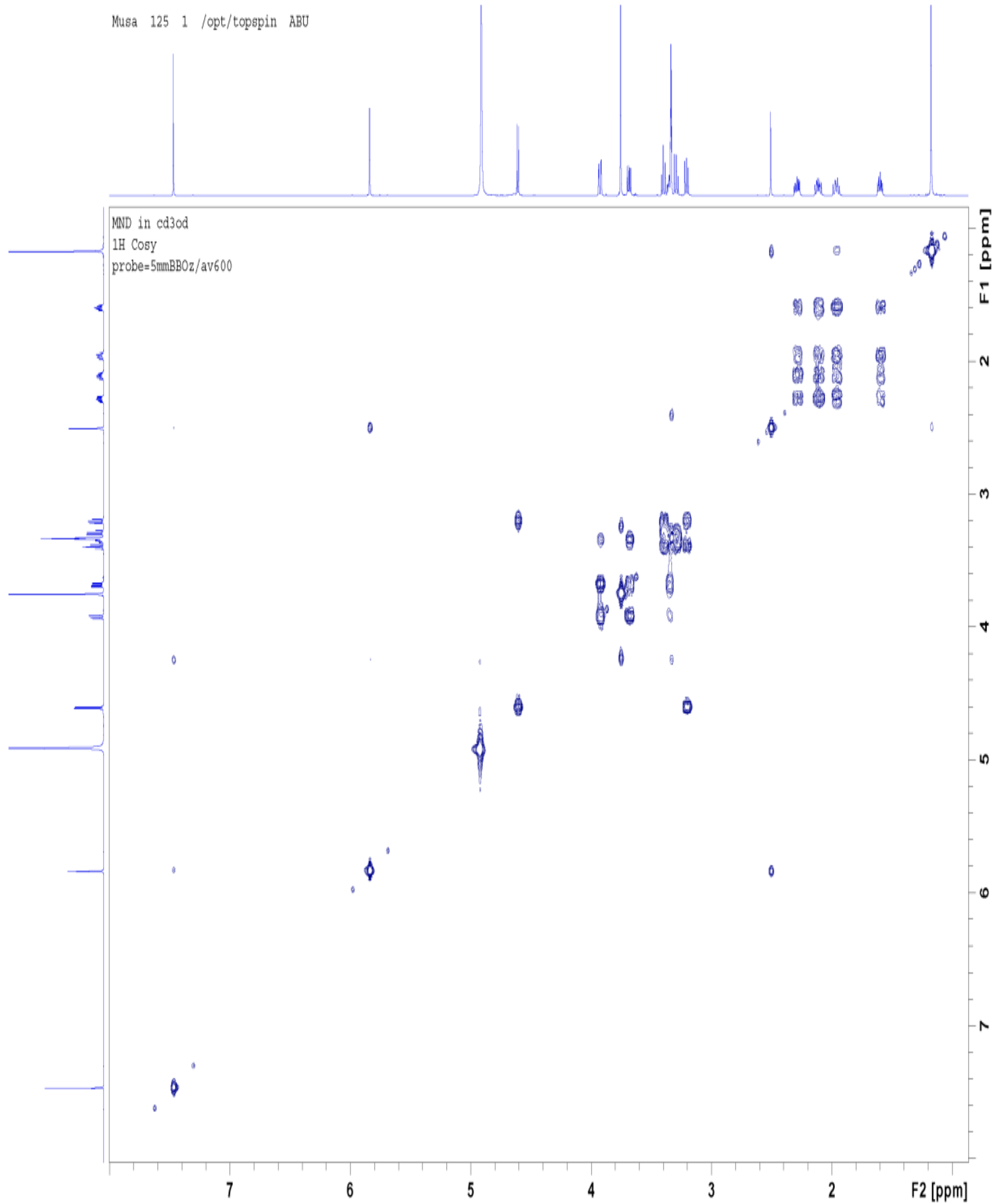


Fig. 4. 17 H-H COSY Spectrum of MND in CD_3OD

4.6.3.7 HMBC of compound MND

Fig. 4. 18 Below shows the HMBC spectrum of compound MND. The HMBC spectrum assists in establishing the connectivity between the various units of the molecules. The following correlations of H-5/ C-1, C-4 and C-11, H-1/ C-5, C-2’, C-1’, C-4 and C-3’, H-1/ C-3’and C-1’, H-6/C-2’, and C-3’, H-9/C-10, C-1 and C-3, H-6/C-10 and C-9, H-7/C-10, C-6, C-9, C-5 and C-1 and H-10/ C-7 and C-3’ were established respectively.

4.6.3.8 NOESY spectrum of compound MND

Fig. 4. 19 Below shows the NOESY spectrum of compound MND. The NOESY Spectrum exhibited the correlation of δ_H 5.8 ppm→δ_H 1-1, and δ_H 5.8 ppm→δ_H 4.6 ppm , δ_H 5.8 ppm→δ_H 3.4 ppm and δ_H3.6 ppm→δ_H 4.6 ppm.

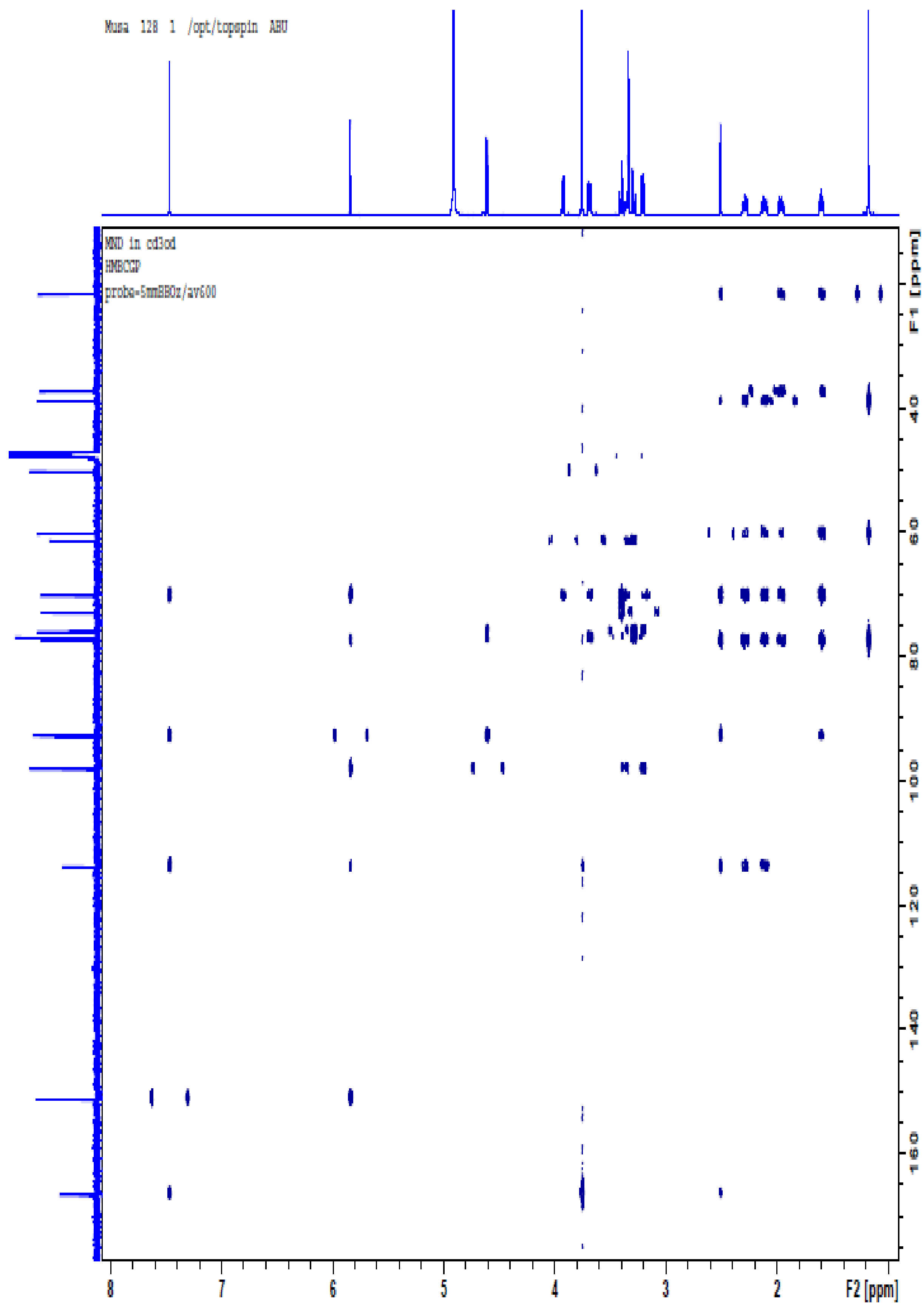


Fig. 4.18 HMBC Spectrum of Compound MND in CD_3OD

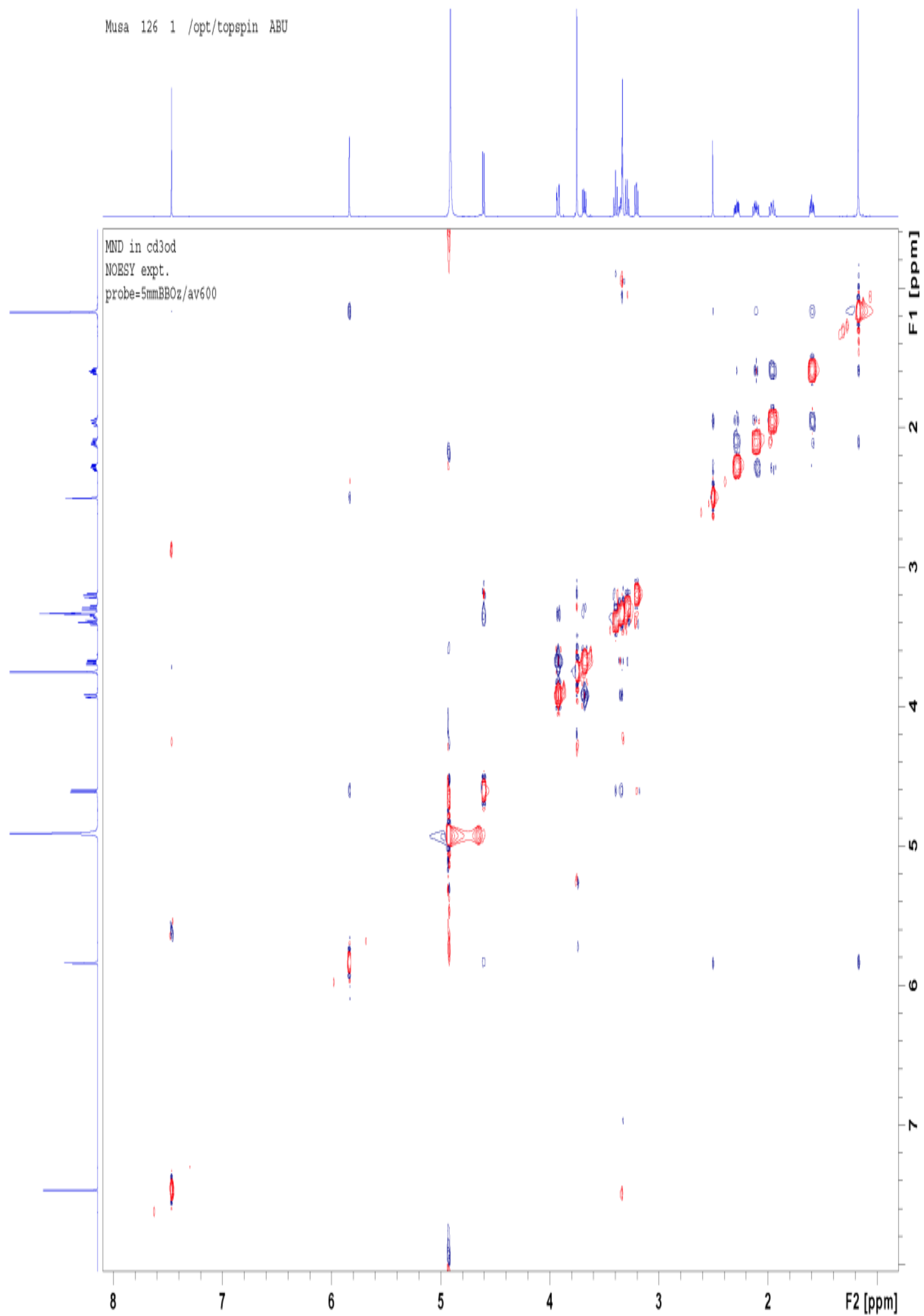


Fig. 4.19 NOESY spectrum of compound MND

Table 4.18 ^{13}C (150 MHz) and ^{1}H (500 MHz) NMR spectra data of Compound MND in CD_3OD, (δ ppm, J in H_Z). ^{1}H and ^{13}C NMR Spectral data of compound MND in CD_3OD (600MH_Z) and ^{13}C (100 MH_Z)

