PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE LEAVES OF *OLAX MANN1* OLIV [OLACACEAE]

BY

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BEING

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE (PHARMACEUTICAL AND MEDICINAL CHEMISTRY)

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CERTIFICATION

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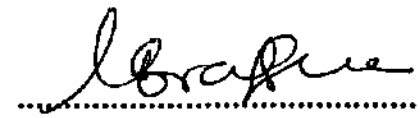
I hereby declare that this thesis has been composed by myself and that it is a record of my own research work. It has not been submitted in any previous application for higher degree. All quotations are distinguished by quotation marks and the source of information are specially acknowledged by means of references.



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DEDICATION

This work is dedicated to:-

my wife, Hadiza

and our children:

Hassan, Hussaini and

Luqman.

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ABSTRACT

The Plant *Olax manni*. is a shrub widely distributed in West Africa and it is employed in traditional medicine in Northern Nigeria to treat poisonous snakebites.

General phytochemical examination of the various crude extracts of the leaves of this plant revealed the presence of tannins, fatty acids, saponins, sterioids/terpenoids. Compound C_1 was isolated from (he petroleum ether extract of the leaves. This was shown to be 5 (0-methoxy phenyl) pent - 2,4 - dienoic aeid using spectroscopic studies (UV, IR, H-NMR, ^{13}C-NMR, COSY and DEPT).

Invivo anti-snake venom studies of the methanolic extract against venom of *Naja nigricollis* and *Echis carinatus* using Swiss albino mice showed significant anti venin activity. The extract was shown to possess protective activity against the venom of these two poisonous snakes. However, the anti-venin activity is more pronounced against the venom of *N. nigricollis*.

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CHAPTER ONE

1.0 INTRODUCTION

Plants continue to be a major source of medicaments either in the form of traditional medicine preparations or as pure active principles. For this reason, it becomes important to identify useful plants with therapeutic properties for possible isolation and chracterization of their active constituent which could be use for possible inclusion in formularies and pharmacopoieas.

There is no accurate data available to asses the value and extent of the use of plants or active principles derived from countries. World Health Organisation (WHO) has estimated that perhaps 80% of more than 4,000 million in habitants of the world rely chiefly on traditional medicines for their primary health care needs, it can be assumed that a major part of traditional therapy involves the use of plant extract or their active principles (Akerele , 1984)

In the developed countries also, plant derived drugs are still used. For example, in the united States of American (USA), 25% of all prescriptions dispensed from the community pharmacies from 1959 to 1980 contained plant extract or active principles prepared from higher plants. (Farnsworth, 1975).

Traditional medicine has been practised for last several thousand years but found place in the WHO programme only twenty-three years ago (Akerele, 1990). Traditional medicine is wide spread throughout the world and the practices are based on belief that were in existence often for hundred of

that are still prevalent today. The acceptance of traditional medicine is largely conditioned by cultural factors and much of traditional medicine therefore may not be readily transferable from one culture to another (Sofowora A. 1982).

The practice of traditional medicine and modern scientific health care side by side can easily be done any where in the world as it is in China and India (Sofowora 1982)

The importance of plants in folkloric medicine may be attributed to interesting historical discoveries and subsequent development of many biologically active compounds from such claims. This includes quinine, reserprine, hyoscyamine, emetine, morphine, strophanthus digitalis, physostigmine. Recently artemisinin, the active constituent of the Chinese anti-malarial medicinal herb 'quinghaosu'. These discoveries led to the intensive search for more medicinal agents discovered from them. This resulted in the increase in the ratio of drugs of plant origin.

In many developing countries, plants are sources of compounds often with unique chemical structures which exhibit a diversity of biological activity most of which may or may not be of immediate interest as drugs, but may be useful as tools of research and they continue to play fundamental role in medical treatment (Olaniyi, 1985).

There may be geographical variation in constituents of related species (cannel 1998). For example Schlittler (1967) discovered that African *Rauwolfia vomitoria* yields higher reserpine than similar species from other continents (Schlitteler and Bein 1967).

Many medicinal plants in this continent remain to be screened. This is a great challenge considering the little resources avilable to researchers.

Plants also remain an untapped reservoir of potentially useful chemical compounds to be used as drugs. Several approaches have been adopted to develop drugs from plants. Among which is phytochemical screening which is often accompanied by biological screening of selected plants based on their folkloric usage.

Synthesis of new drug agents is very tedious, expensive and often non-productive. This should make us re-evaluate our traditional practices in the treatment of disease which usually involve medicinal plants used over many generations. These plants contain chemical constituent which in most cases offer rationale for their uses as medicines. It is therefore strongly recommended that if one is searching for an agent used to treat a certain disease to screen plants that are used locally for such disease. Primitive man out of curiosity and in search for food ate most plants around him. Some plants nourished him, some made him sick, others even killed him. Through the gradual process of trial and error, he was able to sort out plants that are edible and those that are not (Thomson, 1978). Gradually man was able to observed and identify medicinal effects of many plants. This knowledge has been successively transcribed to modern man through various means such as the pictograph of the Egyptians, the clay tablet ideogram of the Babylonians or even the verbal communication. The great contributions of Hippocrates (460 - 377BC), Dioscorides (100 AD) and Galen (131-200 AD) together with the early Arab contributors notably Ibn-al-Baifar (13th century AD) and many others (Stern 1974).

Northern Nigerian plants may offer a virgin field for phytochemical works many plants considered as medicine by the Hausas in Northern

Nigerian are yet to receive attention of phytochemists (Mugu 1982). Among these plants, is *Olax manni* oliv. (Olacaceae) used in the treatment of poisonous snake bites (Rabiu , personal communication).

In Savannah region of West Africa, notably Nigeria, Benin, Cameroon and Ghana, poisonous snake bite continue to be a major medical problem especially among rural communities. The saw-scaled or carpet viper., *Echis C. ocellatus* has proved to be the most important cause of snake bite mortality and morbidity in this region. Farmers, hunters, herds men and children are the groups at risk. The main clinical features of *Echis C. ocellatus* envenoming are haemorrhage, in coagulable blood, shock and local necrosis. In Nigeria where untreated mortality appear to be 10 - 20%, this species cause thousands of death annually. Some communities have resulted to the usage of traditional herbs some of which are of unproven value.

Recently, ethnopharmacologic studies from various part of the world have confirmed the usefulness of some herbal preparations in snake bites. The herb "cobeca de negra" which is an unidentified Amazon plants is used by plantation workers in the jungle as an effective oral antidote against the snake venom of *Bothrops atrox Aristolochia* spp has been studied and found to affect the effect of venoms of *Naja n. atra* (cobra), *Trismeresurus mucrosquamatus* (Chinese habu) *T. gramaineus* (Indian green tree viper), *Agkistrodon acutus* (sharp nosed pit viper) and *Bungarus multicinctus*. Preliminary studies has confirmed the protective effect of *Aristolochia albida* in mice against the venom of two Nigerian poisonous snakes *Bitis arietans* (puff adder) and *Naja nigricollis* (African Spitting cobra) (Haruna 1995) Ethnopharmacological reports from the Nigerian plant *Schummaniophyton*

magnificum claimed to be used against snake bites have prompted series of phytochemical and pharmacological screening on the plant. It was shown to contain active principles which offer *in-vivo* protection against *Naja naja* and *Naja nigricolis* venom. Although the effect of herbs to the venom of vipers is less promising mainly because some vipers have an additional unique mechanism in affecting blood clotting cascade, Vishwanath (1987) has reported on the interaction of *Aristolochia* spp with venom of Russel's viper, that also affects the clotting cascade.

1.2 LITERATURE REVIEW

1.2.1 *Olax manni* Oliv. Olacaceae [Hausa name - Tsadan Biri]

Olax manni Oliv, is a plant specie in the family Olacaceae which are widely distributed in the tropics especially Nigeria, Sierra Leone and Ghana. *Olax manni* is a climbing shrub up to 2 metres high. Leaves are lanceolate to ovate or elliptic up to 6 x 3 inches with 5 - 6 pairs of lateral looped nerves. Flowers are greenish white in axillary racemes. Fruits are orange when ripe about ½ x ¾ inches. Natural habitat is closed forest (Daziel 1963).

1.2.2 Traditional Medicinal Uses of *Olax manni* And Other Plants In The Olacaceae Family

Powdered leaves of *Olax manni* is administered orally with water as antidote for poisonous snake bite. Decoction of the leaves and roots of the plant is use for yellow fever (Irvine 1961).

A related specie, *Olax susbscorpoidea* is use as enema for stomach pain and jaundice. A decoction of root and leaves is use in Nigeria for yellow

fever. Leaves are use as ingredient in snake bite remedies in Cote d'Ivoire. The leaves decoction is also use for guinea worm infection (Irvine, 1961).

Other plants in the same family, *Ximenia gabonensis* Bail are applied as antidote for snake and other poisonous bites (Irivine, 1961). *Ximenia americana* Linn is taken orally against fever, diarrhoea and jaundice. The crushed bark is applied to sores of domestic animals in Angola. Crushed leaves and roots are applied locally to febrile headache. Roots are also use for veneral diseases and cures vomiting and purging. The decoction of the leafy twigs is use for feverish cold and cough, as a laxative, mouthwash for toothache and eye lotion (Irvine 1961; Watt and Breyer - Brandwijk 1962).

Another plant of same genus, *Olax dissitiflora* Oliv is known to have an emetic activity (Watt and Breyer - Brandwijk, 1962)

1.2.3 Chemical Constituent of *Olax manni* And Other Plants In The Olacaceae Family

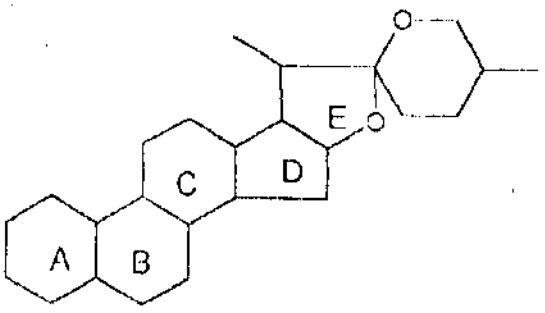
There is no documented work done on *Olax manni* (NAPRALERT report, 1997). However, the presence of saponin have been reported in the leaves of related specie, *Olax subscorpoidea*, Tannins *in Ximenia americana*, cyanogenetic principles in *Ximenia gabonensis* (Irvine, 1964) and fatty acids in the leaves of *Heisteria pervifolia* (Mabry, 1968). Generally the chemical constituent of olacaceae is variable and includes the following:-

SAPONINS

The term saponin applied to a group of natural product which have in common the property of foaming when shaken with water. They are widely distributed in the vegetable kingdom and have been reported to be present in

at least 500 genera of plants (Basu, 1967) Chemically they are glycosides which yields on hydrolysis one or more sugar units and a non-sugar aglycone which are derived from polycyclic ring system and are commonly referred to as sapogenins. Saponins cause haemolysis of red blood cell (Haborne, 1973) and sapogenin which result from hydrolysis are alcohol soluble and does not froth (Basu, 1967).

They can be divided into two based on the structure of aglycone or saponxgenin, these are sapogenins sapogenin which are described as having a spiroketal side chain (I) and triterpenoid sapogenin (II)



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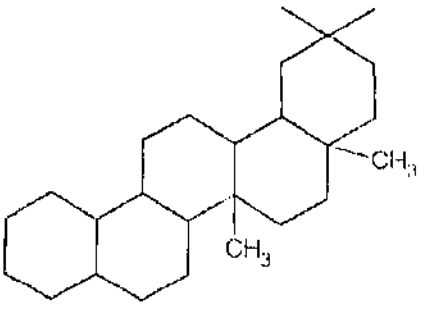


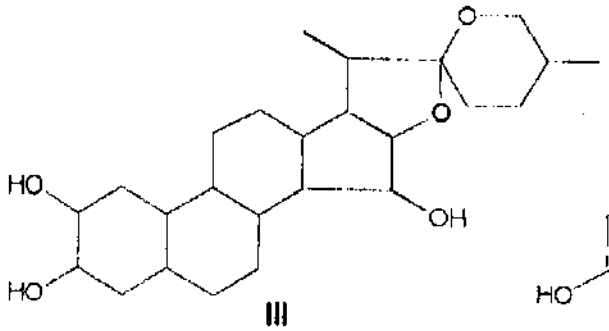
II

The triterpenoid saponin may have as aglycones like oleanolic acid combined with sugars like glucose.