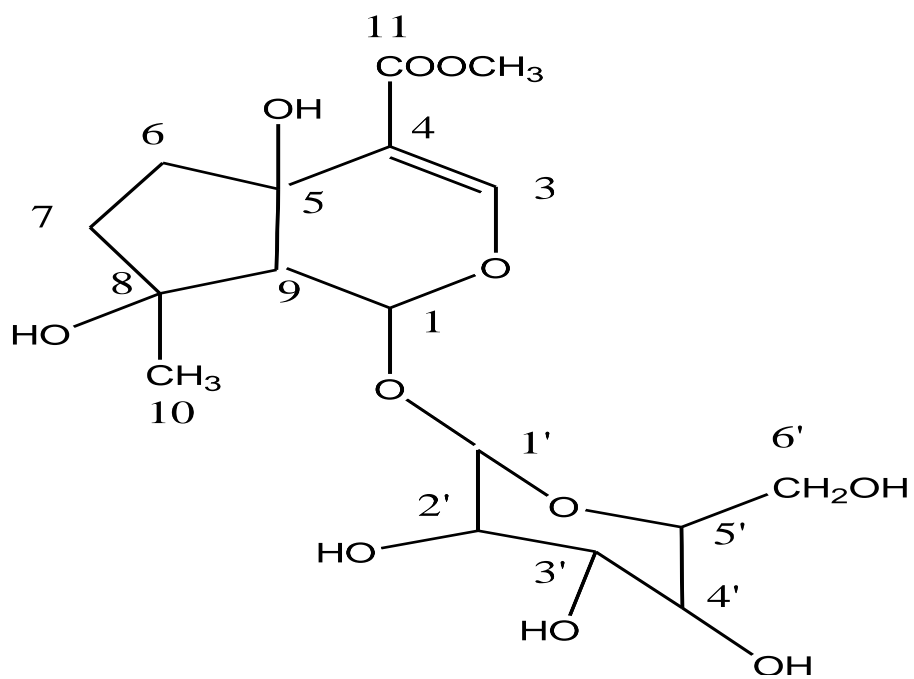
Position	δ_c	δ_H (Jin H_Z)
1	93.0	5.8 (1H, d 1.1 H_Z)
3	151.2	7.4 (1H, s)
4	114.0	-
5	70.3	-
6	38.0	1.9 (1H, m)
7	39.0	1.6 (1H, m), 2.05 (1H, m)
8	77.0	-
9	60.3	2.5 (1H, brs)
10	22.0	1.2 (3H, S)
11	167.0	-
OCH_3	50.3	3.7 (3H, s)
1'	98.2	4.6 (1H, d, 7.8 H_Z)
2'	73.0	3.2 (3H, m)
3'	76.0	3.3 (3H, m)
4'	70.3	3.4 (3H, m)
5'	77.0	3.4 (3H, m)
6'	61.4	3.6 (1H, dd 6.0)

Table: 4.19 H-H Correlation Spectral summary data (NOESY) for compound MND

Position	δ ^{1}H	δ ^{1}H (Jin H_Z)
1	5.8	1.2, 4.6
3	-	-
4	-	-
5	-	-
6	1.9	1.6
7	1.6	1.2
8	-	-
9	2.5	1.2, 1.6
10	-	-
11	-	-
OCH_3	-	-
$1^{'}$	4.6	3.6
$2^{'}$	-	-
$3^{'}$	-	-
$4^{'}$	-	-
$5^{'}$	-	-
$6^{'}$	-	-

Table: 4. 20 ID and 2D NMR Spectral data Summary for compound MND in CD_3OD (600MHZ, 125MHZ).

Position	δ H(J in HZ)	δC	DEPT	COSY	HMBC (C-H)
1	5.8 (1H, d, 1.1 HZ)	93.0	CH	H-9	C -3, C -9, C -1'
3	7.4 (1H, s)	151.2	CH		C -1, C -5
4	-	114.0	C		
5	-	70.3	C		C -3, C -6, C -7
6	1.9 (1H, m)	38.0	CH2		C -5, C -7, C -9
7	1.6 (1h, m)	39.0	CH		C -9, C -1"
8	-	77.0	CH		C -7, C -9, C -1"
9	2.5 (1H, brs)	60.0	CH	H-10	C -4, C -5, C -1"
10	1.2 (3H, S)	22.0	CH3		C -5, C -6
11	-	167.0	-		-
OCH3	3.7 (3H, S)	50.3			-
1'	4.6 (1H, d, 7.8HZ)	98.2	CH	H-2'	C -1, C -2'
2'	3.2 (3H, m)	73.0	CH		C -3'
3'	3.3 (3H, m)	76.0	CH		C -2'
4'	3.4 (3H, m)	77.0	CH		C -3'
5'	3.4 (3H, m)	70.3	CH		C -6'
6'	3.6 (1H, dd, 6.0)	61.4	CH2		C -1'



XVII

4.7 PHARMACOLOGICAL STUDIES

4.7.1 Result of Acute toxicity study.

The intreperitonial LD_{50} of the Leaf, Stem bark and Root bark extract were found to be 118.32 mg/kg ,150 mg/kg and 145 mg/kg respectively.

4.7.2 Results of Gastrointestinal activity study.

Fig. 4.20. Below shows the effect of acetylcholine on rabbit jejunum.

Acetylcholine produces concentration dependent increase in spontaneous contraction of the rabbit jejunum at the concentration of $4.0x10^{-3}$, $8.0\ x10^{-3}$, $1.6\ x10^{-3}$, $3.2x10^{-3}$and $6.4x10^{-3}$ug/ml.

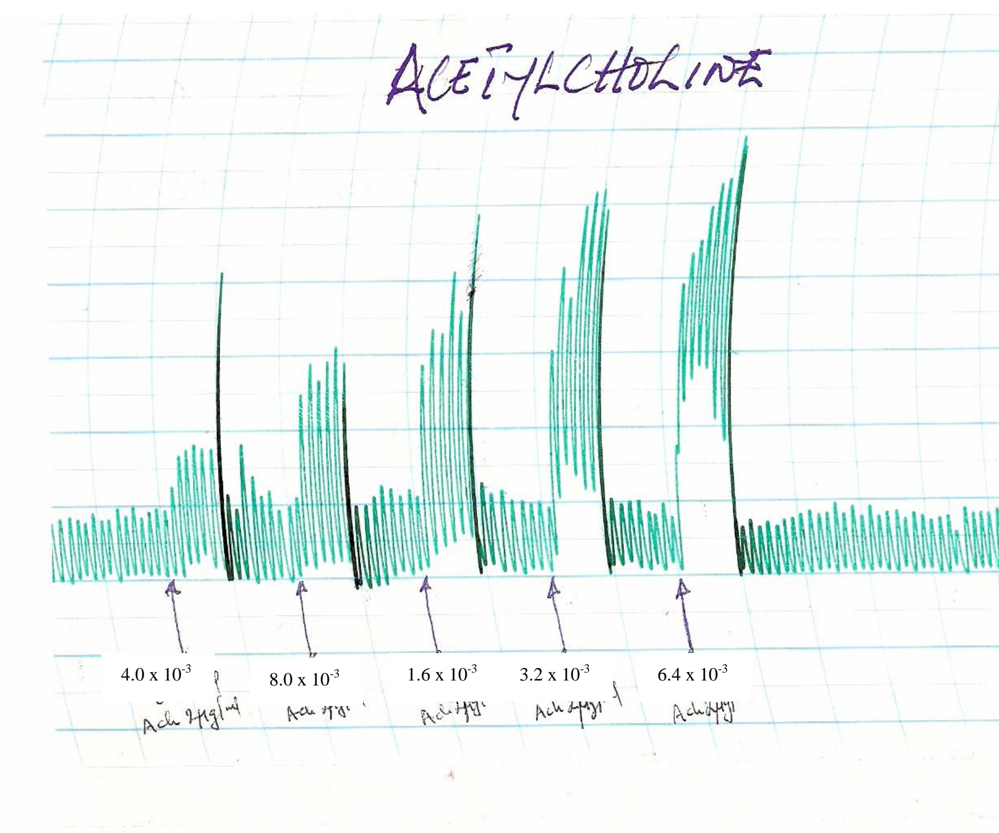


Fig :4. 20 Effect of acetylcholine on contraction of rabbit jejunum.

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Fig. 4.21 Effect of the whole plant ethanol extract on rabbit jejunum. The whole plant ethanol plant extract produces a concentration dependent increase in spontaneous contraction of the rabbit jejunum ($4.0x10^{-3}$, $8.0\ x10^{-3}$, $1.6\ x10^{-3}$, $3.2x10^{-3}$mg/ml).

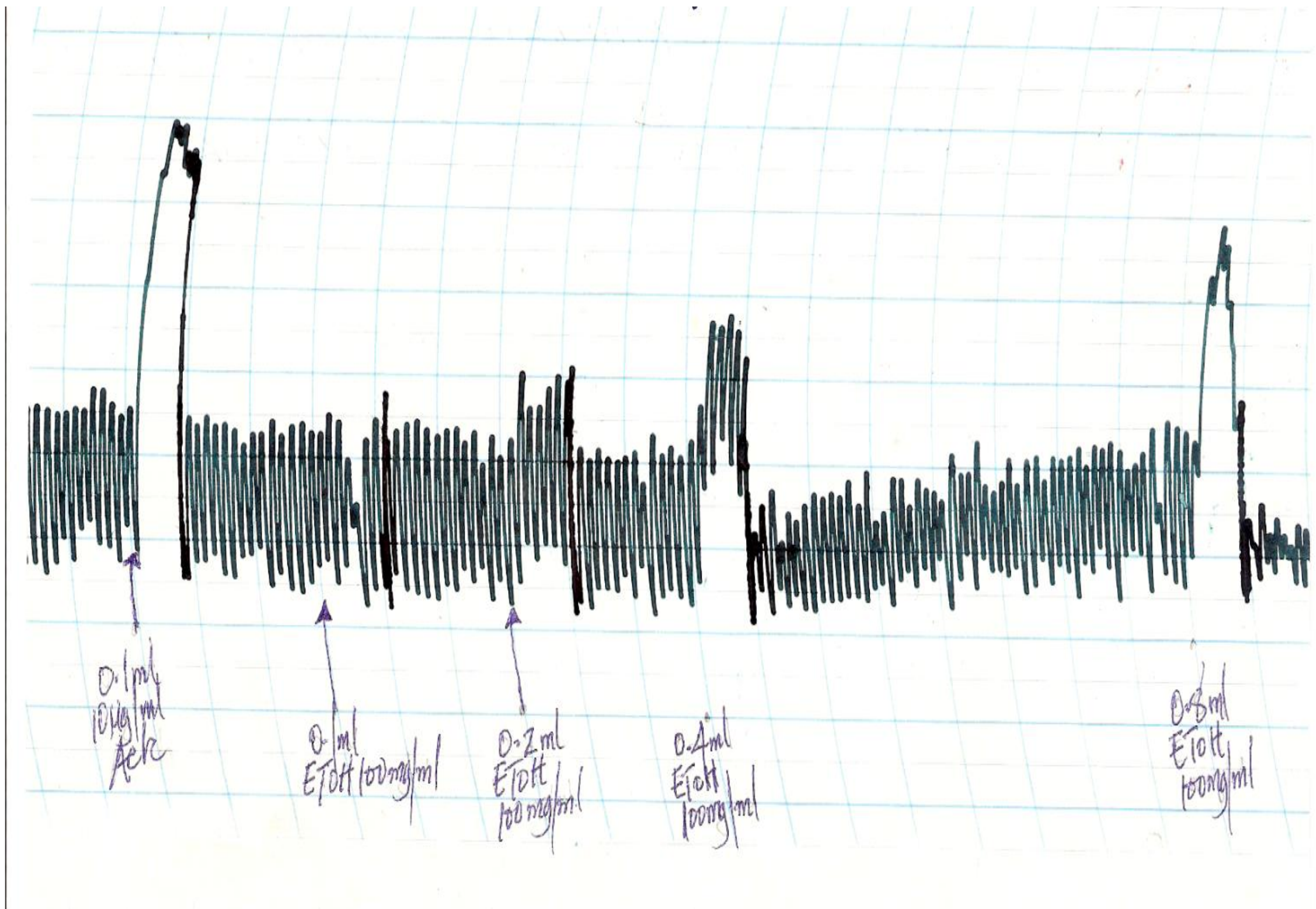


Fig. 4.21 Effect of the whole plant ethanol extract on rabbit jejunum

Fig. 4 . 22. Effect of the ethanol leaf extract on rabbit jejunum. The ethanol leaf extract of *S. angustifolia* produces concentration dependent increase in contraction of the rabbit jejunum ($4.0 x 10^{-3}$, $8.0 x 10^{-3}$, $1.6 x 10^{-3}$, $3.2 x 10^{-3}$ mg/ml).

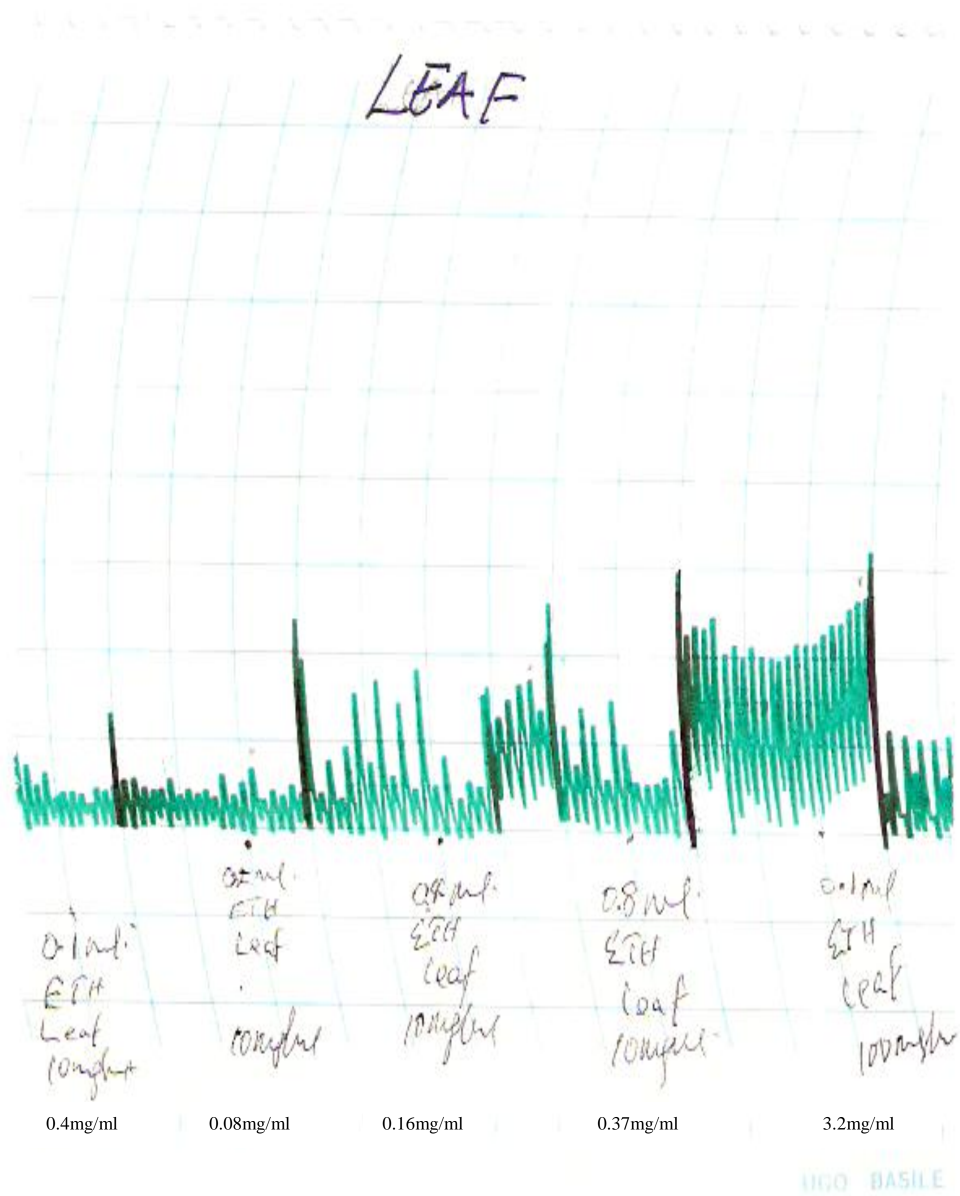


Fig: 4.22 Effect of ethanol Leaf extract on the rabbit jejunum.

Fig. 4.23 Effect of the ethanol stem bark extract on rabbit jejunum. The ethanol stem bark extract of *S. angustifolia* did not have a marked effect on rabbit jejunum at concentration tested ($4.0x10^{-3}$, $8.0\ x10^{-3}$, $1.6\ x10^{-3}$, $3.2x10^{-3}$mg/ml).

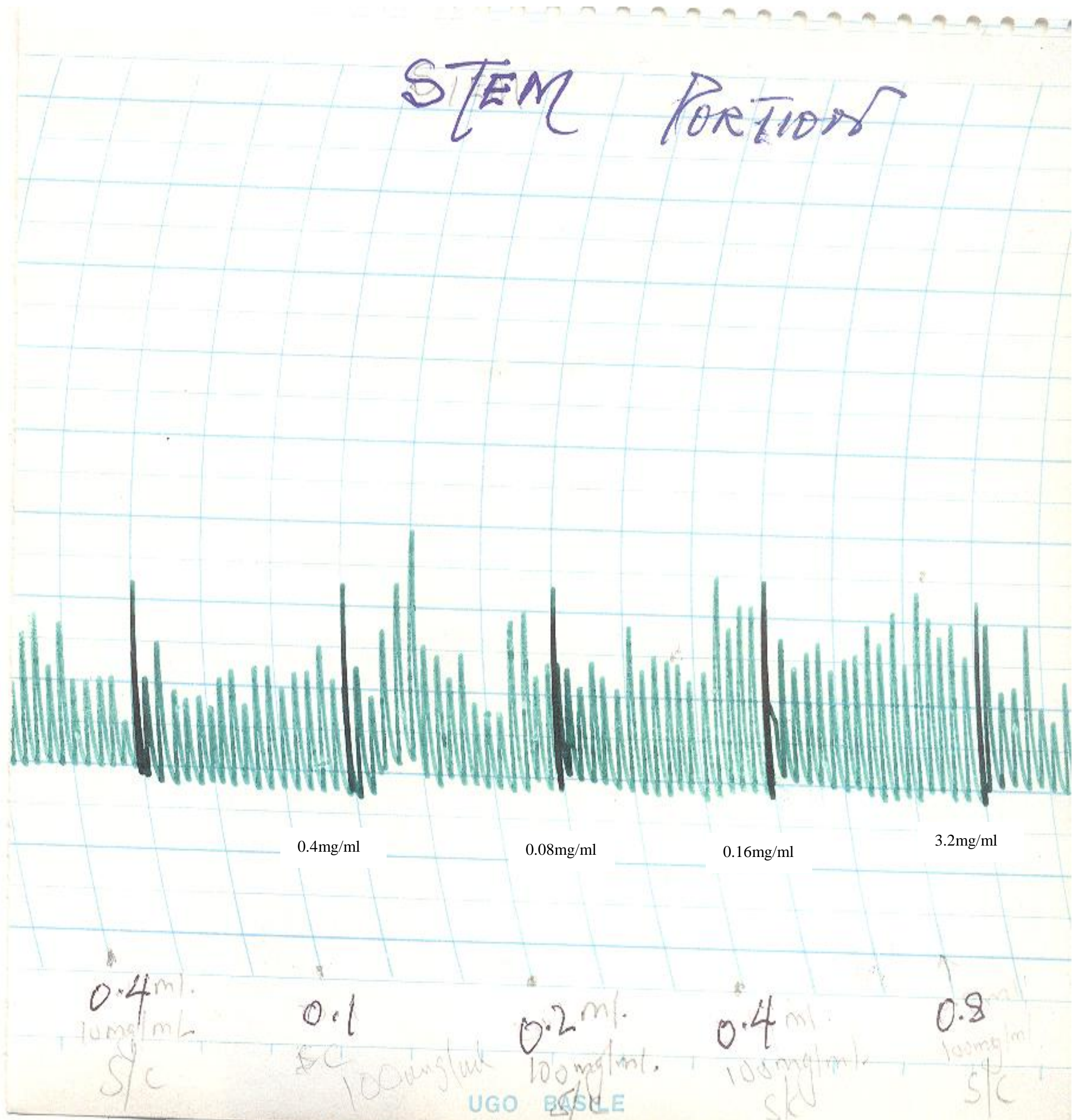


Fig. 4.23 Effect ethanol Stem bark extract on the rabbit jejunum.

Fig. 4.24. Effect of the ethanol root bark extract on rabbit jejunum. The ethanol root extract produced a concentration dose dependent ($4.0 x 10^{-3}$, $8.0 x 10^{-3}$, $1.6 x 10^{-3}$, $3.2 x 10^{-3}$ mg/ml) relaxation of the rabbit jejunum.

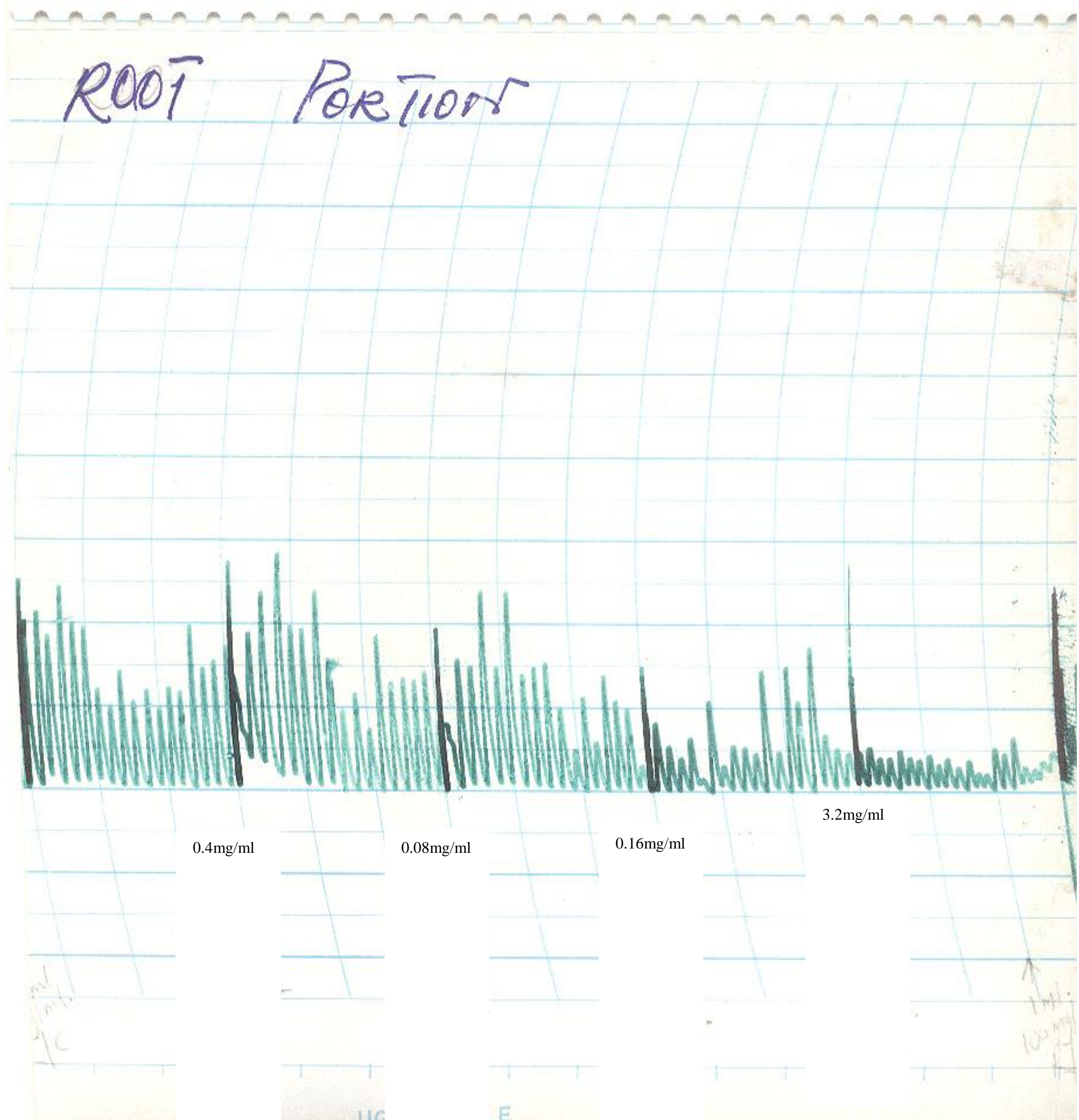


Fig: 4.24 Effect of the effect of Ethanol root extract alone on rabbit jejunum.

Fig. 4.25 Effect of the effect of leaf chloroform fraction on rabbit jejunum. The chloroform fraction of the ethanol leaf extract produced a dose concentration dependent ($4.0x10^{-3}$, $8.0 x10^{-3}$, $1.6 x10^{-3}$, $3.2x10^{-3}$ mg/ml) relaxation of the rabbit jejunum.