Both type of saponins are soluble in water and alcohol but insoluble in ether and non-polar solvent while their aglycones have solubility characteristics of other sterols. Examples of sapogenin structures are digitogenin (III) from digitalis, hacogenin (IV) from *Agave* spp, hederagenin (V) from *Hedera helix*, glycyrrhetic acid (VI) from *Glycyrrhiza glabra*.

Aglycones from steriodal saponin glycoside are used for partial synthesis of cortisone and sex hormones (Foye, 1976).

It will be observed that steroidal saponins are derived from the same tetracyclic ring system as in sterols, bile acids and cardiac aglycones and are also called as saponins of cholane group. These sapogenin are C_{27} steroids carrying spiroketal side chain which exhibit several characteristics strong IR bands in the region of 1350 to 875 cm^{-1} (Silvestein, 1974).



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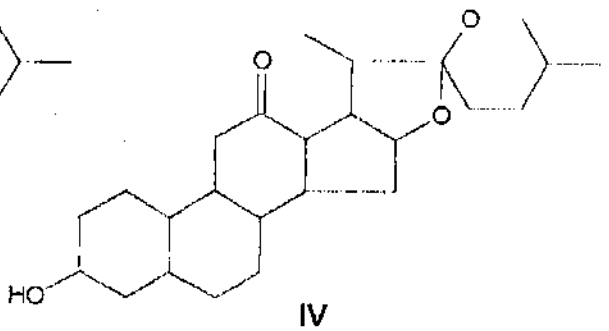

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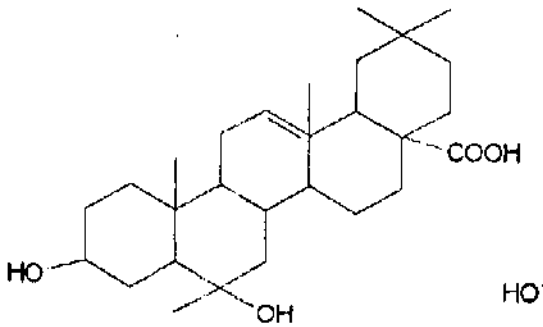

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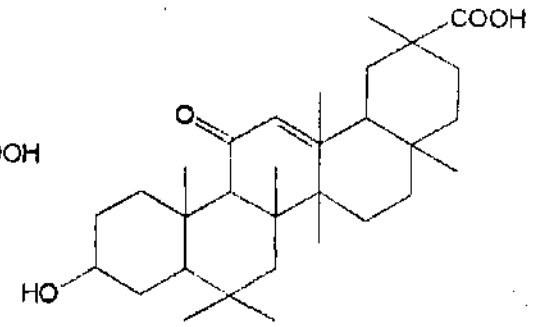


VI

A great variety of these saponins differ only in the number and type of units in the carbohydrate moiety linked to a particular sapogenin. The triterpenoid sapogenins with a few exceptions belong to β-amyrin group and are usually simple alcohols and acids. Occasionally a sapogenin is encountered having aldehydic and ketone functions (Basu 1967).

Triterpenoid saponins have been known for over 100 yrs and a few efforts have been made more than a decade ago to review their chemical and biological properties. However, in the last 20 yrs a note worthy progress has been made in this field as a result of the development of improved techniques for the isolation and detection of natural products and the elucidation of the finer details of triterpenoid chemistry.

They are usually detected by the means of the characteristic colours they produce with various reagents e.g. Lieberman-Burchard, thionyl chloride, phosphomolybdic acid, silico tungstic acid etc. and the haemolysis of blood (Basu, 1967). For the purpose of detection on paper chromatograms, some of the above reagents have been modified. A qualitative and highly sensitive micro method in which filter paper disc wetted with saponin are embeded in blood gelatin has been devised. Other reagents use for detection are sodium metaperiodate, alkaline potassium permanganate mixture, antimony trichloride chloroform, chlorofom vanillin. Various solvent system have been reported to provide satisfactory separating viz: ethyl acetate:Pyridine:water (3:1:3), butanol: acetic acid:water (6:1:3), and butanol:1M NH_4OH:95% alcohol (60:30:5:13) etc. Chloroform:tetrahydrofuran:pyridine (10:10:2) saturated with formamide and chloroform:methanol:water (65:35:10) have proved to be particularly good for paper chromatography, TLC as well as separation of saponins on cellulose and silica gel. (Stahl 1969)

The ease with which saponins are hydrolysed into sapongenins and sugars (or which there may be up to 12 units) vary from one saponin to another. Usually refluxing with 5 - 10% mineral acid is necessary for complete

breakdown. Sometimes hydrolysis can be carried out with enzymes e.g. hydrolysis of alfalfa has been achieved with fungus preparation (Aspergillus)

The sugar component have been identified by the conventional method of paper chromatography in various solvent systems. The sugar units are linked to the genin by glycosidic linkages, an ester linkage is rarely encountered and only D-glucose has so far been found in such combinations. The following eight sugars have almost exclusively been found to be involved in glycosidation. D-glucose, D-galactose, D-galacturonic acid, D-furanose, L-rhamnose. The structure of sugar chains in a few cases have been elucidated by the usual method of methylation followed by hydrolysis and the identification of the individual methylated units. It has been found that the sugar unit is often linked at C-3 OH of the aglycone but in some this linkage is at other positions e.g. In nausemin at C-16 OH.

Glycoside A has two sugar chains one linked at C-3OH and the other of C_{28}-COOH.

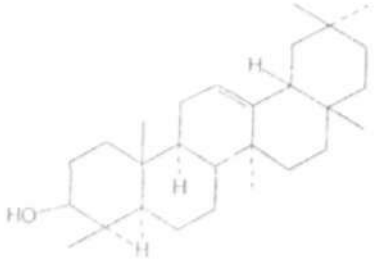
Intensive investigations on the chemistry of triterpenes commenced around 1930 but until 1950, these were mainly confined to establishing the gross structures and relationship of member of this group. The stereochemistry is very complex. There are eight centres of a symmetry giving rise to 256 possible configurations which will be further augmented when substituent were introduced in the alicyclic system. Some simplification of the problem became apparent with the realization that the mode of looking of rings A and D was common to α, β-amyrins and lupane derivatives. The situation was however resolved by the exposition of the principles of

conformational analysis and by the application of rules to the shifts of the molecular optical rotation of polycylic compounds.

The stereo chemistry of β-amyrin was derived from the chemical and X-ray evidence that the lupeol family followed from its relationship with β-amyrin family by inter convesion.

The gross structure of (-amyrin was determined in 1949 and the first direct inter conversion between the two amyrin groups was accomplished in 1955 with the conversion of Ursa-11:13 (18) - dienyl acetate to olean - 11:13 (18) dienyl acetate. Almost simultaneously the complete stereostructure of α-amyrin was established on the basis of extensive chemical and physical data.

Both β-amyrin **(VII)** and α-amyrin **(VIII)** posses *trans-anti-trans-syn-cis* arrangement of the rings A, B, C, D and E and lupeol **(IX)** formulae are given below.



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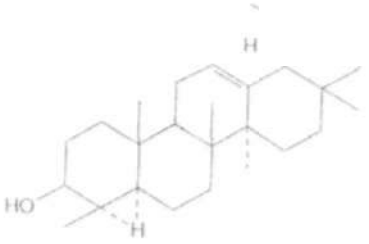
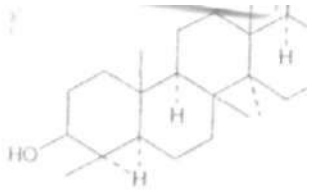


VIII



IX

TANNINS

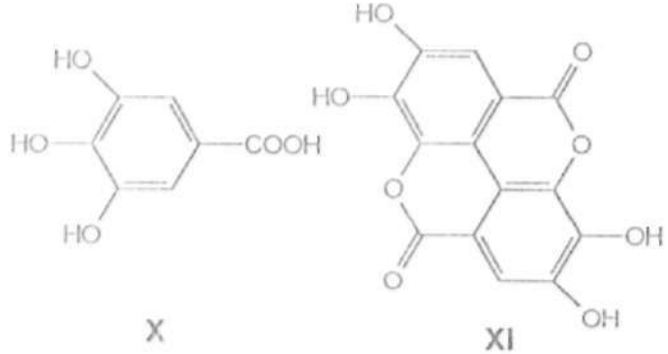
These are phenolic compounds which are found abundantly in plant kingdom. They are water soluble, high molecular weight and made up of simple phenolic nuclei. They are use to convert animal hides into leather and

make it less permeable, prevents putrefaction and swelling. They occur as mixture of polyphenols. They give positive test with ferric chloride. They are classified as true and pseudo tannins.

A. ***True tannins*** - These give all the reactions of tannins and are capable of converting hide into leather. There are two types of true tannins. These are the hydrolysable and the non-hydrolysable tannins.

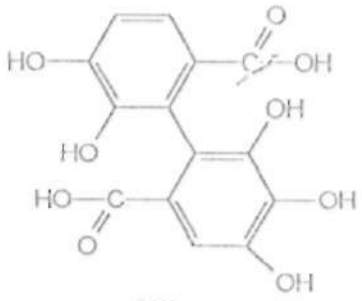
(i) ***Hydrolysable tannins*** - these contain ester linkages which may be hydrolyse by boiling with dilute hydrochloric acid the alcoholic component of the ester is usually sugar. They are also called pyrogalol tannins.

Example of phenolic acids found in tannins are:-



X

XI

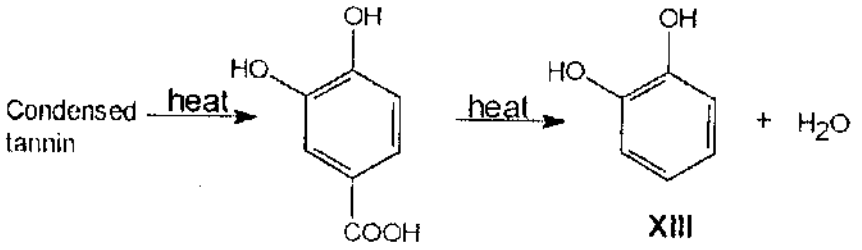


XII

Gallic acid **(X)** is the one occurring most. Ellagic acid **(XI)** is a secondary product formed on hydrolysis of some tannins which are actually esters of hexa oxydiphenic acid **(XII).** Hydrolysoble tannins are often complex mixtures containing several different phenolic acids esterified to different

positions with sugar molecule and these render it more water soluble. They are sometimes named after the acid derivatives e.g. gallitannins for tannins from gallic acid and ellagitanin for tannins from ellagic acid. They can also be hydrolyse by an enzyme called tannose. They are soluble in water, dilute alkalis, acetone, alcohol but insoluble in chloroform, benzene.

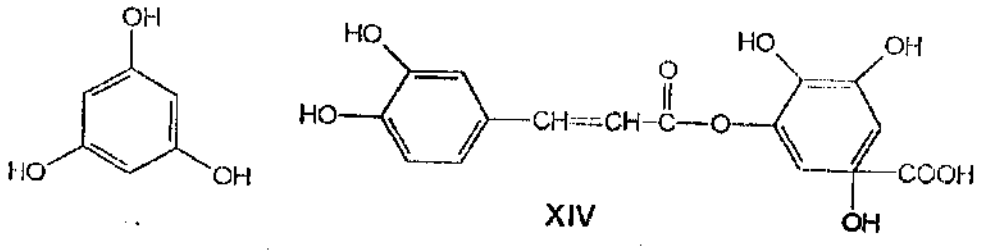
(ii) Condensed tannins - This have no ester linkage with sugar molecules. They consist of large phenolic group chemically linked directly by carbon - carbon (C-C) linkage. They are also called catechol tannins. Their biological activity is limited by their high molecular weight and their relative immobility. The absence of any protective groups an the hydroxylated matrix of these polymers is probably the reason why they are so important in plant - animal interactions but is also limited by their immobility. On boiling with dil. mineral acids e.g. dil HCl they give polyhydroxyphenol which on further heating yields catechol (XIII) and so are also called catechol tannins.