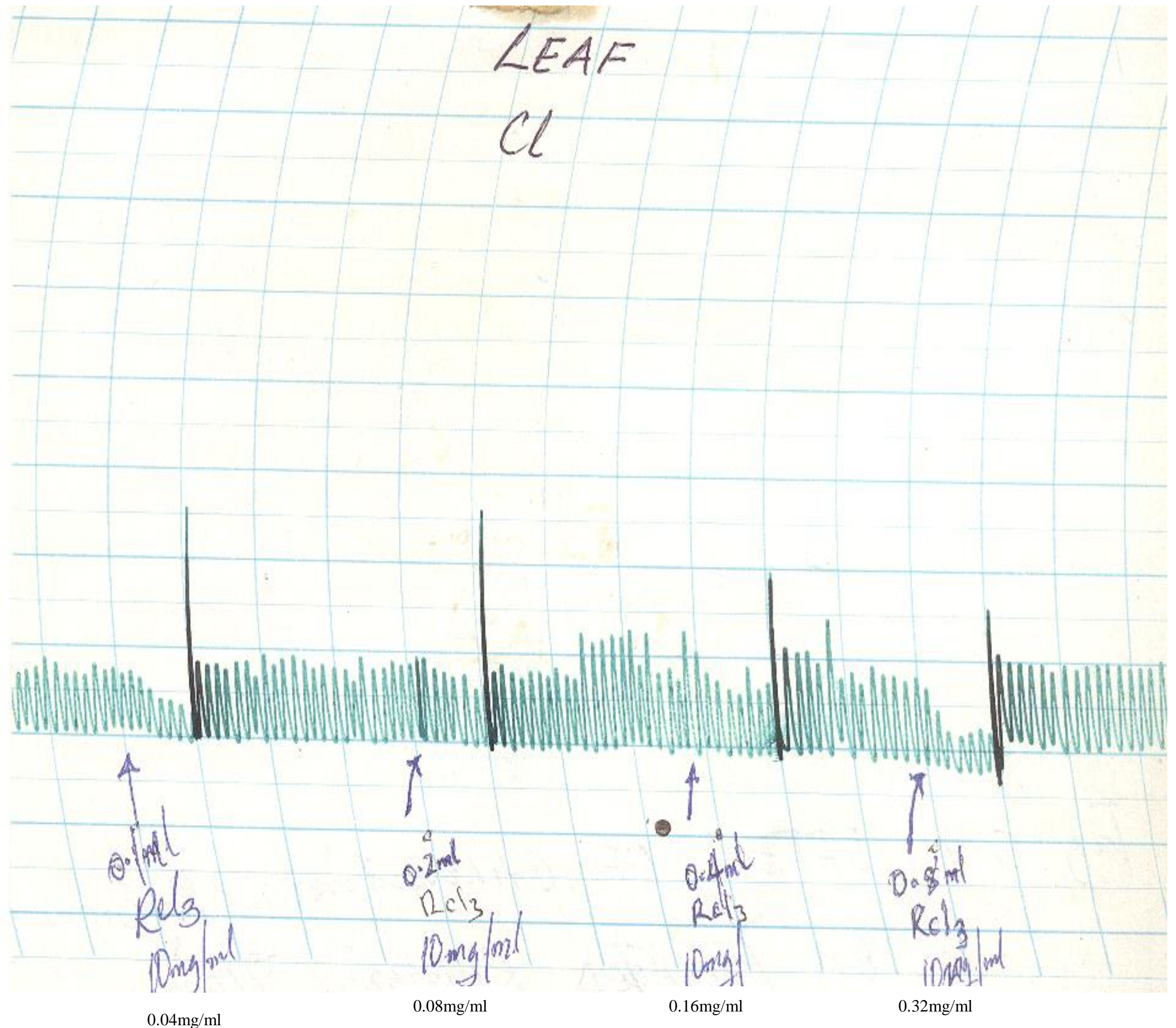
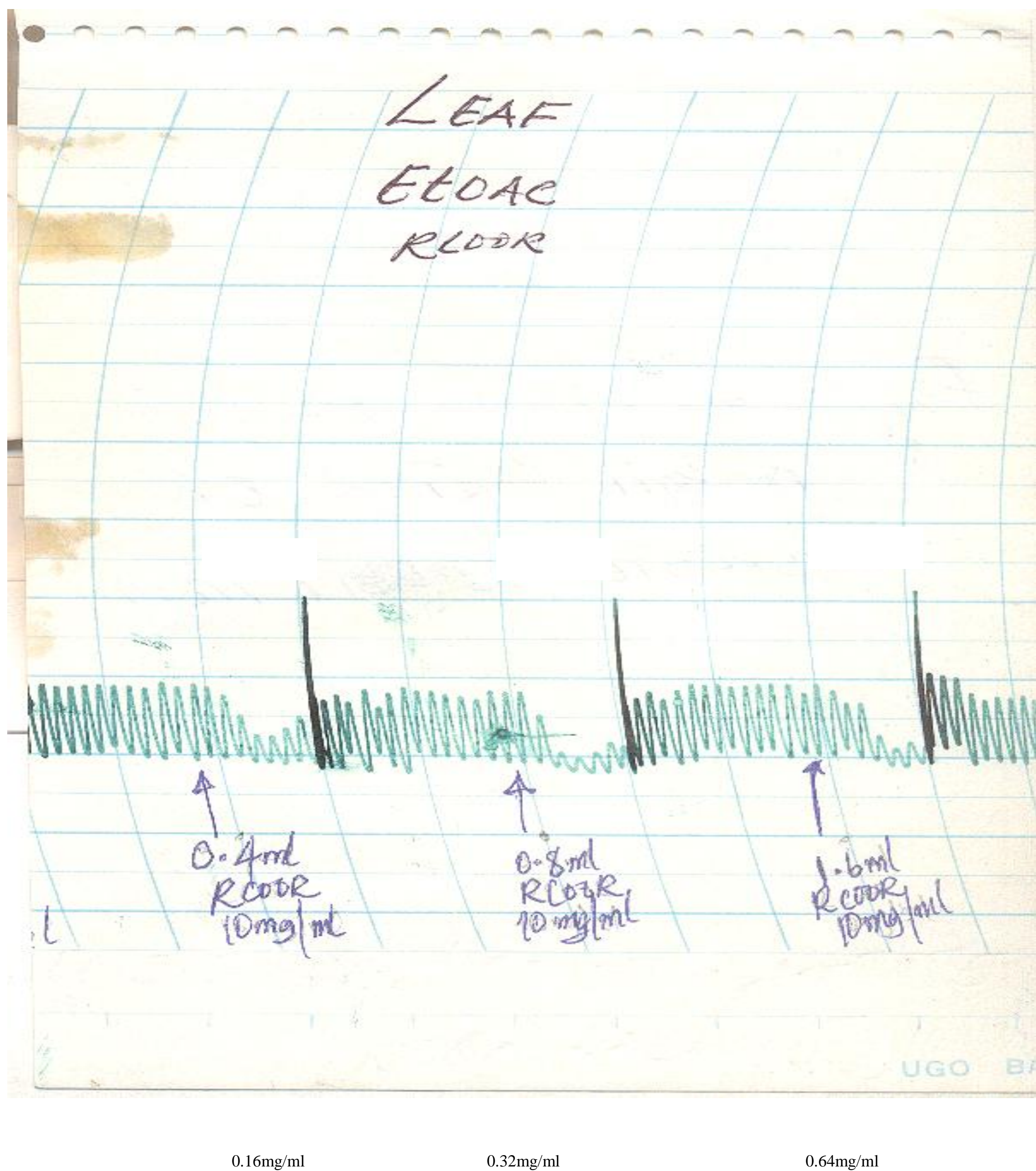


Fig: 4.25 Effect of the chloroform leaf fraction on rabbit jejunum.

Fig. 4.26 Effect of the ethyl acetate leaf fraction on rabbit jejunum. The ethyl acetate fraction of the leaf extract of *S. angustifolia* also produces dose concentration dependent relaxation of rabbit jejunum at ($4.0x10^{-3}$, $8.0 x10^{-3}$, $1.6 x10^{-3}$ mg/ml).



0.16mg/ml 0.32mg/ml 0.64mg/ml

Fig 4.26 Effect of the ethyl acetate Leaf fraction on isolated rabbit jejunum.

Fig. 4.27. Effect of the n-butanol leaf extract fraction on rabbit jejunum. The n-butanol solubl tion of *S. angı* roduced a concentration dependent increase in

spontaneous contraction of rabbit jejunum at ($4.0x10^{-3}$, $8.0 x10^{-3}$, $1.6 x10^{-3}$, $3.2x10^{-3}$ mg/ml).

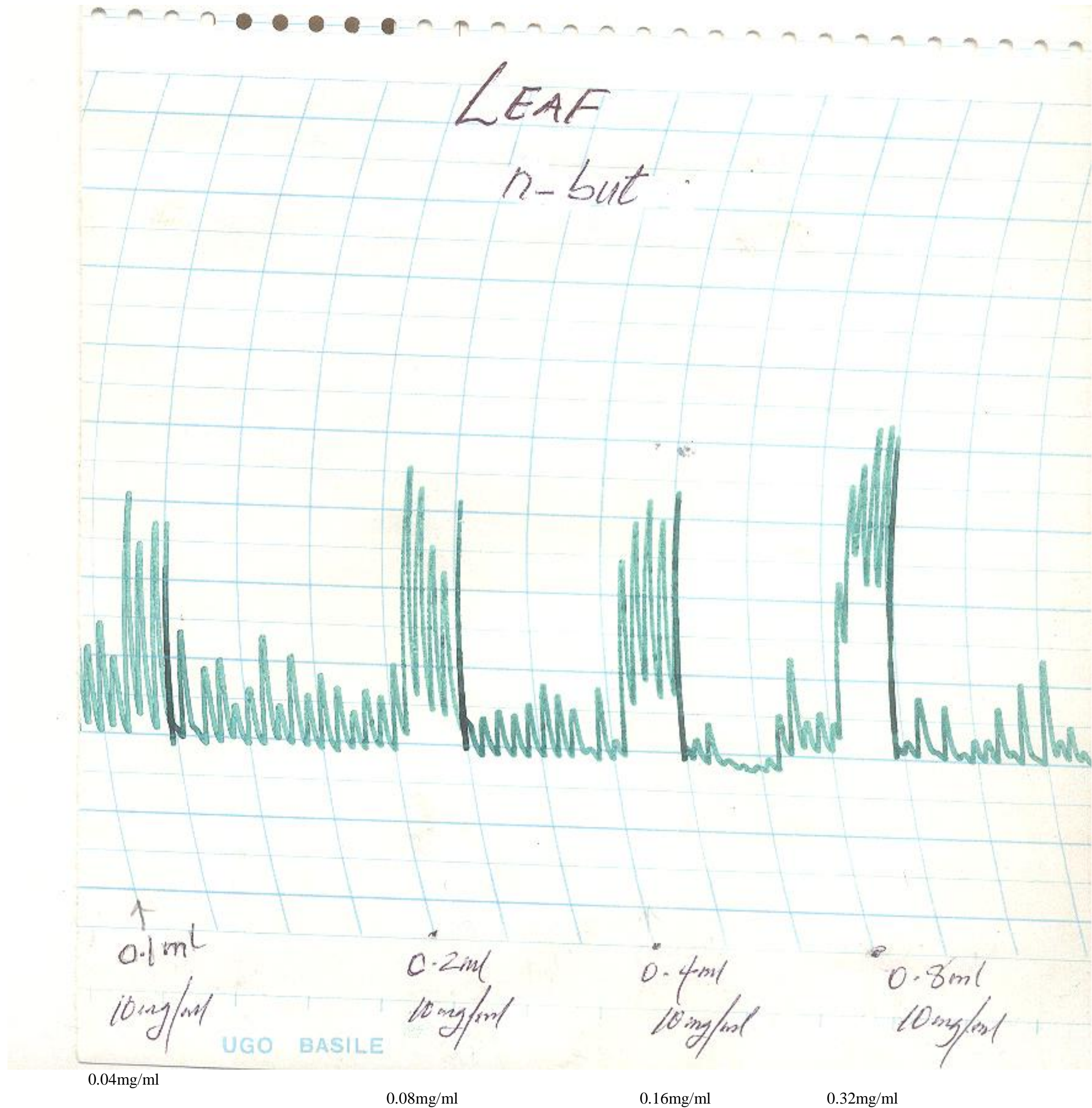


Fig 4.27. Effect of Leaf n-butanol fraction on rabbit jejunum.

Fig. 4.28 Effect of the residual aqueous fraction of the leaf extract on rabbit jejunum. The residual aqueous fraction of the ethanolic extract S. *angustifolia* produced

a concentration dependent increase in spontaneous contraction of rabbit jejunum at ((4.0x10^{-3}, 8.0 x10^{-3}, 1.6 x10^{-3}, 3.2x10^{-3} mg/ml).

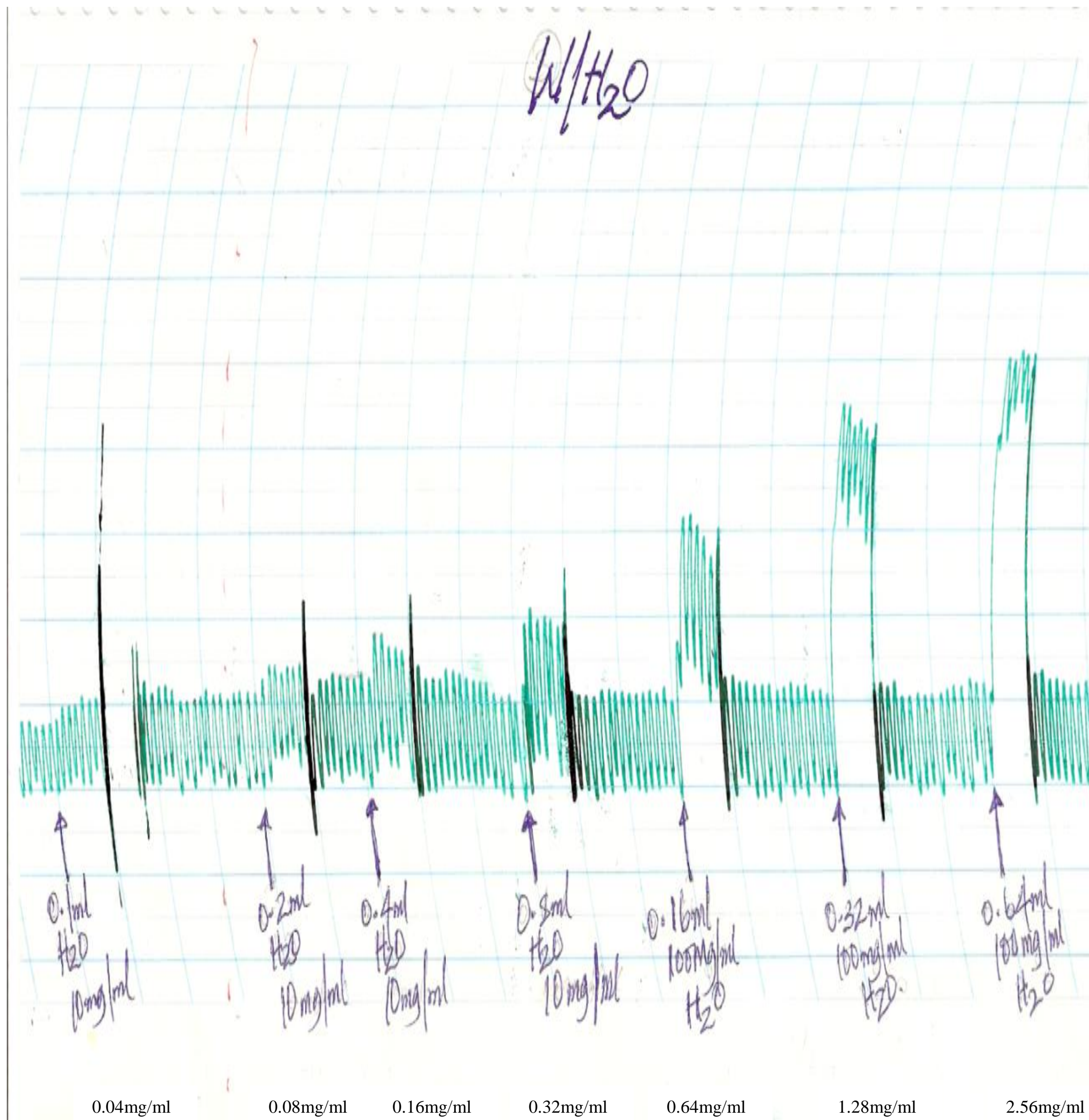


Fig. 4.28. Effect of the residual Leaf aqueous fraction on rabbit jejunum.

Fig.4.29. Effect of atropine with acetylcholine induced contraction of rabbit jejunum. The effect of concurrent administration of atropine with acetylcholine at (0.4 mg/ml) was found to blocked the spontaneous contraction amplitude of the rabbit jejunum.

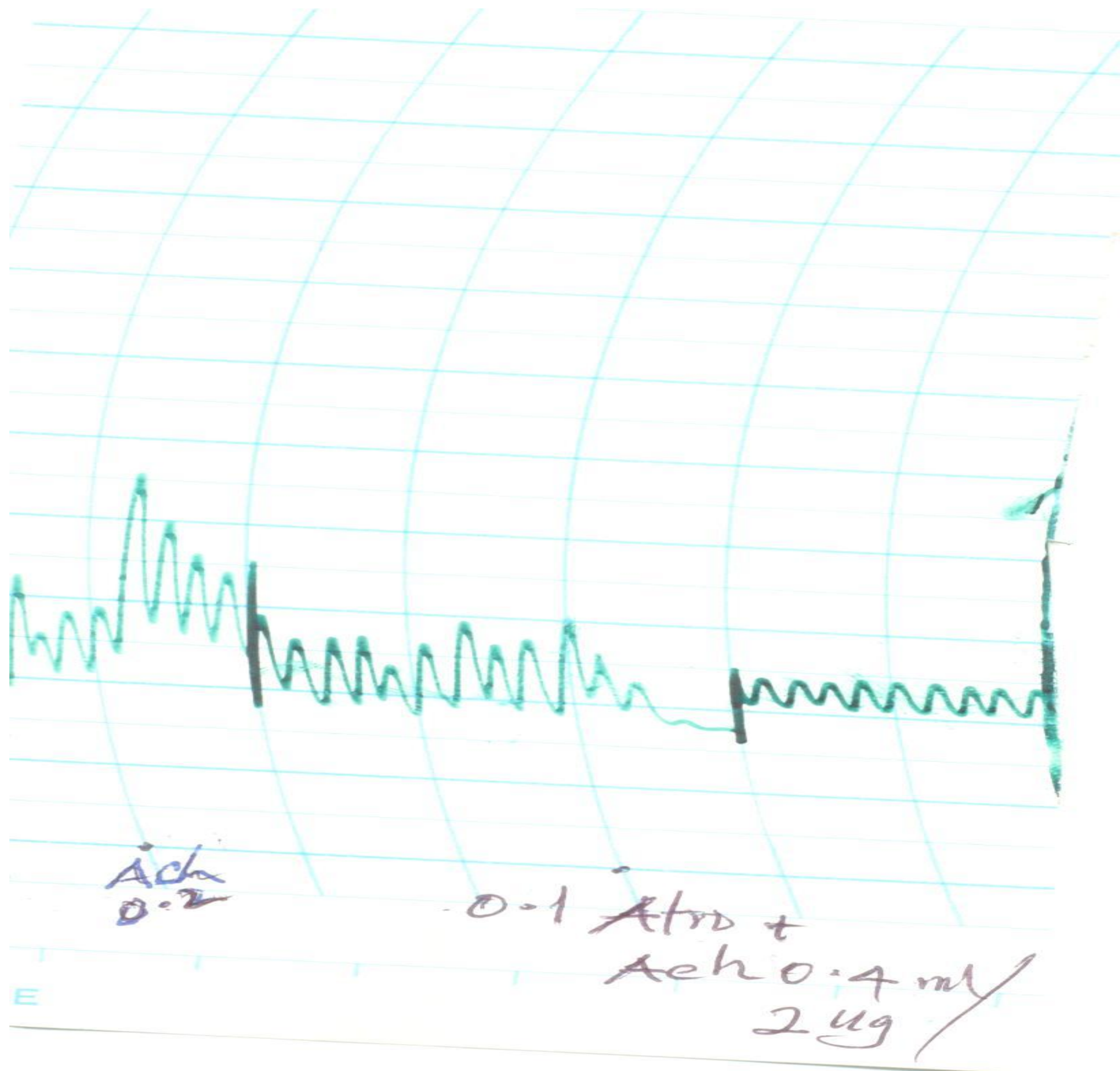


Fig: 4.29 Effect of Atropine with acetylcholine induced contraction of rabbit jejunum.

Fig. 4.30. Effect of the atropine on contraction induced by the ethanol leaf extract on rabbit jejunum. The contraction produced by the leaf ethanol extract at (0.032mg/ml) was blocked or antagonized by atropine at (0.1mg/ml).

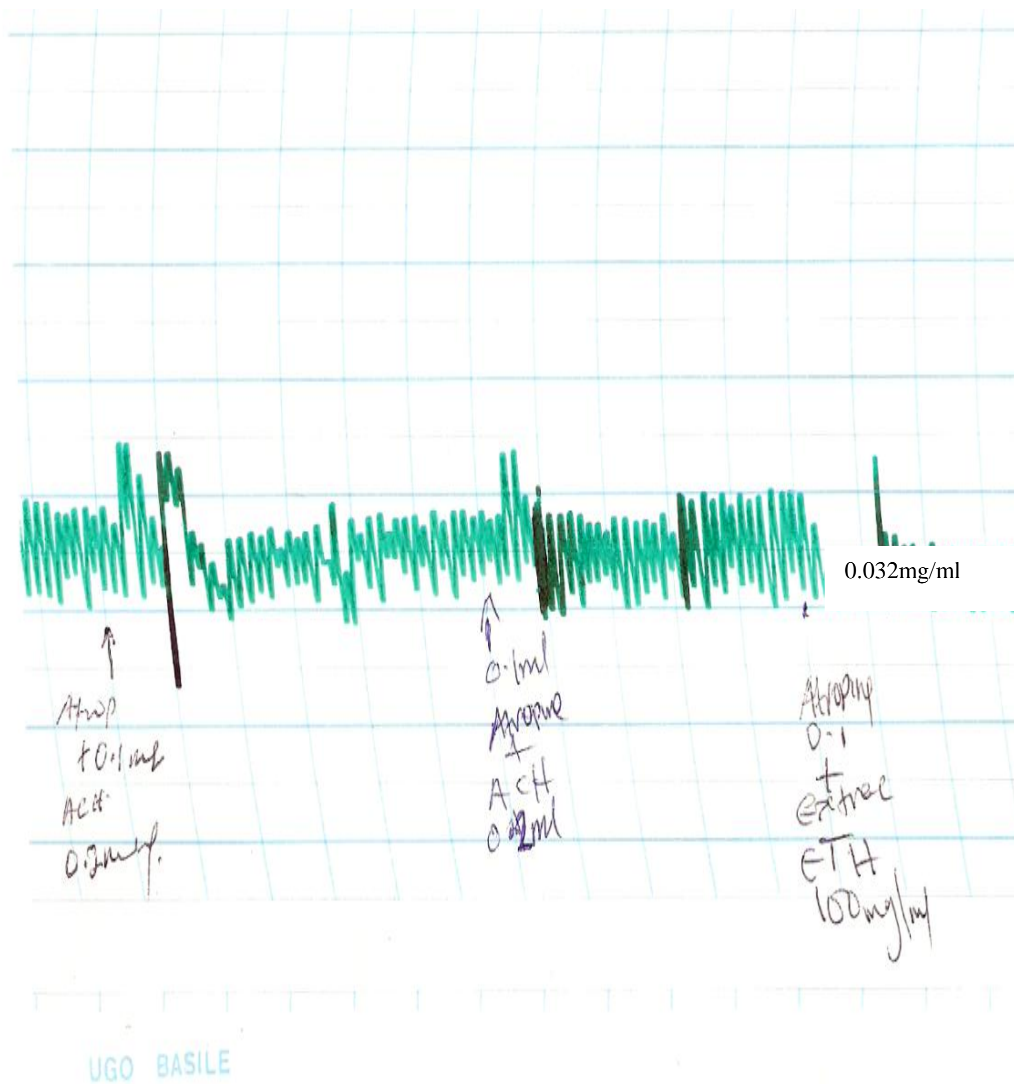


Fig. 4.30 Effect of Atropine on contraction induced by the (n-butanol) fraction on rabbit jejunum.

Fig. 4.31. Effect of the Atropine on contraction induced by the the n- butanol leaf extract. This was found to block or antagonized the atropine at (0.4mg/ml) .

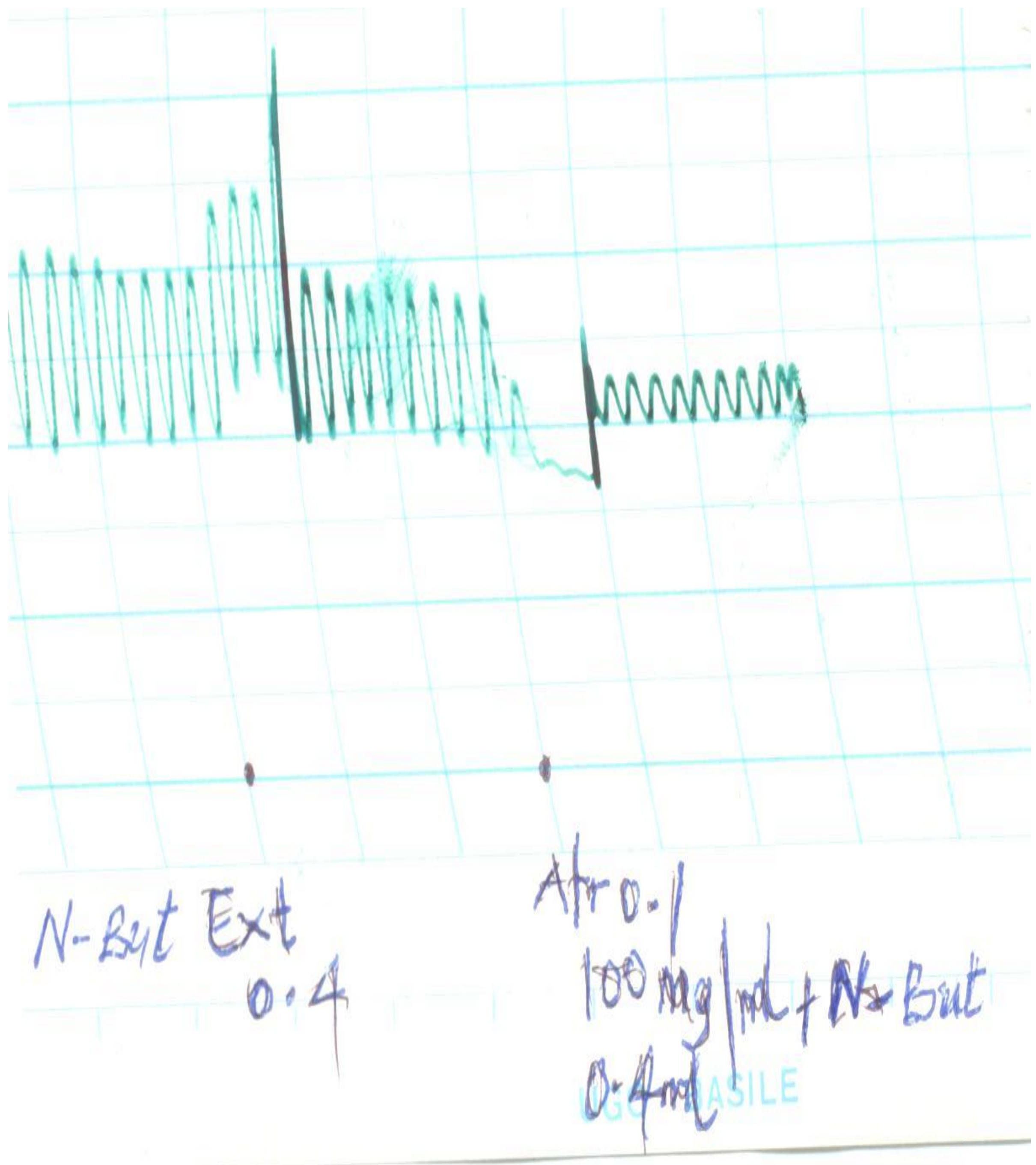


Fig : 4.31 Effect of Atropine with Leaf (n-butanol) fraction on rabbit jejunum.

4.8 RESULTS OF ANTIMICROBIAL ACTIVITY STUDIES

Antimicrobial sensitivity test indicated that the leaf crude ethanol extract and the partitioned fractions inhibited the growth of all microorganism to varying degrees with exception of *S. aureus* and *E. coli.* The highest zone of inhibition ranging from 24-30mm was exhibited by the ethanol extract. The least minimum inhibition concentration of 5mg/ml was exhibited by the n-butanol fraction with a minimum bactericidal concentration of 2.5mg/ml. Ampicillin-cloxacilin, a standard antibiotic at concentration of 5mg/ml inhibited the growth of all the microbes with zone of inhibition ranging from 23-28 mm (Table 4..21, 4.22 and 4.23).

Table. 4.21 Below shows the result of the Susceptibility study of the test microorganisms against ethanol leaf extract of *S. angustifolia*

Test Organisms	Zone of inhibition (mm)					
	P_S	E_S	C_L	n-But	Aq	Ampicillin/ cloxacillin(5mg/ml)
S. aureus	O	12	18	27	21	28
S. pyogenes	10	10	12	22	12	26
E. coli	O	14	17	21	15	23
S. typhi	14	11	10	17	14	25

KEY. R= Resistance, E_S =Ethanol extract, P_s = Petroleum ether extract, Aq= Residual aqueous fraction,

C_L = Chloroform fraction and n-But = n – Butanol fraction.