XIII

B. Pseudo tannins

This give many reaction of tannins but cannot convert hide into leather e.g. phloroglucinol tannins, chlorogenic acid tannins **(XIV).**



XIV

Traditional method of separation of plant tannins is extraction with hot water, salting out with sodium chloride, re-extraction of precipitate into acetone and removal of lipids from acetone - extractable material with ether (Robinson,1964).

Lead or zinc acetate (10%) are often use also to precipitate tannins which may be recovered by decomposing the precipitate with hydrogen sulphide. Ethanol can be use to redissolve tannins from this precipitate. Precipitation by adding alcohol solution of potassium acetate to an alcoholic solution of tannins is often use in tannin isolation. Tannins can also be precipitated with caffein and then recovered by continuous extraction of the caffein in chloroform (Ciulei, 1981). Tannins are characterised by their colour reaction will ferric chloride and their ability to precipitate gelatin (Robinson, 1964).

To distinguish hydrolysable from the condensed tannin, a mixture of 10% acetic acid:lead acetate (2.1) is used. The former precipitate while the latter does not (Robinson, 1964).

FATTY ACIDS

There are over 200 different fatty acids isolated from higher and lower plant but the majority of these maybe classed as unusual a uncommon as some differ from me the usual fatty acids and do not confirm to the homologous series of the organic acids (Galliard and Mercer, 1975).

Fatty acids in plants could be saturated, unsaturated, polyunsaturated or monomeric fatty acids. Fatty acids have been reported in Malvaceae, Boroginaceoe and Olacaceae families (Galliard and Mercer, 1961).

Saturated fatty acids:- These are straight chain consist of an even number of carbon atoms and conform to the homologous series $CH_3(CH_2)_nCOOH$ in which n is even but there has been reports of odd-numbered fatty acids. At present, there is evidence that most of the possible saturated fatty acids between butanoic, $CH_3(CH_2)_2COOH$ and hexacosanoic $CH_3(CH_2)_{24}COOH$ occur in plants. However, while the even - numbered fatty acids from C_{10} to C_{18} comprise more than 50% of total fatty acids in plants, the odd-numbered rarely exceed 1% of the total fatty acids (Miller 1973).

The major fatty acids in plant are only sparingly soluble in water. Most are hydrophobic and are readily soluble in organic solvents such as hexane, benzene, chloroform and chloroform - methanol mixtures. Usually the solubility of fatty acids decrease with increasing chain length both in aqueous and in organic solvents. However, odd - numbered fatty acids are soluble to the same extent as the preceding even - numbered fatty acids.

Unsaturated fatty acids:- These comprise a greater percentage than the saturated. The predominant unsaturated fatty acids are 16 and 18 - carbon acids. The introduction of unsaturated centers into fatty acid chains leads to the possibility.

of positional isomerism of the double bonds. Examples of common unsaturated 18 carbon fatty acids of plants are:

Oleic (**XV**), linoleic (**XVI**), linolenic (**XVII**), γ - linolenic (**XVIII**) acids.

$CH_3(CH_2)_7CH{=}CH\ (CH_2)_7COOH$ (*Cis*) (**XV**)

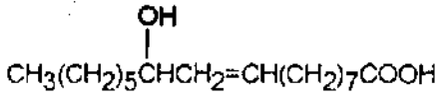
$CH_3(CH_2)_3\ (CH_2CH{=}CH)_2(CH_2)_7COOH$ (*Cis*) (**XVI**)

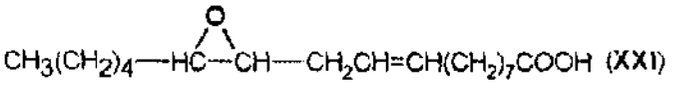
$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOH$ (*Cis*) (**XVII**)

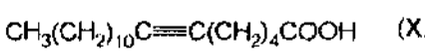
$CH_3(CH_2)_3(CH_2CH{=}CH)_3(CH_2)_4COOH$ (*Cis*) (**XVIII**).

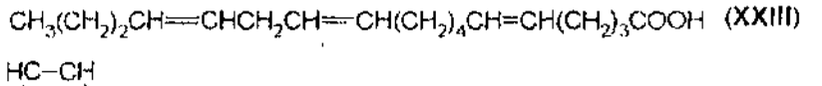
Examples of uncommon unsaturated 18 carbon fatty acids of plant origin are: eleostearic (**XIX**), ricinoleic (**XX**), 12,13-epoxyoleic (**XXI**) tariric (**XXII**), octadeca- 5, 11, 14- trienoic (**XXIII**), Chaulmoogric (**XXIV**) acids, (**XXIII**), chaulmoogric (**XXIV**) acids.

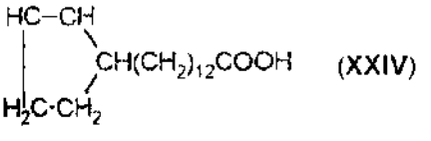
$CH_3(CH_2)_3(CH{=}CH)_3(CH_2)_7COOH$ (*cis*, *trans*) (**XIX**)


(**XX**)


(**XXI**)

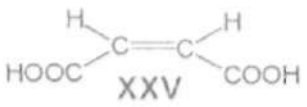

(**XXII**)


(**XXIII**)

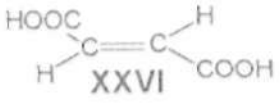

(**XXIV**)

The presence of double bond in a fatty acid chain leads also to the possibility of geometrical isomerism. Thus fatty acids substituent on the two

carbon atoms involved in the double bond can be arranged cis or trans to each other in a manner that is reminiscent of maleic **(XXV)** and fumaric acids **(XXVII)**



XXV



XXVI

1.2.4 SNAKES AND SNAKE - BITES

Snakebites are major cause of morbidity and mortality affecting particularly the rural population as an occupational risk to farmers and plantation workers. Snakebite is largely a problem of remote rural areas where the victims resort to traditional herbalist due mainly to inaccessibility to hospitals and professional health care.

There are five families of venomous snakes comprising of more than 200 species (TU. 1977). The five families of these venomous snakes are:

i) **Colubridae**

This is the largest snake family. Most species are harmless except two notorious species that are clearly venemous this are *Dispholidus typus* (boom slang) and *Helotormis kirtlandic* (bird snake). Both are natives of Africa. They posses short non-hinged fangs situated far back in the mouth. The colubrid *Dispholidus typus* is less common and bites by this species is very rare except when handled.

Elapidae

These are popular snakes of Africa and Asia. They include Asian *Naja naja* (cobra), *Bungarus caeruleus* (common kraits), Australian *Acanthopis antarctica* (death adder) *Notechis scuatus* (tiger snake) and African *Naja nigricollis* (spitting cobra) and *Dendroaspis polylepsis* (black mamba). Elapids

are generally longer with short non-hinged front fangs. In Nigeria, the spitting cobra is very common in Northern parts of the country. They posses an opening near the apex of their fangs with which they spit with stertling accuracy into the opponent's eyes.

iii) Viperidae

These are distributed in Africa, Europe and middle east. They comprised

i) viperinae, the typical vipers and adders of the world. Vipers are relatively shorter and thicker snakes than others. they posses long hinged front fangs. Examples are *Echis carinatus ocellatus* (carpet viper), *Bitis arietans* (Puff adder), *Viper ruselli*

ii) crotalinae. These are distinguished by an accessory infra red sensing organ in a pit between the eyes and the nose. They include *Trimeresurus arboralis* (arboreal great pit viper), *Colloselasma rhobdastoma* (Malayan pit viper), *Crotalus sistrurus* (rattle snake).

iv) Atractaspididae

This include the genus Atractospis viper. They are found in Africa.

v) Hydrophidae (sea snakes)

These are distributed in Indo pacific West coast of America. They are venomous and bites are rare and limited to people engaged in fishing and marine expenditure. Their venoms are reported to be the most toxic and induces paralysis and rhabdomyolysis (TU, 1977).

Snakebites from poisonous snakes usually result in massive envenomenation leading to neurotoxicity in the case of elapidae, myotoxicity

in the case of hydrophidae and cytolytic plus haemotoxicity/vasculo toxicity in the in case of viperidae.

Snake Venom

The cobra venoms are said to be neurotoxic and carpet viper venoms are haemotoxic. Snake venoms consist of about 90-95% proteins - mainly enzymes, non-enzymatic polypeptides and non-protein component consisting of nucleotides, carbohydrates, lipids, biogenic amines, metal ions (TU, 1977).

Protein fraction are biologically more important than non-protein ones as most of the biological activities resides in the protein fraction contain major and minor toxins, non-toxic proteins and enzymes. Most enzyme are hydrolytic in nature. Because of this property. It is thought that they facilitate tissue damage on the prey to help eventual digestion (exodigestion).

Specific chemical compounds have been isolated from snake venoms some having both pre and post synaptic activity. The post synaptic toxins attach to the acetylcholine (Ach) receptor site, giving a myasthenia-like syndrome, while the presynaptic toxins exhibit phaspholipase A activity (TU 1977). α-bungaro toxin is a post synaptic toxin that attach to the Ach receptor on the motor end plate. Acetylcholinestrases reverse the effect of α-Bungarotoxin but not those of presynaptic poisons.

The various enzymes in the snake venom play different role. About twenty - six have been identified out of which ten are well understood. The most important snake venom enzyme is called phospholipase-A_2 (lecithinase) present in venoms of all the snake families and are found to increase membrane permeabilities (both nervous and muscular tissues), disrupt electron transport chain and the integrity of mitochondrial structure. Hydrolytic

enzymes hyalurinidase facilitates toxin diffusion into tissues of victims and is called spreading factor.

The organic non-protein fraction such as the biogenic amines e.g. bradykinin, spermine, histamine, serotonin also have their toxic effects such as hypersensitivity.

Individual components may affect many different tissues e.g. an enzyme dubbed cadiotoxin in the venom of *Naja* spp exert effect on venom tissue causing irreversible cell membrane depolarisation. It blocks neuromuscular transmission and axonal conduction, cause haemolysis and is cytotoxic (TU 1977).

Snake venoms most frequently causes coagulopathy and bleeding. A number of snake venoms were reported to activate various blood clotting factors causing thrombosis while others causes in coagulable bleeding leading to profuse haemorrhage. Some venoms have these complementary properties by possessing both procoagulant and anticoagulant actions (TU 1977). Some snake venoms contain compounds that cause vasoconstriction like sarafotoxins which mimics potent endogenous vasopressor peptides. Other snake venoms cause local pains, swelling and necrosis.

Anti-snake Venoms

The use of antivenin continue to be the main stay of therapy in the management of snake venom poisoning all over the world. Antivenin efficacy resides in providing high affinity antibody to combine with venom proteins with objective of enhancing toxin elimination.

The antivenoms are prepared by hyper immunising horses or other animals like sheep as in the recent case of Warrel (1993) and Theakson

(1994). The immunogens stimulate, the production of hyper immune serum which is harvested and suitably precipitated and then digested using enzymes pepsin or papain which is also recently used (Theakson 1994). The final product is separated by extensive chromatographic separation, it is then purification and concentrated and standardised. The anti venoms can either be lyophilised or prepared in thermolabile liquid form to be stored with uninterrupted cold chain at temperature ranges of 2-8°C with shelf life of about three years.

Anti-snake venoms are highly specific, this implies that a particular anti venom will neutralise the venom of the snake used in its preparation only. Monovalent serums are prepared using the venom of a single snake while polyvalent anti snake venoms are generally intended to include all the venomous snake found in a particular region.

Traditional Treatment Of Snake Bites

This vary from one society to another These includes plant, animal and mineral products which includes the black stone reported in some societies (Garba, personal communication), Israel stone (Hanya, personal communication), plants etc.

In many instances plants are used as antidote to snake venoms, a claim that differ from region to region and nature of the snake fauna. Sudarsanam and Prasad (1995) reported the use of 22 plant species as antidote to snake venoms use among the Yanadi tribe in South India, Walter et al., (1977) reported over 45 plant species that one known to be beneficial against snake bites in various part of the world.

Haruna and Choudhary (1995) reported effectiveness of furanoid diterpene (Albidine) isolated from *Aristodochia albida* against the venoms of *Naja nigricallis* and *Bitis arietans* in Swiss albiono mice.

Some of these plants contain active compounds which may be synonyms of antisera which directly and symptomatically produce actions opposing those of the venoms. The plants compounds may act like agents above or specifically reverse or block the neutrotoxic actions on the adrenergic or cholinergic receptor sites.

In Northern Nigeria *Olax manni* is claimed to be benefit in all forms of snakebites. *Naja* nigricollis and *Echis carinatus* (Plates 2-3 and 2-4) are the two common venomous snakes in this region.

1.25 Scope Of The Present Study

Olax manni Oliv. is a shrub growing wild in Nigeria and other West African countries. The plant is used extensively by herbalist in treatment of yellow fever and is also claimed to be beneficial in the management of snake bites but there is no documented work done on the plant (NAPRALERT 1997).

Photochemical screening will be carried out to determine the various classes of chemical constituent in the plant. Chromatographic and other separative techniques will be employed for isolation of constituent of the plant which will be characterise by spectroscopic techniques. Pharmacological screening of the plant extracts would also be undertaken to justify the claimed biological actions.

CHAPTER TWO

2.0 METHODOLOGY

2.1 PHYTOCHEMICAL METHODS

2.1.1 MATERIALS

Plant Material

The fresh plant material bearing fruits and leaves growing wild were collected around Samaru, Zaria

The plant was authenticated in Herbarium, Ahmadu Bello University, Zaria in July 1997. A herbarium specimen is kept at Herbarium Department of Biological Sciences A.B.U. Zaria, Nigeria, and the plant was planted in the botanical garden, Faculty of Pharmaceutical sciences, Ahmadu Bello University, Zaria (Plates 2-1 and 2-2).

Solvent / Reagent / Equipment

Solvent and reagent GPR used were distilled twice before use. All equipment used and capacity are quoted accordingly and operated according to standard procedures using standard solvents.

2.1.2 EXTRACTION OF LEAVES OF *OLAX MANNI*

Powdered leaves of *Olax manni* (1.0 kg) was continously defatted with petroleum ether (60-80 °C) using Soxhlet extractor for 36 hours until the draining solvent was clear. Solvent was recovered at reduced pressure to afford a yellowish - green waxy mass (35 g) which was kept in the refrigerator at about 8 °C and labeled light petroleum extract and was coded "PE".

Plates 2 - 1 Olax manni in the natural habitat

Plate 2-2 Leaves and stem of Olax manni

The marc after defatting was air-dried at room temperature and then extracted continuously with methanol using the Soxhlet extractor. The methanol was evaporated at reduced pressure to afford greenish gummy mass (42 g) this greenish mass was labeled methanolic extract 'ME'. Marc was again dried and extracted continuously with water using soxhlet extractor. Water was evaporated at reduced pressure to afford greenish gummy mass(12g) water extract, WE.

2.1.3 PRELIMINARY PHYTOCHEMICAL SCREENING OF LEAVES EXTRACTS OF *OLAX MANNI*

The above fractions, PE, WE and ME were separately subjected to preliminary phytochemical tests using standard techniques (Ciulei 1981).

2.1.3.1 PHYTOCHEMICAL SCREENING OF PETROLEUM ETHER EXTRACT OF *OLAX MANNI*

(a) Test for sterols/triterpene (Ciulei, 1981)

About 1.0 g of the extract was dissolved in acetic anhydride and then in chloroform (0.5 ml). The solutions were transferred into a dry test tube and by means of a pipette, conc. H_2SO_4 (1-2 ml) was added to the bottom of the tube (Lieberman - Buchard reaction).

(b) Test for flavone aglycone (Ciulei, 1981)

About 0.5 g of extract was taken in a test tube. 1 - 2 ml of methanol was added and heated. Magnesium chips (1 - 2) and 4 - 5 drops of conc. HCl was added (Shinoda test).

(c) Test for Coumarins (Ciulei, 1981).

About 0.5 g of extract was taken in a test tube. Water was added and boiled. After cooling, the solution was divided into two tubes. One tube is the

reference and the other was made alkaline with 0.5 ml ammonia solution (10%). These were observed under UV light.

(d) Test for fatty acids (Ciulei, 1981).

2 g of extract was dissolved in 5 ml chloroform. 5 ml of alcoholic potassium hydroxide was added and refluxed for 30 minutes. The mixture was cooled, diluted with purified cold water and extracted with chloroform.

The chloroform layer will contain non-saponifiable oil and the aqueous layer will contain the saponifiable lipids including fatty acids. This was acidified with dil. HCl to pH 3 - 4. Under these condition, the fatty acids are released from their alkaline (potassium) salts and are extracted by shaking the aqueous layer with ethyl ether repeatedly. The ether solutions were evaporated to dryness. Residue obtained above was spotted on filter.

(e) Test for volatile oils (Ciulei 1981).

About 2 g of extract was dissolved in alcohol and 2 - 3 drops of Sudan IV was added.

(i) Test for basic alkaloids (Brain and Turner 1975).

2 g of extract was extracted with chloroform. To the extract, 2 - 3 drops of Dragendorff's reagent was added and observed for any precipitate formation.

This was repeated with Mayer's and Wagner's reagents.

2.1.3.2 PHYTOCHEMICAL SCREENING OF THE METHANOLIC EXTRACT OF *OLAX MANNI*

(a) Test for Sterol / Triterpene glycoside (Ciulei 1981).

To 10 ml of extract, 0.5 ml acetic anhydride and 0.5 ml chloroform were successively added. This was transferred into a dry test tube. By means of a pipette, 1 - 2 ml conc. H_2SO_4 was added to the bottom of the tube.

(b) Test for saponins (Brain and Turner, 1975).

(i) Frothing test

2 ml of the diluted solution (1:1) was taken in a test tube and shaken.

(ii) Haemolytic test

To 2 test tubes, 2 ml of 1.8% sodium chloride was added. To one of the tube, 2 ml distilled water was added and to the other, 2 ml of the methanolic extract was added. 5 drops of fresh blood was added to each of the 2 tubes and the tubes inverted gently to mix.

(c) Test for Tannins (Brain and Turner, 1975)

To 1 ml of extract, 2 - 3 drops of ferric chloride was added.

(d) Test for Flavone glycoside (Haborne 1973).

To 2 ml of extract, magnesium chips (1 - 2) and 5 - 6 drops of conc. HCl were added (Schinoda test).

(e) Coumarin derivatives (Ciulei 1981)

4 ml of extract , was divided into two tubes. To one of the tube, 0.5 ml of 10% ammonia was added and the two were observed under UV light.

(f) Test for Alkaloidal salts (Ciulei, 1981).

To 1 g of extract, 5-10 ml hydrochloric acid (10%) was added. The alkaloids now become salt of the mineral acid. The alkaloids are now precipitated with ammonia solution and then extracted with chloroform. These were tested with Dragendorff's, Mayer's and Wagner's reagents.

(g) Test for free carboxylic group (Brain and Turner, 1975).

To 2 ml of extract, 1 ml of 15% $NaHCO_3$ was added and the solution was warmed on water bath.

2.1.3.3 PHYTOCHEMICAL SCREENING OF THE AQUEOUS EXTRACT

This was screened for all group of compounds screened under methanolic extract (3.1.3.2) above (a - g). Reducing substances were tested as below.

(h) Test for reducing substances (Ciulei 1981).

To 1 ml of the water extract, 2 - 3 drops of Fehling's solution (I and II) were added.

2.1.4 CHROMATOGRAPHIC STUDIES OF THE LEAVES EXTRACT OF *OLAX MANNI*

2.1.4.1 Thin Layer Chromatographic Studies Of Petroleum Ether Extract Of *Olax manni*

Preliminary Thin Layer Chromatographic of crude pet ether extract was carried out System use are as follows:

1. Technique - ascending
2. Adsorbent and plates - glass plates, size 10 x 4 cm, precoated with silica gel, G. thickness 0.25 mm and activated at 105°C for I hour before use.
3. Solvent system - Pet ether. chloroform: acetone (6:3:1)
4. Detection - (a) Day light
   (b) Iodine vapour
   (c) Ultraviolet light

2.1.4.2 Thin Layer Chromatographic Studies Of The Methanolic Extract Of *Olax manni*

This was carried out using the following system:

1. Technique - ascending

2. Adsorbent and plates - glass plates, size 10 x 4 cm, precoated with silica gel, G. thickness 0.25 mm and activated at 105°C for I hour before use.
3. Solvent system: methanol: acetone: water (6:3:1)
4. Detection - (a) Day light
   (b) Iodine vapour
   (c) Ultraviolet light

2.1.4.3 Column Chromatographic Studies Of Petroleum Extract Of *Olax manni*

About 5 g of the petroleum - ether extract was weighed out and subjected to column chromatography. Thin Layer Chromatography was used to monitor fractions/eluates. The following column conditions were employed.

a. Technique - gradient elution
b. Column - glass column with sintered disc. at the bottom. Dimension - 60 cm length x 1.5 cm width.
c. Adsorbent - 200 g silica gel 60 - 120 mesh
d. Column packing - wet slurry method.

50ml pet ether was added to 200g silica gel and stirred with glass rod. The slurry was poured slowly and steadily into the column.

e) Sample application - 5 g of the pet ether extract was dissolved in pet ether and mixed thoroughly with small quantity of silica gel. This was allowed to dry and then deposited on top of the settled column using spatula.
f) Solvent for elution - Gradient elution using the following solvent:
   i) - Petroleum ether (60 - 80°C)
   ii) - Chloroform

iii) - Methanol

g) A total of 265 fractions of 10 ml each were collected and monitored by TLC. Similar fractions were pooled together. Results are recorded in table 3.6.

Eluent XXII (fraction 260 – 263) from methanol gave almost single spot (major) on TLC. This fraction was worked up to afford a colourless crystals coded 'C' (mp 139-140 ^{0}C) which on repeated recrystalization gave "C1" mp 139-140 ^{0}C.

2.1.5 Thin Layer Chromatographic Studies Of The Crystals (C_1) Isolated From The Petrolleum Ether Extract Of *Olax manni*

This was carried out to ascertain the purity. System use are as follows:-

1. Technique - ascending
2. Adsorbent and plates - glass plates, size 10 x 4 cm, precoated with silica gel, G. thickness 0.25 mm and activated at 105°C for I hour before use.
3. Solvent system: acetone: chloroform: Pet ether (5:4:1).
4. Visualisation - daylight, ultraviolet light Iodine vapour and ferric chloride spray results are recorded in table 3 - 7.

2.1.6 Determination Of Spectral And Other Physical Data Of "C_1" Isolated From The Petroleum Ether Extract Of *Olax manni*

2.1.6.2 Determination Of Solubility Of C_1

This was determine in methanol, chloroform, acetone, petroleum ether and water results recorded accordingly (table 3.8).

2.1.6.2 Determination of Melting Point of C_1

Melting point was determined using the Gallenkamp melting point apparatus using sealed capillary tube. Results are uncorrected.

2.1.6.3 Spectroscopic Analysis Of C_1 Isolated From The Petroleum Ether Extract Of *Olax manni*

The crystal, C_1, was subjected to the following spectral analysis.

Spectra are shown in figures 3: 1-7

a) UV spectra was recorded in methanol (AnalaR grade) using Pye Unicam spectrometer SP 8 - 100.

b) Infrared spectra was recorded using Perkin Elmer 1710 FT IR spectrometer, sample was prepared in chloroform.

c) Proton nuclear Magnetic resonance (^{1}H-NMR) spectra was recorded using Bruker WM 400 NMR spectrometer. Sample was prepared in deutero chloroform ($CDCl_3$) with tetra methyl silane (TMS) as internal standard. The H-NMR in $CDCl_3$ and chemical shift are expressed as δ-values (ppm) downfield from TMS.

d) Carbon -13 Nuclear magnetic resonance (^{13}C-NMR) spectra was recorded using Bruker WM 400-^{13}C spectrometer in $CDCl_3$ with TMS as internal standard.

e) Two - Dimensional proton - NMR (^{1}H-^{1}H 2D) COSY spectra was recorded with Bruker WM 400 ^{1}H-Cosy spectrometer in $CDCl_3$.

f) Two dimensional ^{13}C-HNMR (^{13}C-^{1}H NMR) spectra was recorded with Bruker WM 400 spectrometer in $CDCl_3$.

g) Carbon -13 Nuclear magnetic resonance (^{13}C-NMR) distortionless enhanced poliazation transfer (DEPT) spectra was recorded using Bruker WM 400-^{13}C spectrometer in $CDCl_3$ with TMS as internal standard.