Table.4.22 Below shows the result of Minimum inhibitory concentration of the test microorganism against the leaf extract and fractions of *S. angustifolia*

TEST	**Ps**					**E_S**					**C_L**					**n-But**					**Aq**				
Organisms						**(Concentrations**					**mg/ml)**														
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
S. aureus	o	o	o	o	o	-	ox	+	++	+++	-	ox	+	++	+++	-	-	ox	+	++	-	ox	+	++	+++
S. pyogenes	o	ox	+	++	+++	-	ox	+	++	+++	-	ox	+	++	+++	-	-	ox	+	++	-	ox	+	++	+++
E. coli	o	o	o	o	o	-	ox	+	++	+++	-	ox	+	++	+++	-	-	ox	+	++	-	ox	+	++	+++
S. typhi	-	ox	+	+ +	+++	-	-	ox	+	++	-	-	ox	+	++	-	-	ox	+	++	-	-	ox	+	++

KEY : - = No growth, o = Not tested, ox = MIC, + = Turbid , ++ = fairly growth, +++ = Highly growth ,Es=Ethanolic, C_L= Chloroform, n-But =n-Butanol, Aq=Residual aqueous and Ps= Petroleum ether extract.

Table 4.23 Below shows the result of the minimum bactericidal concentration of the test microorganism (MBC) against the leaf extract and fractions of *S. angustifolia.*

Test Organisms	Concentration of extract in mg/ml																								
	P_S					E_S					C_L					n-But					Aq				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
S. aureus	0	0	0	0	0	ox	+	++	+++	++++	ox	+	++	+++	++++	-	ox	+	++	+++	ox	+	++	+++	++++
S. pyogenes	ox	+	++	+++	++++	ox	+	++	+++	++++	ox	+	++	+++	++++	-	ox	+	++	+++	ox	+	++	+++	++++
E. coli	0	0	0	0	0	ox	+	++	+++	++++	ox	+	++	+++	++++	-	ox	+	++	+++	ox	+	++	+++	++++
S. typhi	0x	+	++	+++	++++	-	ox	+	++	+++	-	ox	+	++	+++	-	ox	+	++	+++	-	ox	+	++	+++

KEY: - = No growth, ox = MBC + =Turbid, ++ = fairly growth +++ = More growth, ++++=Highly growth, o= Not tested Es = Ethanolic extract, C_L = Chloroform fraction, n-But = n – Butanol fraction, Aq = Aqueous fraction, P_s = pet ether extract

CHAPTER FIVE

DISCUSSION

5.1 Phytochemical Studies

Preliminary phytochemical screening of the ethanol extract of the leaf, stem bark and root bark of *S. angustifolia* revealed the presence of Flavonoids, tannins, steroids, carbohydrate terpenoids, cardiac glycoside and saponins. These phytochemical constituents have been reported to be associated with pharmacological activities of plants (Cowan, 1999). The medicinal properties of drug plants are dependent on the presence of these chemical substances known as the secondary metabolites. They are constituents synthesized by the plant in addition to its basic metabolites, which may be concentrated in different part of the plant. Therefore, medicinal plants are define as those in which one or more of their parts can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Farnsworth, N. R. 1996).

COMPOUND MNF

MNF was isolated as a light brown amorphous solid. The IR frequency of 3419cm^{-1} observed on MNF indicate the presence of a hydroxyl group, while resonance of 1648cm^{-1}(s) could be attributed to C=O in esters of aliphatic acid. An intense band at 1027cm^{-1}(s) could be attributed to C-O while absorption band at 2848cm^{-1} could be attributed to C – H. The absorption band at 1486cm^{-1} indicated the presence of -C=C- (stretch) (Kemp 1991).

The ^{1}HNMR spectrum of MNF (Fig 4.2) showed signal at δ_H 5.6ppm corresponding to H – 1, 7.3ppm/ H – 3, δ_H 0.96ppm/ H – 10 and δ_H 3.65ppm/ H – 12. The signal at δ_H 1.40ppm corresponds to H – 7, δ_H 1.9 ppm/ H – 6 and δ_H 3.7 ppm/ H – 9. The signal at δ_H 7.3/ H – 3 could be attributed to an Iridoid proton while signal at δ_H 4.4 (1H, d, J = 9.9H_Z) /H – 1' could be attributed to an anomeric proton respectively (Shengmin *et al.,* 2003; Shu-Hua *et al.,* 2004). The over lapping signals ranging from δ_H 3.4ppm - 3.6ppm / H – 2' – H – 6' could be attributed to protons on the sugar nucleus. Signal

observed at δ_H 0.96ppm/ H – 10 and δ_H 3.65ppm/(OCH_3) could be attributed to a tertiary methyl and a methoxy protons respectively. The signals observed at δ_H 1.9 ppm (d)/ H – 6, δ_H 1.4ppm / H – 7 could be attributed to methylene and a methine proton while signal observed at δ_H 3.6ppm indicated the presence of an oxymethylene of the sugar moiety (Kemp, 1991; Yong and Peng 2003). The structural elucidation of MNF was also accomplished with the aid of ^{13}CNMR spectrum and DEPT experiment. ^{13}CNMR spectrum experiment, indicated the presence of 19 carbon atom signals, with 13 carbon atoms assigned to the aglycone, 6 to the sugar moiety and 3 carbon signals attributed to the cyclo propyl ring. The signal observed at δc 151.6ppm(C-3) and signal at δc 152.0 (C-1”) were found to resonate within the same region with a single peak. The down field signal observed at δc 167.1ppm could be attributed to a carbonyl group in esters while signal at δ_H 23.50ppm could be attributed to the methyl group (Nan-zhang, *et al.,* 2008 ; Shu-xiang, *et al.,*2003).

The DEPT experiment (fig 4.4) indicated the presence methylene carbon at δc 36.0ppm/C-6 and δc 38.0 ppm/C-7 while signal at δc 61.8 ppm/C-6’ could be attributed to an oxymethylene of the sugar moiety. The methine carbon at δc 151.6 ppm/C-3 confirms the presence of an iridoid carbon on the aglycone moiety. The signal at δc 23.50ppm/C-10 could be attributed to the presence of a methyl group while signal at δc 51.9ppm/C-12 is for a methyl carbon due to methoxy considering its chemical shift. The HSQC (Fig. 4.5) has further assisted in the assignment of the protons to the respective carbons. The signals at δc 93.4ppm (C - 1) corresponds to δ_H 5.60ppm (H-1), δc 152.0ppm (C – 3)/ δ_H 7.30ppm (1H, s), δc 36.0ppm (C– 6)/ δ_H 1.90ppm (1H, d), δc 38.0ppm (C – 7)/ δ_H 1.4ppm (H – 7), δc 104.5ppm (C - 8), δc 61.1ppm (C – 9)/ δ_H 3.7ppm, δc 23.5ppm (C – 10)/ δ_H 0.96ppm (3H, S, H – 10) and δc 51.9ppm (OCH_3)/ δ_H 3.70ppm (3H, s) are all attributable to the aglycone moiety.

The signals at δc 98.6 ppm (C – 1')/ δ_H 4.40 ppm (1H, d, j=9.85 H – 1'), δc 73.5 ppm (C – 2') /δ_H 3.50 ppm (3H, m, H – 2'), δc 70.8 ppm (C – 3')/ δ_H 3.10 ppm (3H, m, H – 3'), δc 77.7 ppm (C – 4') /δ_H 3.20 ppm (3H, m, H – 4'), δc 76.4 ppm (C – 5')/ δ_H 3.4 (3H, m, H – 5') and δc 61.8 ppm (C – 6')/ δ_H 3.60 ppm/ (1H, dd, 6.0 H – 6') are all attributed to the sugar moiety (Kemp, 1991).The signals at δc 152.0 ppm/C-1", δc 82.8 ppm(C-2")/ δ_H 3.6 ppm/H-2"and δc 74.8 ppm(C-3")/ δ_H 3.5 ppm/ H-3" could be attributed to the cyclo propyl ring.

The resonance observed at δc 98.6 ppm (C- 1') which correlates with δ_H 4.40 ppm (1H, d, j= 9.85Hz/ H – 1") suggest the presence of an an anomeric carbon while signals between C – 2'– C – 6' are for the sugar nucleus. The resonance at δc 61.8ppm (C – 6')/ δ_H 3.6 (H – 6') is a signal due to oxymethylene of the sugar moiety. The aforementioned values are in conformity with the –β–D- glucopyranosyl moiety (Kemp, 1991). The anomeric proton resonating at δ_H 4.4 ppm (1H, d, J = 9.85 H_Z) is in accordance with – β – configuration, because J – values > 7H_Z signifies a – β – configuration at the anomeric proton. Signal at δ_H 7.3 (1H, s, H – 3) also indicated the presence of an Iridoid proton (Ihsan *et al.,* 2001; Shengmin *et al.,* 2001). Signal observed at δ_H 0.96ppm (3H, s, H - 10) suggest the presence of a tertiary methyl proton (Wei-li, *et al.,* 2008 ; Jian – ming *et al.,* 2003). The relative down field chemical shift of C-1 (δ_C 93.4ppm) confirms that, the glycosidation of the sugar unit is attached to the carbon at C-1' (Masaki, *et al.,* 2001). The glycosidation of the ether linkage between C-5 and C-8 were observed from the COSY spectroscopy correlation, so also the esterfication at C – 4 (HSQC), were indicated by the down field shift respectively. The COSY correlation between C-3" and C-3' of the sugar were observed to be in the same environment (Hiroyuki, *et al.,* 2004 ; Naobumi, *et al.,* 1995).

On the basis of 1D and 2D spectrum analysis, compound MNF was proposed to have the molecular formula of $C_{19}H_{26}O_{11}$. Thus, named as 1-[3,4-dihydroxy-5-(3-hydroxy-cycloprop-1-enyloxy)-6-hydroxymethyl-tetrahydro- pyran- 2-yloxy]- 7-methyl-1, 4a, 5, 6, 7, 7a-hexahydrocyclopenta [c] pyran -4- carboxylic methyl ester and 4a, 7 – dihydroxy – 7 – methyl – 1 – (3, 4, 5 – trihydroxy – 6– hydro methyl – tetrahydropyran – 2 – yloxy) – 1 -, 4a, 5, 6, 7, 7a, hexahydro – cyclopentan [C] pyran – 4 – carboxylic acid methyl ester.

COMPOUND B

The IR spectrum of compound B (Fig. 4.9) exhibited absorption attributable to a hydroxyl group at 3447cm^{-1}, - CH_2 – CH – at 2954 cm^{-1}, a carboxylic acid at 2923cm^{-1}, 1462 for aromatic absorption and 1201cm^{-1} for C = O stretch (Robert, 1989).

The ^{1}H NMR spectrum of compound B (fig. 4.10) displayed signals at δ_H 7.1, δ_H 6.5ppm, δ_H 6.8 ppm, δ_H 7.5 ppm, δ_H 6.3ppm, δ_H 6.7 ppm, δ_H 6.5 ppm, δ_H 6.7 ppm, δ_H 3.8 ppm and δ_H 3.8 ppm respectively. The signal observed at δ_H 6.8 ppm, δ_H 7.5ppm and δ_H 6.7 ppm could be attributed to olefinic protons (Kemp, 1991; Xi-Hao *et al.,* 2003). The signal displayed at δ_H 4.5ppm, δ_H 3.6 ppm, δ_H 3.9 ppm, δ_H 4.8 ppm, δ_H 3.4 ppm and δ_H 3.6 ppm could be attributed to a sugar protons possibly glucose while signals at δ_H 5.3ppm, δ_H 3.7 ppm, δ_H 3.3 ppm, δ_H 3.1 ppm, δ_H 3.4 ppm and δ_H 1.1 ppm could also be attributed to a sugar protons possibly rhamnose (Kemp, 1991).

The signals displayed at δ_H 4.5 ppm and δ_H 5.3ppm could be attributed to the anomeric protons. The signal observed at δ_H3.6 ppm could be attributed to the oxymethylene proton of the sugar moiety (Ya-Ching *et al.,* 2004). The anomeric proton signal at δ_H 4.5 ppm and the signals at δ_H 3.3 ppm – 3.8 ppm are in conformity with the – β – glucopyranosyl protons (Jun-mian, *et al.,* 2008 ; Ihsan, *et al.,* 2001).

Signals observed at δ_H 5.3ppm and the resonance at δ_H 3.4ppm – 3.7ppm also corresponds to another sugar nucleus. The signal at δ_H 1.1ppm corresponds to the methyl group which could be attributed to a rhamnose protons. The signals at δ_H 7.5ppm and 6.3ppm could be attributed to an α and β vinylic protons (Ya-Ling, *et al.,* 2003 ; Yong-Qin *et al.,* 2008).

The ^{13}CNMR spectrum (Fig 4.11) exhibited 29 carbon signals. The spectrum displayed signals at δc 128.0 ppm/C-1, δc 115.2 ppm/C-2, δc 147.0 ppm/C-3, δc 150.0 ppm/C-4, δc 115.0 ppm/C-5, δc 123.0 ppm/C-6, δc 148.0 ppm/C-6, δc 117.1 ppm/C-7 and δc 168.3 ppm/C-8. The signals observed at δc 148.0 ppm and δc 117.1 ppm could be attributed to an α and β vinylic carbons while signal at δc 168.3ppm to be a carbonyl group. These resonances are in great conformity with the values of Caffeoyl moiety (Andary *et al.,* 1982 ; Andary *et al.,* 1985). The signals observed at δc 132.0ppm, δc 117.0ppm, δc 146.0ppm, δc 145.0 ppm, δc 116.0 ppm, δc 121.3ppm, δc 37.0 ppm, and δc 72.1ppm could be attributed to the –β-(3 ,4-dihydroxyphenyl) ethyl- as the aglycone moiety (Andary *et al.,* 1982 ; 1985).