2.2 PHARMACOLOGICAL SCREENING OF THE METHANOLIC EXTRACT OF THE LEAVES OF *OLAX MANNI*

2.2.1 Materials and Methods

a) Animals

i) Swiss albino mice of both sex (Plate 2 - 5) weighing between 25 - 30 g bred in animal house, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The mice had full access to clean drinking water and were fed on standard pellets.

ii) - Snakes and snake venoms.

The two snake species, *N. nigricollis* and *E. carinatus* (Plates 2 - 3 and 2-4) were obtained from local snake catchers.

Venoms were obtained by milking process (Plate 2-6). This is normally done by holding the snakes over small beaker covered with clean sheet of polythene and the fangs were made to penetrate the polythene to release the venom into the beaker. The venom were lyophilised into amorphous.

Plate 2 - 3 Naja nigricollis

Plate 2 - 4 : Echis carinatus

Plate 2 - 5: Swiss Albino mice

Plate 2 - 6: Milking process of venom from *Naja nigricollis*

The methanolic extract of *Olax manni* was used for the preliminary screening of the anti-snake venom effects as this is related to the ethno medical use.

2.2.2 In-vivo antisnake venom activity of the methanolic extract of *Olax manni* against the venom of *N. nigricollis* and *E. carinatus*

a) Preparation of stock solutions

0.2 g and 0.1 g of lyophilised *N. nigricollis* and *E. carinatus* venoms were dissolved in 0.9% normal saline to produce stock concentration of 0.5 mg/ml of each venom. Other concentrations were prepared by serial dilution.

b) Determination of LD50 and LD99

LD50 (Median lethal dose) and LD_{99} (Maximum lethal dose, MLD) are those doses of a substance that kill 50% and 99% of a given population respectively in specified period, usually 24 hours.

Pilot studies were carried out to find the highest tolerated safe doses of the venom and the lowest lethal dose. Within this range, five doses were selected for each of the venoms (Tables 3.12 and 3.13) five groups of mice (fifteen in each group) kept separately were injected with these different doses of the venom intraperiotoneally. Another group was injected with 0.9% normal saline and this group served as the control. Mortality was recorded after 24 hours. LD_{50} and LD_{99} were determined by probit analysis (Theakson and Reid 1983). This are shown in figures 3.1 and 3.2 for *Naja nigricollis* and *Echis carinatus* respectively.

In another experiment, increasing doses of the methanolic extract of *Olax manni* were injected intraperitoneally. Symptoms of toxicity were

observed and recorded so as to determine the maximum tolerated dose of the methanolic extract of *Olax manni*.

(c) Determination of protective effect of the methanolic extract of *Olax manni* an Swiss Albino mice against LD99 of *N. nigricollis* and *E. carinatus.*

Five groups of mice (fifteen in each group) wee pre treated by giving increasing doses of the methanolic extract (IP) 30 min. before injecting the LD_{99} of the venom of *N. nigricollis* (IP) and the survival of mice was observed within 24 hours. This was repeated with LD_{99} venom of *E. carinatus*. symptoms of acute toxicity and mortality after 24 hours were recorded. The log dose against mortality was plotted and median effective dose, ED_{50} of the methanolic extract against the LD_{99} of the venoms *N. nigricollis and E. carinatus* were calculated respectively (figure 3-3 and 3-4)

CHAPTER THREE

3.0 RESULT

3.1 PHYTOCHEMICAL METHODS

3.1.1 Materials and Methods as described in Chapter 2.2.1

3.1.2 Extraction

a) % yield of the pet. ether extract = 3.5% w\w

b) % yield of the methanol extract = 4.2% w\w

c) % yield of the aqueous extract = 1.2% w\w

3.1.3 Preliminary Photochemical Screening Of The Leaves Extract Of *Olax manni*

3.1.3.1 Photochemical Screening Of Petroleum Extract Of The Leaves Of *Olax manni*

a. Test for sterols/triterpenes.

At contact zone of the H_2SO_4 deposited at bottom of test tube with the solution violet ring was formed and green in the supernatant liquid. This indicated presence of sterols and triterpenes.

b. Test for flavone aglycone

There was no red or orange colour indicating the absence at flavone aglycone.

c. Test for coumarins

The solution in the tube made alkaline with ammonia solution gave a more intense fluorescence under UV light than the other tube indicated the presence of coumarins.

d. Test for lipids/fatty acids

Oily residue was obtained after evaporating diethyl ether which turns paper transparent. This indicated presence of lipid /fatty acid.

e. Test for volatile oils

Absence of red colour on addition of Sudan IV indicated absence of volatile oils.

f. Test for alkaloids

No red precipitate with Dragendorff's and no brown precipitate with Mayer's and Wagner's reagents. This indicated absence of alkaloids.

TABLE 3.1 Results of Preliminary Photochemical Screening of Pet Either Extract of the Leaves Of *Olax manni.*

	Compound /group	Test	Observation	Inference
1	Steroid/ triterpene	Lieberman- Buchard	Pink-red ring	+
2	Alkaloid free Base	Dragendorff's	reddish brown Solution	-
		mayer's	Yellowish solution	-
		Wagner's	Yellowish solution	-
3	Flavone aglycone	Schinoda	Brown solution	-
4	Coumarins	UV	Blue Fluorescence with UV	+
5	Volatile oils	Sudan IV	No red colour	-
6	Lipids/fatty acids	Saponificaation and spotting on paper	oily residue obtained and paper turned transparent	

Key

\+ = present

\- = Absent

3.1.3.2 Photochemical Screening of methonolic Extract of the leave of *Olax manni*

a) Test for steroid/triterpenoid glycoside

Violet -brown ring and superior layer greenish violet indicated the presence of triterpenoid glycoside.

b) Test for saponins

i) Frothing test

Upon shaking, copious froth was formed up to 1.6cm height and persisted for up to 15 mins indicated the presence of saponins.

ii) Haemolytic test

Haemolysis was observed in the tube with extract but not in the control

c) Flavone glycoside

No red or orange colour was produced on addition of magnesium chips and conc. HCl (Schinoda test). This indicated absence of flavone glycoside

d) Coumarin derivatives

Blue fluorescence under UV which was deeper for the alkaline solution indicated the presence of Coumarin derivatives

Table 3.2 Result Of Preliminary Photochemical Screening of Methanolic Extract of the Leaves of *Olax manni*

	Compound /group	Test	Observation	Inference
1	Steroid/ triter period glycoside	Lieberman-Buchard	Pink -red ring	+
2	Saponins	frothing	Copious froth	+
		Haemolytic	Haemolysis	+
3	Tannins	Ferric chloride	Green -black	+
4	Flavone glycoside	shinoda	Brown solution	-
5	Coumarin derivs	UV	blue fluorescence under UV, deeper with alkalinised solution	+
6	Alkalodial salts	Dragendorff's	reddish brown solution	-
		Wagner's		
		Mayer's	Yellowish brown solution	-
7	Free carboxylic acid	Sodium bicarbonate	evolution of gas	+

KEY

\+ = Present

\- = Absent

f) Test for Alkalodial salts

No red precipitate with Dragendorff's and no brown precipitates with Wagner's and Mayer's reagents. This indicated absence of alkaloid salts.

g) Test for free carboxylic group

Evolution of colourless gas indicated the presence of free carboxylic group.

3.1.3.3 Photochemical Screening of the Aqueus extract of the Leaves of *Olax manni*.

The results 1 - g in 3.1.2.2. are the same here.

h) Test for reducing substance

Red colour was produced, indicated the presence of reducing substances.

Table 3.3 Result Of Preliminary Photochemical Screening of Aqueous extract of leaves of *Olax manni*

	Compound /group	Test	Observation	Inference
1	Steroid/ triter period glycoside	Liebermon-Buchard	Pink -red ring	+ve
2	Tannins	Ferric chloride	greenish-black	+
3	Saponins	Frothing	Froth form	+
4	Free Carboxylic group	sodium bicarbonate	evolution of gas	+
5	Free reducing sugars	Fehlings solution	Brick red ppt	+
6	Alkaloidal salts	Dragendorffs Mayers Wagners	Reddish brown solution Yellowish solution Yellowish solution	- - -
7	Flovone glycosides	shinoda	brown solution	-
8	Coumarin derivatives	ultraviolet	Blue fluorescence which was deeper with alkalinised solution	+

KEY
+ = Present
- = Absent

3.1.4 Chromatographic studies of the leaves extract of *Olax manni*

3.1.4.1 Thin Layer chromatographic studies of the Petroleum ether extract of *Olax manni*

Seven distinct spots were obtained. They were visualised and respective R_f calculated. These are shown in table 3.4

Table 3.4 Results of Chromatographic Separation of the Petroleum Ether Extract

	Visualization (Colours)				R_f
Spots	**Daylight**	**UV(366 nm)**	**Iodine vapour**	**Potassium Permanganate**	
I	Green	Red	Brown	Yellow	0.20
II	Green	Red	Brown	Yellow	0.25
III	Orange	Yellow	Brown	Yellow	0.40
IV	Orange	Yellow	Brown	Yellow	0.45
V	Orange	Yellow	Brown	Yellow	0.60
VI	Yellow	Yellow	Brown	Yellow	0.65
VII	Yellow	Yellow	Brown	Yellow	0.70

3.1.4.2 Thin Layer Chromatographic Studies of the methonolic extract *Olax manni*

Four distinct spots were obtained. These were visualised and their respective R_f calculated. There are shown on table 3 - 6.

Table 3.5 Results of Chromatographic seperation of the methanolic extract.

	Visualization (Colours)				R_f
Spots	**Daylight**	**UV(366 nm)**	**Iodine vapour**	**Potassium Permanganate**	
I	Yellow	Red	Brown	Yellow	0.30
II	Yellow	Yellow	Brown	Yellow	0.50
III	Orange	Red	Brown	Yellow	0.70
IV	Green	Red	Brown	Yellow	0.80

3.1.4.3 Column Chromatographic Separation of Petroleum ether extract of *Olax manni*

A total 265 fractions of 10ml each were collected. Results is summarised on table 3.6

Table 3.6 Results of Column Chromatography of Pet Ether Extract

Eluent	Eluting solvents	Fraction	developing solvent	Visulization I_2 vapour	Nos. Of spots
I	Pet ether	1 - 10	Pet ether (PE): Acetone (8:2)	Brown	4
II	Pet ether: $CHCl_3$ (9:1)	11-21	Pet ether (PE): acetone (AC) (8:2)	Brown	5
III	Pet ether: $CHCl_3$ (8.2)	22-35	PE: Benzene: AC (3:21)	Brown	6
IV	Pet ether: $CHCl_3$ (7:3)	36-50	PE: Benzene: AC (4:2:2)	Brown	6
V	Pet ether: $CHCl_3$ (6:4)	51-65	PE: Benzene: AC (4:2:2)	Brown	6
VI	Pet ether: $CHCl_3$ (5:5)	66-97	PE: $CHCl_3$: AC (4:1:5)	Brown	7
VII	Pet ether: $CHCl_3$ (6:4)	83-97	PE: $CHCl_3$: AC (3:2:5)	Brown	5
VIII	Pet ether: $CHCl_3$ (7:3)	98-111	PE: $CHCl_3$ AC (3:2:5)	Brown	4
IX	Pet ether: $CHCl_3$ (8:2)	112-124	PE: $CHCl_3$: AC (2:3:5)	Brown	4
X	Pet ether: $CHCl_3$ (9:1)	125- 134	PE: $CHCl_3$ AC (2:3:5)	Brown	3
XI	$CHCl_3$	135-140	$CHCl_3$ AC (6:4)	Brown	3
XII	$CHCl_3$	141- 142	$CHCl_3$ AC (6:4)	Brown	Nil
XIII	$CHCl_3$ MeOH (9:1)	143-152	$CHCl_3$ AC (6:4)	Brown	4
XIV	$CHCl_3$ MeOH (8:2)	153-164	$CHCl_3$ (5:5)	Brown	4
XV	$CHCl_3$ MeOH (8:2)	165-176	$CHCl_3$ AC (5:5)	Brown	4
XVI	$CHCl_3$ MeOH (6:4)	177-190	$CHCl_3$:AC:EtoH (3:2:3)	Brown	5
XVII	$CHCl_3$ MeOH (5:5)	191-214	$CHCl_3$:A:EtOH (2:2:4)	Brown	6
XVIII	$CHCl_3$ MeOH (4:6)	215-227	$CHCl_3$:A:EtOH (3:2:3)	Brown	6
XIX	$CHCl_3$ MeOH (3:7)	228-243	$CHCl_3$:AC:EtoH (3:2:3)	Brown	4
XX	$CHCl_3$ MeOH (2:8)	244-250	$CHCl_3$:A:EtOH (2:2:4)	Brown	4
XXI	$CHCl_3$ MeOH (1:9)	250-259	$CHCl_3$:A:EtOH (2:2:4)	Brown	3
XXII	MeOH	260-263	MeOH:AC:n-But (4:2:3)	Brown	3
XXIII	MeOH	264-265	MeOH:AC:n-But (4:2:3)	Brown	Nil

KEY
Nil = no spots

On evaporation of solvent and work-up procedures from XXII, needle shaped crystas were formed. This were recrystallized several times in methanol to obtain C_1 (2mg)

3.1.5 Thin Layer Chromatographic Studies of C_1

Thin layer Chromatography using several systems, C1 (Table 3.7) was shown to be homogenous. The structure elucidation was carried out by combination of physical and spectroscopic methods.

Table 3:7 TLC Result of Crystals C_1

Solvent System	Visualization (Colours)				R_f
	Daylight	UV(366)	Iodin vapour	Ferric Choride	
Acetone: $CHCl_3$: PE (7:2:1)	Nil	flouresce (Orange)	brown	Blue Black	0.75
$CHCl_3$: Benzene:Acetone (5:4:1)	Nil	flouresce (Orange)	brown	Blue Black	0.70

3.1.6 Spectroscope studies and other Physical Data of C_1

3.1.6.1 Melting Point

Cystals have sharp melting point of 139 - 140°C

3.1.6.2 Solubility

This was determined and results represented in table 3.9

Table 3.8 Solubility of C_1 in different organic solvents

Solvent	Solublity
Methanol	+
Choloform	+
Petroleum ether	-
Acetone	+
Water	Sparingly Soluble

KEY

\+ = Soluble

\- = Insoluble

3.1.6.3 Spectroscopic Studies

This are presented in Tables 2:10-12 and figs 3:1-7

(a) UV (MeOH) 248, and 288nm (fig 3-1)

(b) IR ($CHCl_3$) - Results is summarised in tables 3-9 and spectrum shown in figs 3-2

Table 3.9 IR Spectral data C_1

Absorption frequency (cm^{-1})	
1453	C=C bond (aromatic)
1659	C=C bond (alkenes)
1729	C=O (carboxylic acid)
732	ortho di-substitute aromatic ring
3424	Intermolecular and weakly bonded OH
2954	OH (carboxylic group)
3148	Aryl C-H

C) **Proton Nuclear Magnetic Resonance (^{1}HNMR) Spectrum is shown In Fig 3-3 and Result Summarized in Table 3.10**

TABLE 3.10 **^{1}H-NMR ($CDCl_3$) Spectral data of C_1 (400MHz, TMS)**

Carbon Number	Chemical shift δ (ppm)	Proton C – H assignment
1	-2 (S)	- COOH
2	7.0 (d)	- CH
3	6.9 (t)	- CH
4	7.1 (d)	- CH
5	7.4 (t)	- CH
1^1	-	- C
2^1	-	- C
3^1	8.1 (d)	- CH
4^1	7.9 (d)	- CH
5^1	7.6 (t)	- CH
6^1	7.5 (t)	- CH
7^1	2.3 (br,s)	- OCH_3

KEY:

S = Singlet

d = Dublet

t = triplet

br = broad

d) Carbon – 13 Nuclear Magnetic Resonance (^{13}C-NMR)

Spectrum is shown in fig 3-4 and result summarized in table 3-11

TABLE 3.11 ^{13}C-NMR Spectral data of C_1 (400MHz,$CDCl_3$ TMS)

Carbon Assignment	Chemical shift δ (ppm)	
1	169.9	- COOH
2	126.3	- CH
3	126.1	- CH
4	122.1	- CH
5	117.6	- CH
1^1	162.0	- C
2^1	151.1	- C
3^1	136.5	- CH
4^1	134.5	- CH
5^1	132.4	- CH
6^1	130.9	- CH
7^1	20.39	- OCH_3

e) Two dimensional proton NMR ^{1}H-^{1}H-2D (COSY) of C_1 spectrum is shown in fig 3-5

f) Two dimensional proton - ^{13}C NMR (CH 2D) of C_1, spectrum is shown in fig 3.6

g) Carbon- 13 Nuclear magnetic Distortionless Enhanced Polarization Transfer (DEPT) spectrum is shown in fig 3.7.