The signals at δc 103.1 ppm/4.5 ppm, δc 76.2 ppm/3.3 ppm, δc 82.0 ppm/4.8 ppm, δc 76.1 ppm/3.4 ppm, δc 70.4 ppm/3.6 ppm and δc 62.4 ppm/3.8ppm are in conformity with – β – glucosyl moiety (Ik-Hwi, *et al.,* 2004 ; Rong *et al.,* 2003). The signalsatδc104.2ppm/5.3ppm,72.4ppm/3.7ppm,72.3ppm/3.1ppm,74.0ppm/3.1ppm71.0 ppm/3.4ppm and 18.5 ppm/1.1ppm could also be attributed to a sugar nucleus. The high shielded value at δc 18.5ppm/1.1ppm is for a methyl group of the sugar moiety possibly rhamnose. The aforementioned signals are in conformity with the values of rhamnose moiety (Andary, *et al.,* 1982 ; Andary, *et al.,* 1985).

Base on the result of 1D NMR (^{1}H and ^{13}C) and comparing the spectral data no's with those of Andary, *et al.,* 1982 and Andary, *et al.,* 1985, (Table 4.24) Compound B was proposed as the same as the reference compound (Verbascoside) with molecular formula as $C_{29} H_{36} O_{15}$ and molecular weight of 624.594. Thus, compound B is named as -β – (3', 4' – dihydroxy phenyl) – ethyl – O - α - L – rhamnopyranosyl (1 → 3) – β – D – (4 – O- Caffeoyl) – glucopyranoside.

COMPOUND MND

MND was isolated as a bluish green amorphous solid. The IR spectrum (fig.4.12) displayed absorptions attributable to a hydroxyl group at 3419 Cm^{-1}, a conjugated carbonyl group at 1648Cm^{-1} and an intense band at 1021Cm^{-1} attributed to $CH_3C – 0$ (Francis, 2003 ; Peter, 1985).

The ^{1}H NMR spectrum of MND (Fig 4.13 and table 4.22) displayed signal at δ_H 7.4 ppm (1H, s) corresponding to H-3 and δ_H 4.6 ppm (1H d, J=7.8H_Z)/ H-1', 1.2 ppm (3H, s)/ H-10 and 3.6 ppm (1H, dd, 6.0H_Z) / H – 6'.The signal at δ_H 3.7ppm (3H, s) is attributable to the (OCH_3). The signals at δ_H 3.2 ppm (1H, dd, J=8.0) /H-2', δ_H 3.3 ppm (3H, m)/ H-3', δ_H 3.4 ppm (3H-m)/ H-4', δ_H 3.4ppm (3H-m)/ H-5'and δ_H 3.6 ppm(1H, dd, J=6.0H_Z)/ H-6' above are assigned to the sugar nucleus (Dharma, *et al.,* 2001;Kemp, 1991). The J value of the anomeric proton at δ_H 4.6ppm (IH, d, 7.8H_Z) corresponding to H – 1' and other resonance at δ_H 3.2ppm (3H, m) → δ_H 3.6ppm (1H, dd, J=6.0H_Z)/ H – 2' →H – 6' is an indication of a β– glucosyl moiety (Hongzhu *et al.,* 2004 ; Li-juan *et al.,* 2008).

The DEPT experiment spectrum (fig.4.15 table 4. 25) exhibited 17 carbons. The DEPT experiment showed 3, methylene, a single methine and a methyl carbon signal.

One methyl carbon signal due to methoxy considering it chemical shift of δc 50.3ppm/ 3.7 ppm was also observed.

The HSQC (Fig.4.16, table 4. 23) has further assisted the assignments of the protons to the respective carbons. The signals at δc 93.0 ppm (C – 1) corresponds to δ_H 5.8 ppm (H-1) , δc 151.2 ppm (C – 3)/ δ_H 7.4 ppm(H-4), δc 38.0 ppm (C – 6) / δ_H 1.9 ppm (H-6), δc 40.0 ppm (C – 7)/ 1.6 ppm(H-7), δ_H 60.3 ppm (C – 9)/2.8(H-9), δc 22.0 ppm (C – 10)/ 2.5 ppm (H-10) and δc 50.3 ppm/3.7(OCH_3). These values are attributable to the ipolamiide aglycone moiety, a cyclopentano pyran ring system (Zuhal, *et al.,* 2005; Tayfun *et al.,* 2001). The resonance observed at δc 98.2 ppm (C – 1') which correlate with δ_H 4.6ppm (H – 1') could be attributed to an anomeric carbon while signals at δc 73.0 (C – 2')/H-2, δc 76.0 ppm (C – 3')/H-3, δc 70.3 ppm (C – 4')/H-4, δc 77.0ppm (C – 5')/H-5 and δc 61.4 ppm (C – 6')/H-6 could be attributed to a sugar moiety. The signals observed at δc 61.4 could be attributed to the oxymethylene carbon Suggesting the presence of a glucopyranosyl moiety (Brown, 2003; Yoshiyasu, et al., 2004.), while signal at δc 22.oppm is assigned to the methyl group. The COSY spectroscopic analysis has exhibited the correlation of δ_H 5.8ppm with δ_H 2.5ppm) and δ_H 4.6ppm with 3.2ppm (Fig.417, table 4. 23).

The NOESY spectrum of MND (Fig.4.19, table 4. 24) has shown that δ_H 5.8 (H – 1) is in the same environment with δ_H 1.2 (H - 10) and δ_H 4.6 (H – 1). This also shows the correlation of δ_H 4.6 (H – 1') with δ_H 3.6 (H – 6'). The HMBC spectrum revealed the correlations between protons and the neighboring carbons up to 3 bonds. The connectivity between the various atoms and other units in the molecules was established (Fig.4.19 and Table 4.). Correlations were also established between δc 93.0 (C – 1) with δc 151.2 (C – 3) δc 60.3 (C – 9) and δc 98.2 (C – 1').

So also correlations were established between δc 22.0 (C – 10), a methyl group of the aglycone with the δc 70.3 (C - 5) and δc 38.0 (C – 6). The correlations between 151.2 (C – 3) with δc 93.0 (C – 1) and δc 70.3 (C- 5) was established. The following correlations were also observed δc 60.0 (C – 9) with δ_H 4.0 (C – 4), δc 70.3 (C – 5) and δc 98.2 (C – 1') respectively. Base on the aforementioned correlations from HMBC, the aglycone was confirmed to be a cyclopentano pyran ring system (Zuhal, *et al.,* 2005; Brown,2003).

The connectivity between the sugar unit and the aglycone moiety was hence established, the anomeric proton δ_H 4.6ppm (H – 1') and the δc 93.0 (C – 1) of cyclopentano pyran ring (aglycone) was ascertain. This firmly confirmed the structural similarity of MND with ipolamiide.

Base on the result of FTIR ,1D , 2D NMR and comparing the spectral data no's with those of (Zuhal, *et al.,* 2005; Tayfun, *et al.,* 2001), MND was proposed to be the same as the reference compound (Ipolamiide), with molecular formula of $C_{17}H_{27}O_{12}$. Thus, named as 4a, 7 – dihydroxy – 7 – methyl – 1 – (3, 4, 5 – trihydroxy – 6– hydro methyl – tetrahydropyran – 2 – yloxy) – 1 -, 4a, 5, 6, 7, 7a, hexahydro – cyclopenta [C] pyran – 4 – carboxylic acid methyl ester.

5.2 PHARMACOLOGICAL STUDIES

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

The whole plant extract, leaf and n-butanol soluble fraction of the leaf extract produced a concentration dependent increase in spontaneous perpendicular movement of the rabbit jejunum.

The contractions observed by the whole plant extract, leaf extract and n-butanol soluble fraction were similar to those produced by Acetylcholine (Pohocha and Grampurohit, 2001). Acetylcholine induces contraction of the smooth muscles via activation of the muscarinic receptors and differences in muscarinic receptors are known to exist (Augustine *et al.,* 2003; Nathanson, 1987; Mitchelson. 1984).

The contraction induced by the plant extract and other fractions of the leaf extract might be mediated via the muscarinic receptors (Bolton, 1979a; Bolton, 1979b ; Bonner, (1989). The contractile responses exhibited by the leaf extract and n-butanol soluble fraction were blocked by atropine suggesting that the observed pharmacological effect, were mediated through the muscarinic receptors. (Amos *et al.,* 2003; Nkeh *et al.,* 1993; Amos *et al.,* 2000). The gastrointestinal activities of the leaf extract, chloroform and Ethylacetate fraction of the leaf were assessed for their pharmacological activities on the rabbit jejunum. The chloroform and ethylacetate fractions were found to relax the jejunum concentration dose dependently. This hence can be use as a laxative in the management of constipation.

5.3 ANTIMICROBIAL ACTIVITY

The various fractions of the ethanolic leaf extract of *S. angustifolia* showed a remarkable antimicrobial activity against all the organisms tested except for the petroleum extract which did not inhibit the growth of *S. pyogenes* and *S. typhi*. This might be attributed to the inactive biological principles present in the less polar extract to inhibit their growth. The activities of the crude ethanolic extracts of the leaf were found to be less than the partitioned fractions.

This could be resolved that, the metabolites responsible for these activities are more active when they are less combine with other principles (Meenakshi, *et al.,* 2001). Activities of the polar fractions against the tested microbes shows that, these fractions contain substance(s) that could inhibit or kill the growth of some micro – organisms (Akujobi, *et al.,* 2004; Osadebe and Ukweze *et al.,* 2004). The observed anti microbial effects could be attributed to the presence of saponins, glycosides, flavonoids, tannins and terpenoids (Draughon, 2004; Cowan 1999). The presences of these metabolites in the plant suggest great potential for the use of the plant as a source of phytomedicines. The zone of inhibition exhibited by the leaf extract against *S. aureus, P. pyogenes, E. coli and S. typhi* justified its use by the traditional medical practitioners in the treatment of sores, boils, open wounds, dysentery, sexually transmitted diseases and other related ailments. *S. aureus* and *P. pyogenes* have been implicated in cases of boils, sores and wounds (Braude, 1982). *S. aureus* is also known to play a significant role in skin diseases including superficial and deep follicular lesion (Srinivessan *et al.,* 2001; Singleton, 1999). The activities of the ethanolic extract and n-butanol fraction on the tested microbes (Table: 4 .28/4.29), suggest that the plant can be a source of compound that can be effective against the related ailments.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

Preliminary phytochemical screening of the ethanol leaf, stem bark and root bark extract of *Stachytarpheta angustifolia* revealed the presence of Tannins, Steroids, Terpenoids, Flavonoids, Saponins and Glycoside.

Extensive phytochemical investigation led to the isolation of two iridoid glycosides as 1-[3,4-dihydroxy-5-(3-hydroxy-cycloprop-1-enyloxy)-6-hydroxymethyl-tetrahydro-pyran- 2-yloxy]- 7-methyl-1, 4a, 5, 6, 7, 7a-hexahydrocyclopenta [C] pyran -4-carboxylic methyl ester from the n-butanol fraction of the leaf extract and 4a, 7 – dihydroxy – 7 – methyl – 1 – (3, 4, 5 – trihydroxy – 6– hydro methyl – tetrahydro pyran – 2 – yloxy) – 1 -, 4a, 5, 6, 7, 7a, hexahydro – cyclopenta [C] pyran – 4 – carboxylic acid methyl ester (Ipolamiide) from the root bark extract, while a caffeic glycoside ester (Verbascoside), as -β – (3', 4' - dihydroxyphenyl) – ethyl – O – α – L – rhamnopyranosyl – (1 – 3) – β – D – (4 – 0 – Caffeoyl) – glucopyranoside from the stem bark extract.

Pharmacological studies on the ethanol leaf, stem bark, root extract and other leaf fractions showed that, the chloroform and ethylacetate fractions were found to relax the jejunum concentration dose dependently. This hence can be use as a laxative agent in the management of constipation.

In addition, the extract showed an antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Salmonella typhi*. The n-butanol fraction shows the greatest activity on the microbes.

6.2 CONCLUSION

Base on the finding in this study, it could be concluded that the use of *Stachytarpheta angustifolia* as a laxative agent in the treatment of gastrointestinal disorder(constipation) and condition related to bacterial infection by the folkloric healers has scientific basis. The laxative properties in the management of constipation observed in our studies and the antimicrobial activities of the leaf extract could be linked to the presence of the isolated iridoid glycosides. This is because, iridoid glycosides have been shown to posses various biological activities such as antimicrobial, antitumor, hemodynamic, choleretic, hepatoprotective as well as been anti inflammatory agents (Sophon, *et al.,* 2002). Iridoid glycosides are also been known to show remarkable activity against anticancer, ant cardiac activity, anti-coagulant activity, ant oxidative activity, anti-protozoal activity, neuroprotective and antinociceptive activity (Biswanath, *et al.,* 2009; Hosny and Rosazza, 1998; Joy, *et al.,* 1998). The compounds isolated will add to data base of the plants. To the best of our search, this is the first time these activities were been reported.

6.3 RECOMMENDATIONS (suggested further studies)

- Futher studies should be carried out to isolate tannins and saponins present in the more polar region of the extract.
- There is need to screen the various part of the plant for, abortificient activity, ant diarrhea, antitumor, hepatoprotective, anti ulcer, anti rheumatism and antiviral activities is highly recommended.
- There is need to carry out more acute toxicity studies on the plant so as to rule out the possibility of any side effect that may arise as a result of the prolong use of the plant.

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APPENDIX

Table: 4.24. A Comparison of the ^{1}H and ^{13}C NMR spectral data for compound B and reference compound (Verbascoside).