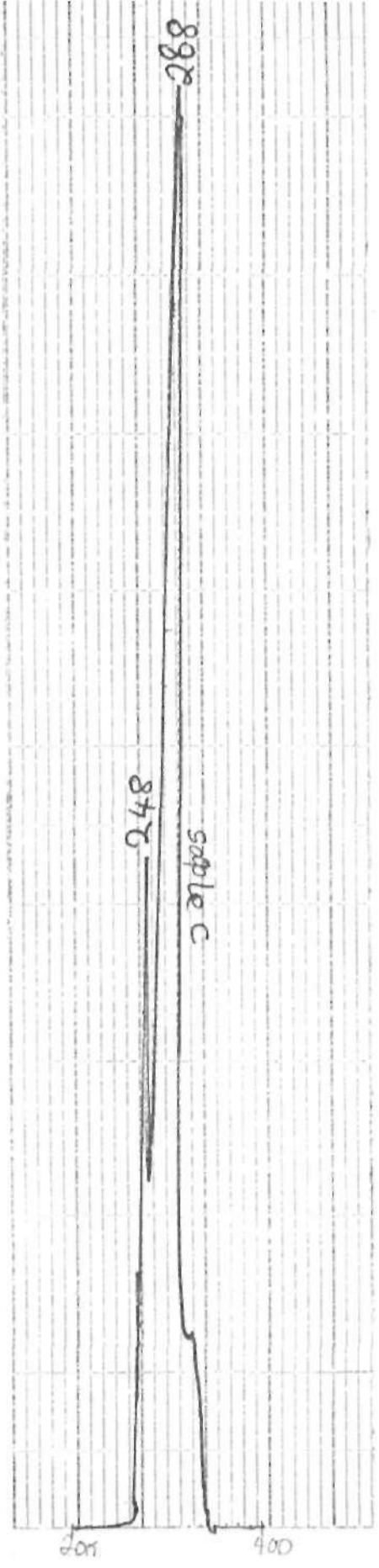


Fig 3 - 1: Ultraviolet Spectrum of C_1

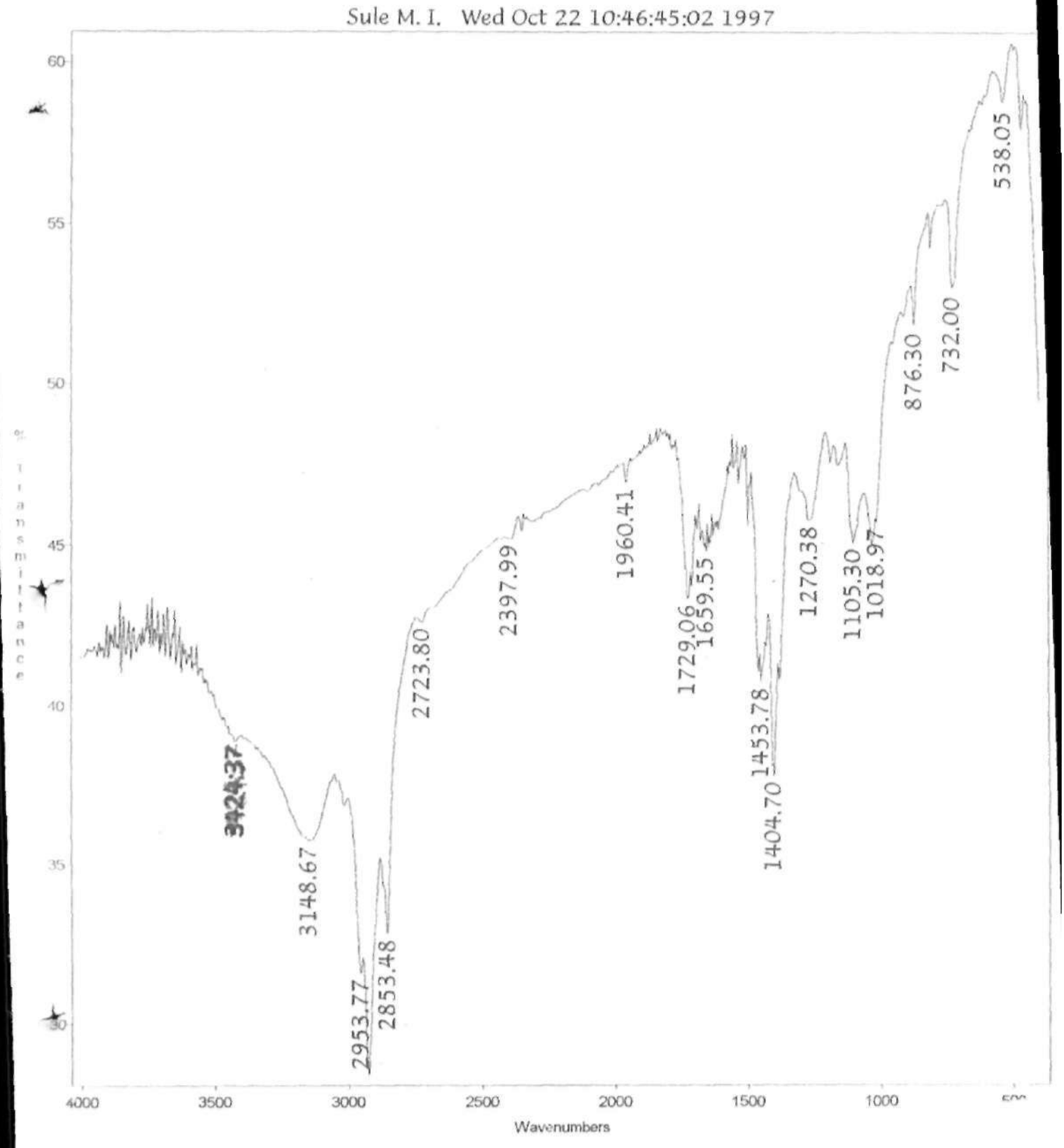


Fig 3 - 2: Infrared Spectrum of C_1

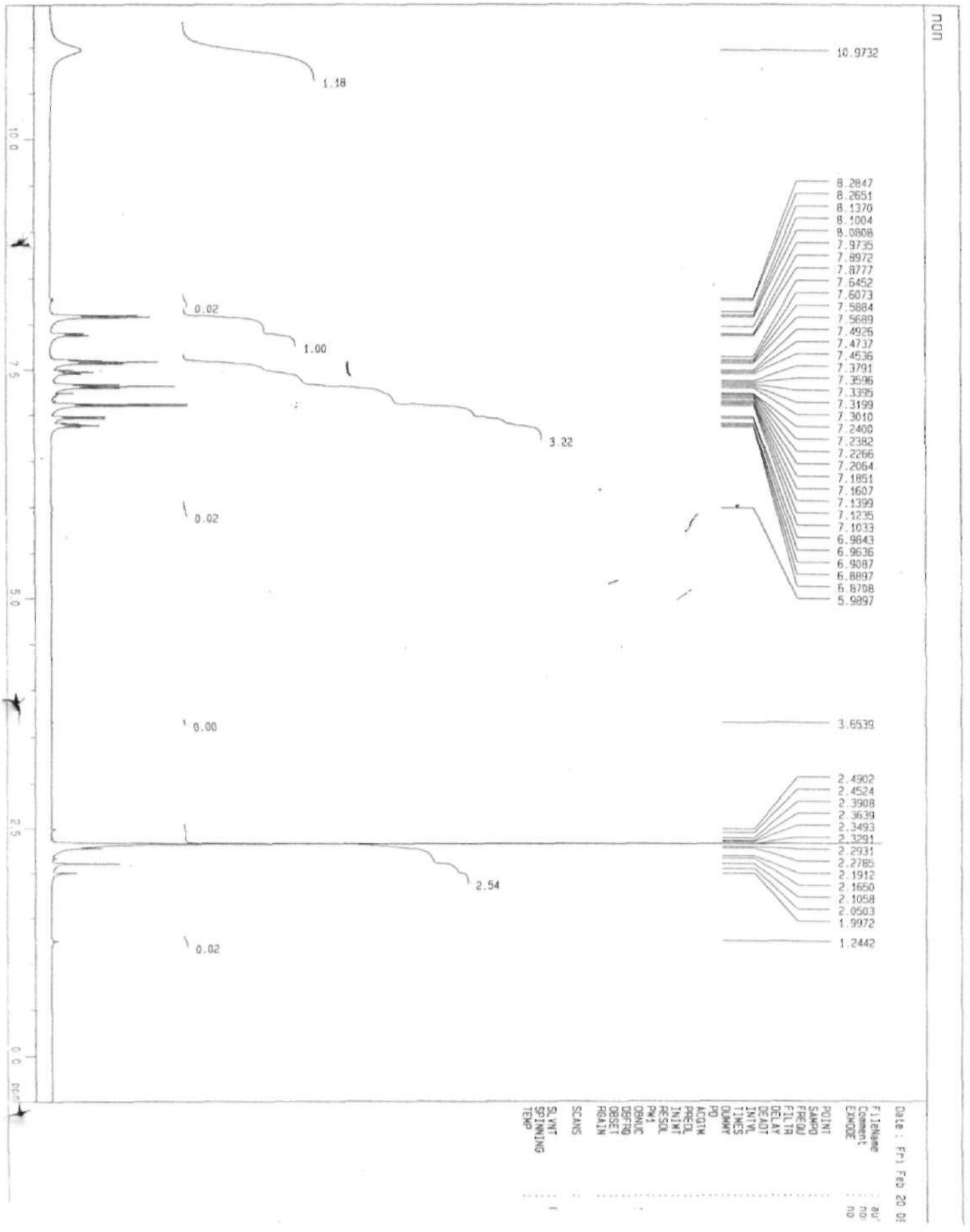


Fig 3 - 3: Proton Nuclear Magnetic resonance (^{1}H-NMR) Spectrum of C_1

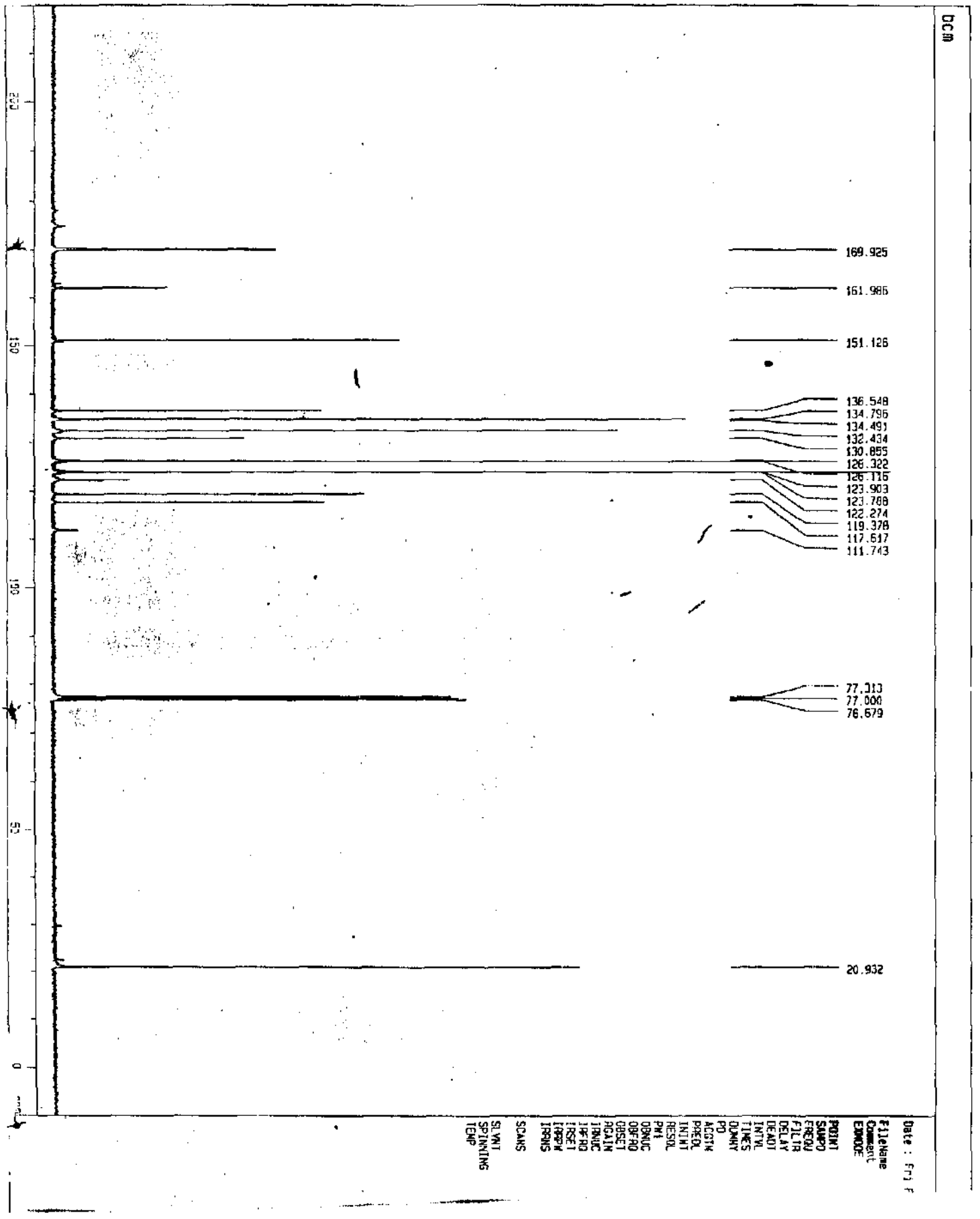


Fig 3 - 4: Carbon - 13 Nuclear Magnetic resonance (^{13}C-NMR) Spectrum of C_1

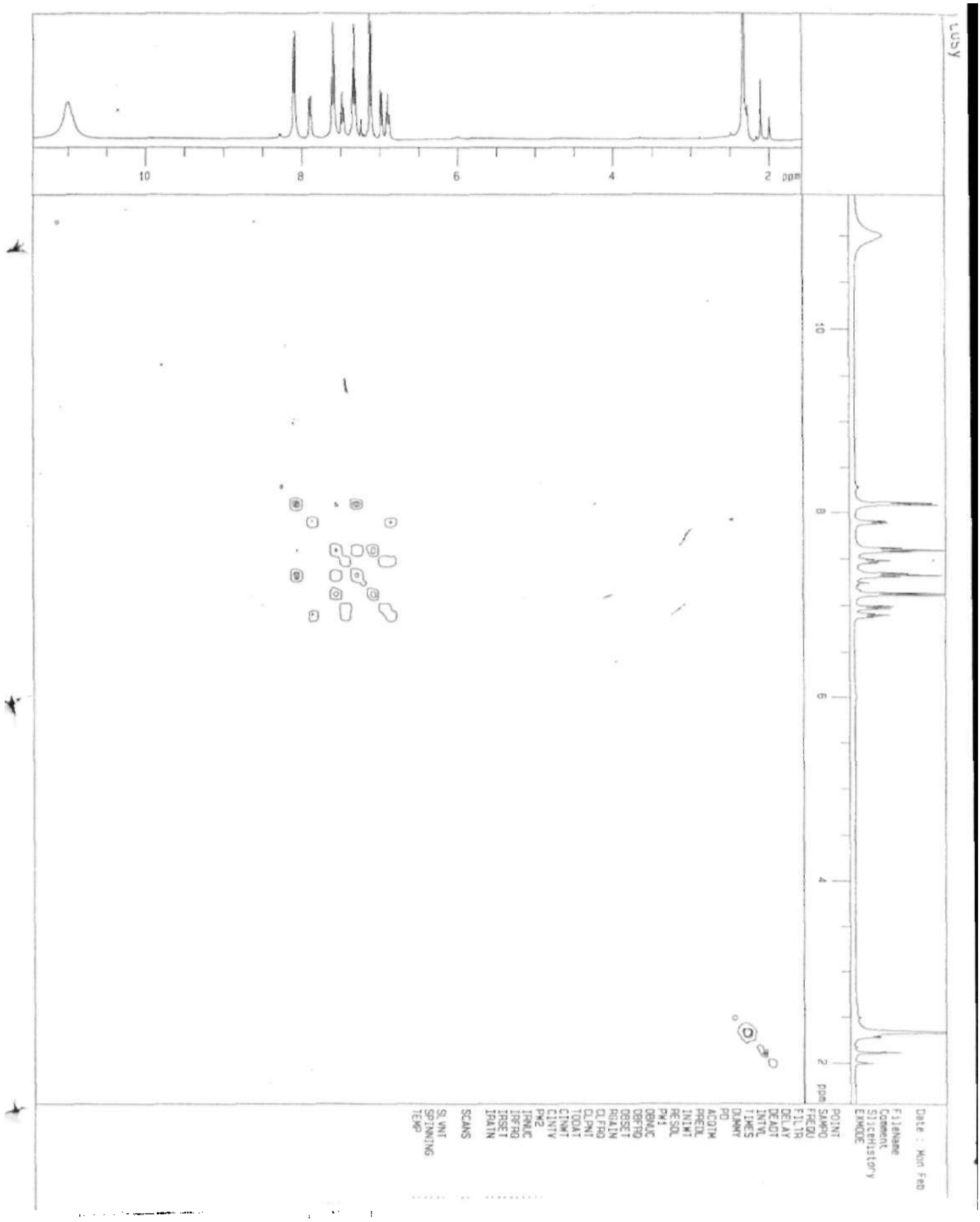


Fig 3 - 5: ^{1}H - ^{1}H COSY 2D-NMR spectrum of C_1

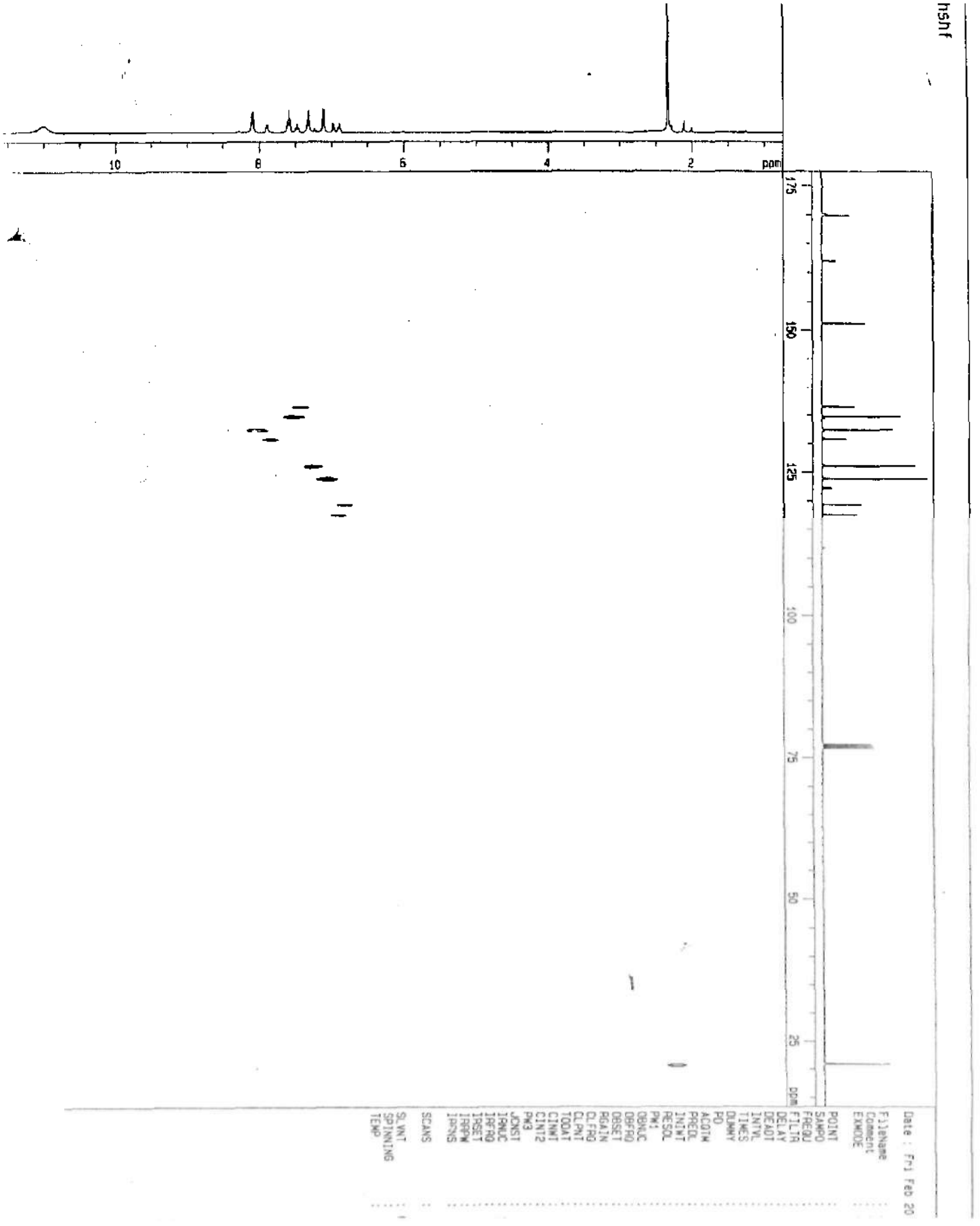


Fig 3 - 6: CH, 2D-NMR Spectrum of C_1

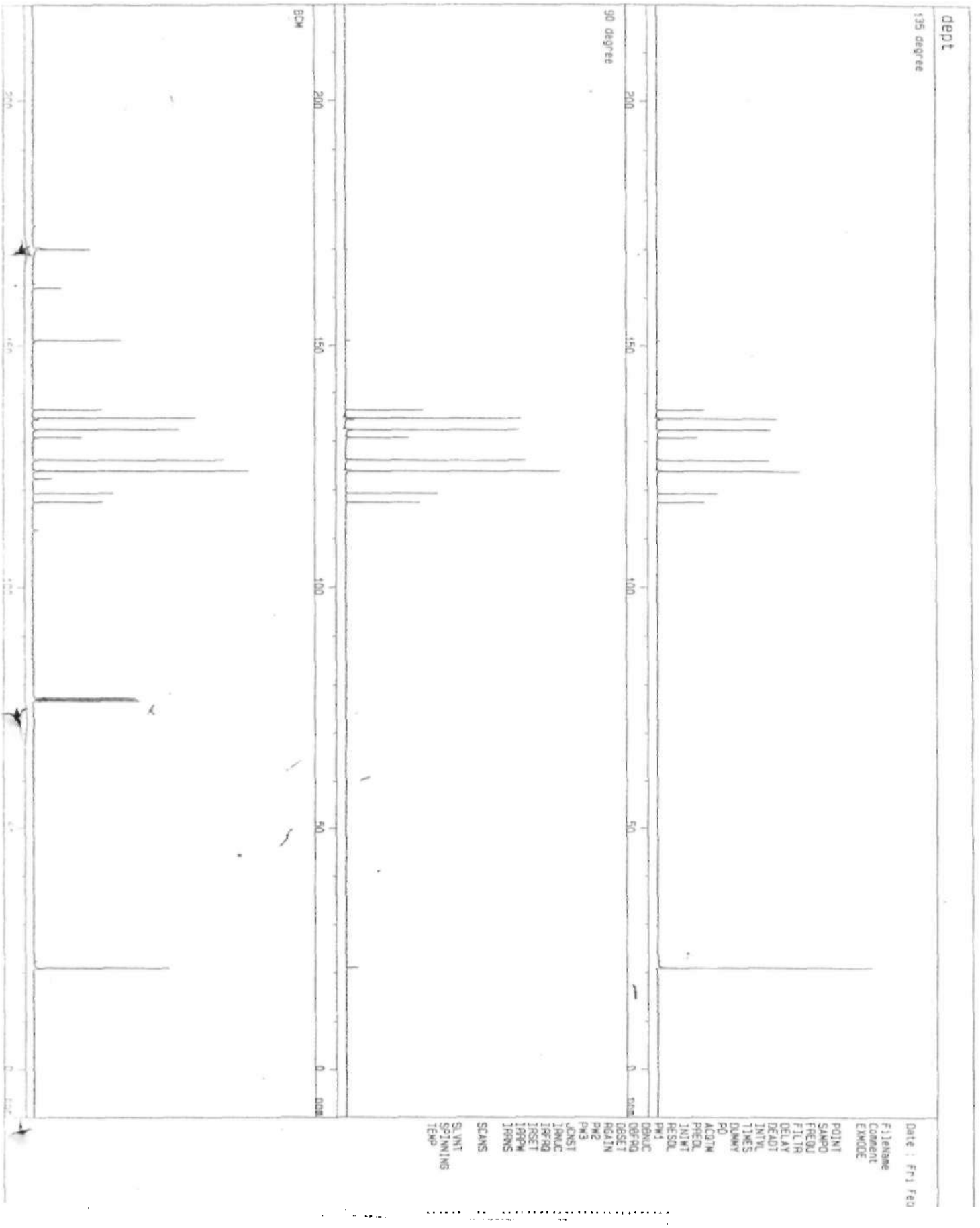
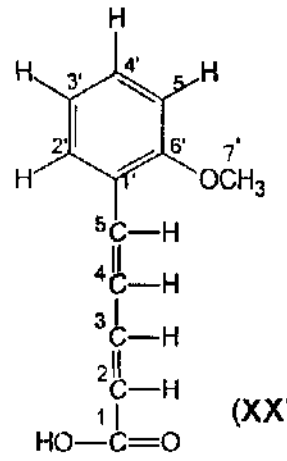


Fig 3 -7: Carbon - 13, DEPT Spectrum of C_1



(XXVII)

5 (*o*-methoxy phenyl)-pent-2,4- dienoic acid

3.2 PHARMACOLOGICAL SCREENING OF THE METHANOLIC EXTRACT OF THE LEAVES OF *OLAX MANNI*

3.2.1 Materials and Method as described in Chapter 2.2.1

3.2.2 Invivo Anti snake Venom activity of the methonolic extract against the venoms of *N. nigricollis* and *E. carinatus*

(1) Determination of LD_{50} and LD_{99} of the venom of *N. nigricollis*

Pilot studies to determine the maximum and lowest tolerated doses of the venom gave 8 mg/kg and 4 mg/kg respectively. Five doses were thus chosen: 4,5,6,7 and 8 mg/kg for the LD_{50} and LD_{99} determination.

Intraperitoneal injection of the venom showed increasing toxicity (lethality) with increasing dose. Number of animals dead per group was recorded (table 3.12) other signs of toxicity observed are respiratory distress and paralysis.

Table 3.12 **Showing lethal effect on group of mice injected (ip) with different doses of lyophillised venom of N nigricollis**

Group	Dose mg/kg	Number of death	% Death	Average Time of Death
I	4	0/15	0	-
II	5	3/15	20	24 hrs.
III	6	9/15	60	20 hrs
IV	7	12/15	80	20 hrs
V	8	15/15	100	18 hrs
VI	N/SALINE	O/15	O	-

N = 15

The % death was converted to probit and the log of the various doses of the venom taken as shown in table 3.13.

Table 3.13 LD_{50} and LD_{99} calculation by probit analysis method for *N. nigricollis* venom

DOSE	LOG DOSE	% DEATH	PROBIT
4	0.602	0	-
5	0.698	20	4.1
6.	0.778	60	5.2
7	0.854	80	5.8
8	0.900	100	-

The above probit values were plotted against log dose (fig 3-8) LD_{50} and LD_{99} were determined from the graph as follows:

LD_{50} = 5.75 mg/kg

LD_{99} = 9.55 mg/kg

(ii) Determination of LD_{50} and LD_{99} determination of the venom of *Echis carinatus*.

Pilot studies to determine the maximum and lowest tolerated doses of the venom gave 4 and 2mg/kg respectively. Five doses were thus chosen - 2.0, 2.5, 3.0, 3.5, 4.0 mg/kg for the LD_{50} and LD_{99} determination.