Position	Cpd. B	Cpd. B	Verbascoside	Verbascoside
	$\delta\ ^{13}C$	δ^{1}_{H}	$\delta\ ^{13}_{C}$	$\delta\ ^{1}H$
1	127.7	-	127.4	-
2	115.2	7.1	115.8	6.7
3	146.9	-	146.4	-
4	149.8	-	149.3	-
5	114.7	6.6	114.7	6.7
6	123.2	6.8	123.4	6.9
7	148.0	7.5	148.1	7.4
8	117.1	6.3	117.0	6.2
9	168.3	-	169.5	-
1’	131.5	-	131.6	-
2’	116.5	6.7	117.5	6.6
3’	146.2	-	145.6	-
4’	144.7	-	144.1	-
5’	116.3	6.5	116.8	6.5
6’	121.3	6.7	121.7	6.6
7’	36.6	2.8	36.0	2.7
8’	72.1	3.8	72.1	3.9
1’’	103.1	4.5	102.0	4.3
2’’	76.2	3.6	74.6	3.2
3’’	81.6	3.9	79.2	3.7
4’’	70.4	4.8	69.2	4.7
5’’	76.1	3.4	74.6	3.5
6’’	62.4	3.8	60.8	3.7
1’’’	104.2	5.3	101.3	5.0
2’’’	72.4	3.7	70.6	3.7
3’’’	72.3	3.3	70.5	3.3
4’’’	73.8	3.1	71.8	3.1
5’’’	70.6	3.4	68.8	3.4
6’’’	18.4	1.1	18.2	1.0

400MH$_{Z}$ ^{1}H, 100MH$_{Z}$ ^{13}CNMR, CD_3OD and Ref. Cpd.(Andary *et al.,* 1982, 1985).

Table: 4.25 A Comparison of the ^{1}H and ^{13}C NMR Spectral data for Compound MNDand Reference compound (Ipolamiide).

Position	δ_C of MND	δ_H of MND j(H_Z)	δ_C of Ipolamiide	δ_H of Ipolamiide
1	92.7926	5.8378 (1H, d, 1.1H_Z)	93.0	5.7 (1H, d, 1.1H_Z)
3	151.2050	7-4682 (1H, S)	151.4	7.4 (1H, S)
4	113.8174	-	114.0	-
5	70.2667	-	70.6	-
6	37.4573	1.9354 (1H,m) ,	37.6	2.0
7	38.9934	1. 5971 (1H,m), 2.05	39.6	1.56 (1H,m), 2.05
8	77.0040	(1H, m)	77.7	(1H, m)
9	60.2769	-	60.5	
10	21.8444	2.5 062 (1H, brs)	22.0	2.79
11	166.6558	1.1732 (3H, S)	166.8	1.14 (3H, S)
Ome	50.2635	-	50.4	-
1’	98..1872	3.7532 (3H, S)	98..4	3.72
2’	72.9915	4.6141 (1H, d, 7.8)	73.2	4.5
3’	76.0267	3.2190 (1H, dd, 8.0)	76.2	3.17
4’	77.0040	3.3366 (3H, m)	70.6	3.23
5’	70.2980	3-3464 (3H, m)	77.1	3.32
6’	61.4333	3.3551 (3H, m)	61.5	3.38
		3.6780 (1H, dd, 6.0)		3.65

600MH$_Z$ ^{1}H and 150MH$_Z$ ^{13}CNMR for MND and Ref. Cpd.((Zuhal, *et al.,* 2005; Tayfun *et al.,2*001

PHYSIOLOGICAL SOLUTION:

The physiological solution used in this experiment are made from Tyrode and De-Jalon solution; others are the Ringer Locke solution as seen from the recipe

Below (Kitchen, 1994). The solution was prepared for 10litre in de- ionized water (Table 4.26).

Table.4.26

Salt	De-Jalon	Ringer Locke	Tyrode
D-glucose	5g	10g	10g
Nacl	90g	50g	90g
Kcl (10% solution)	42ml	42ml	20ml
$NaH_2Po4\ 2H_2O$ (10%)			5ml
$NaHCo_3$	5g	5g	10g
$Mgcl_26H_2O$			1ml
Aerating gas	5% CO_2 195% O_2	Pure O_2	O_2 or Air
$Cacl_2$ (10% solution)			20ml
$Cacl_2$ (molar solution)	2.7ml	10.8ml	

The above formula solution was prepared for 10 litre de-ionized water (Kitchen, 1994)

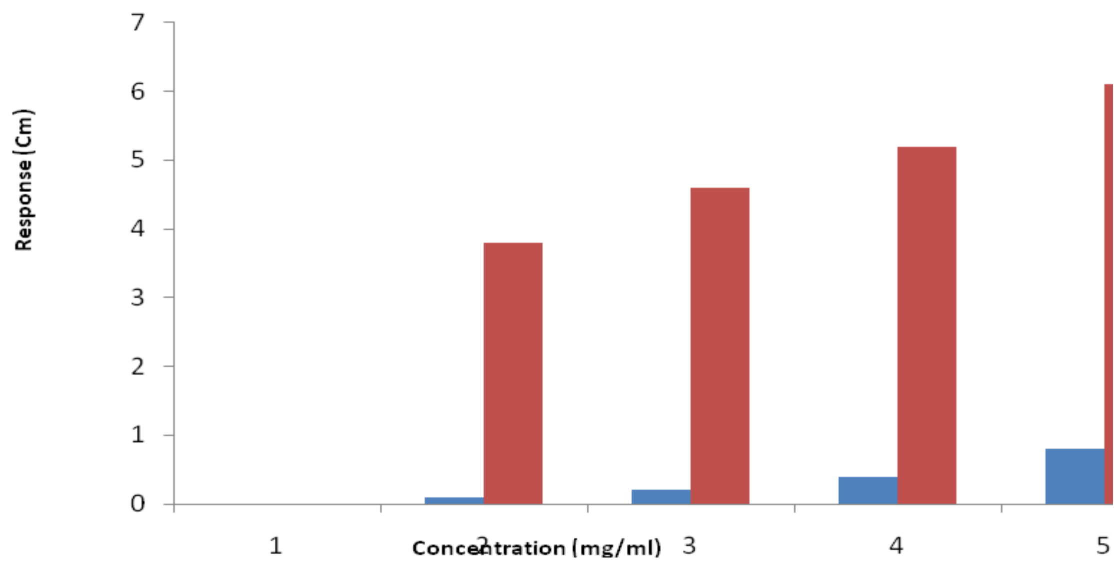

Fig.32 Effect of Acetylcholine on rabbit jejunm

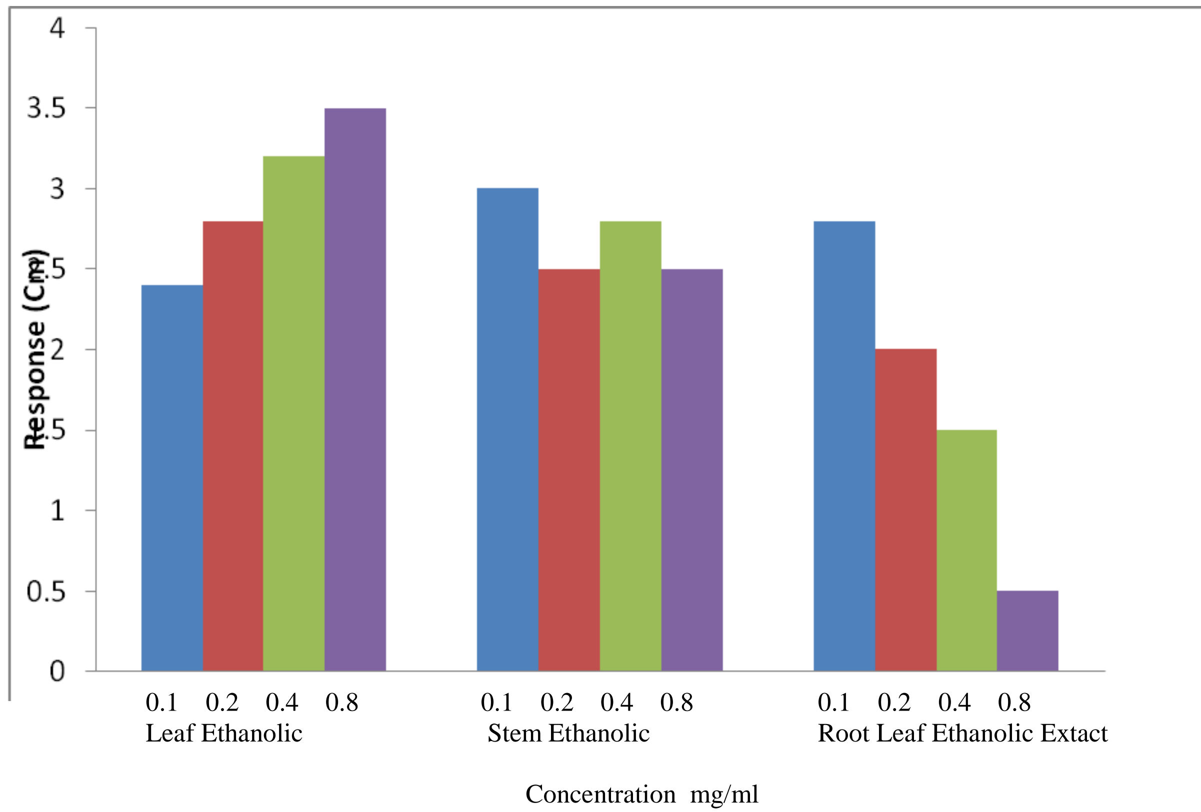

Fig: 33 Effect of ethanolic leaf, stem bark and root extracts on the rabbit jejunum

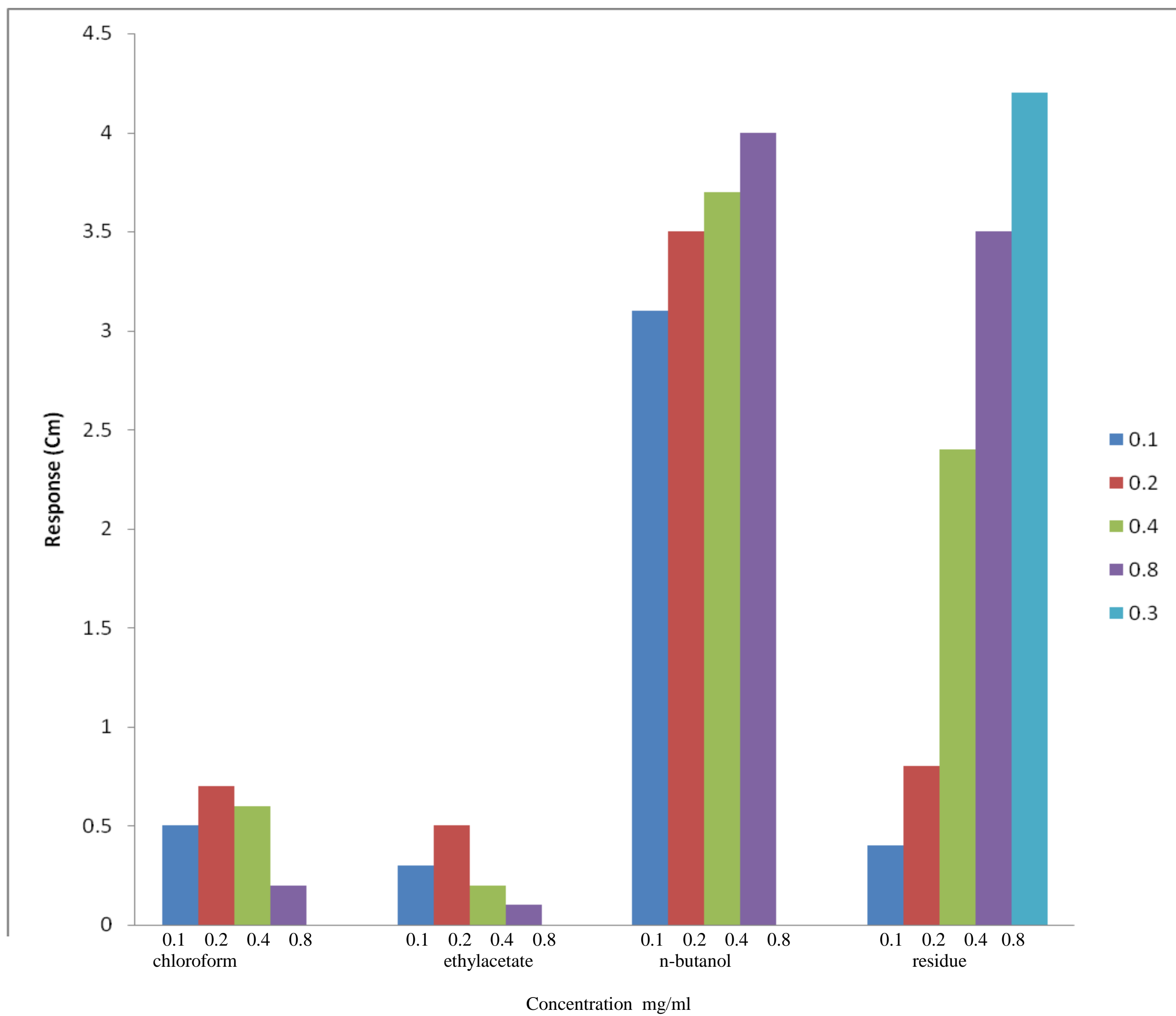

Fig:. 34 Effect of chloroform, ethylacetate, n-butanol and aqueous residue of the leaf fraction on rabbit jejunum.

Table 4.27 Below shows the result of acute toxicity studies.

TABLE: 4.27 TOXICITY STUDIES

S/No	Weight	Doses	Sign of toxicity	Result
1	14.5 gram	1000mg/kg	calm	Sudden death
2	26.30gram	1000mg/kg	Difficulty in breath	Sudden death
3	23.40gram	1000mg/kg	Neurological deficit	Sudden death
4	17.00gram	100mg/kg	Neurological deficit	saddened
5	26.30gram	100mg/kg	Weak	Survive and die
6	23.50gram	100mg/kg	weak	Survive and die
7	25.00gram	10mg/kg	Active	Survive and die
8	16.40gram	10mg/kg	Active	Survive and die
9	32.00gram	10mg/kg	Active	Survive and die