Intraperitoneal injection of the venom showed increasing toxicity (lethality) with increasing dose. Other signs of toxicity observed are necrosis at site of administration of venom (Plate 3 - 1), sedation, respiratory distress. Number of animals dead per group was recorded (table 3-14).

Table 3 - 14: Showing lethal effect on group of mice injected (ip) with different doses of lyophillised venom of *Echis carinatus*

Group	dose (mg/kg)	Number of death	% Death	Average time of Death
I	2.0	0/15	0	
II	2.5	3/15	20	24 hrs
III	3.0	6/15	40	22 hrs
IV	3.5	9/15	60	20 hrs
V	4.0	15/15	100	10 hrs
VI	N/SALINE	O/15	-	

N = 15

The % death above was converted to probit and the log dose of the various doses of the venom taken as shown in table 3.15.

The probit values were plotted against the Log dose (fig. 3.9) and the LD_{50} and LD_{99} were determined from the graph to be as follows:

LD_{50} = 3.09 mg/kg, LD_{99} = 4.8 mg/kg.

Table 3.15 LD_{50} and LD_{99} calculation by probit analysis method for *E. carinatus* venom.

DOSE	LOG DOSE	% DEATH	PROBIT
2.0	0.30	-	-
2.5	0.397	20	4.15
3.0	0.48	40	4.75
3.5	0.54	60	5.25
4.0	0.60	100	-

(iii) Determination of Invivo antivenin Activity of Methanolic Extract of *Olax manni*

(a) Determination of Maximum Tolerated Dose of the Methanolic Extract of *Olax manni.*

Intraperitoneal injection of the extract showed increasing toxicity (lethality) with increasing dose.

Other signs at toxicity observed includes excitation followed by sedation, respiratory distress. Number of animals dead per group were recorded (table 3.16).

Table 3.16 Toxic effect of methanolic extract of *Olax manni* on Swiss Albino mice.

GROUP	DOSE (g/kg)	NUMBER OF DEATH	% DEATH	AVERAGE TIME OF DEATH
I	O.1	0/15	-	-
II	0.2	0/15	-	-
III	O.3	0/15	-	-
IV	0.4	0/15	-	-
V	O.5	5/15	33	24 hrs.
VI	0.6	10/15	67	20 hrs.
VII	0.7	15/15	100	18 hrs.
VII	0.8	15/15	100	15 hrs.
IX	0.9	15/15	100	13 hrs.
X	1.0	15/15	100	12 hrs.

N = 15

Plate 3 - 1 Necrotic region at site of administration of venom of *E. carinatus* in Swiss Albino mice.

(b) **Determination of protective Activity of the Methanolic Extract of *Olax manni* venomenated with LD_{99} of *Naja nigricollis* and *Echis carinatus* Venoms.**

Decreasing lethality was observed with increased dose of extract especially in mice envenomenated with the LD_{99} venom of *Naja nigricollis.* This are presented in table 3.17.

Table 3.17 **Showing protective effect on different group of mice protected by different doses of the methanolic extract of *Olax manni* and then challenged separately with lethal doses of the venoms of *N nigricollis* and *E carinatus***

Experime -ntal Group	Dose of extract g/kg	Envenomenation with LD_{99} of Naja nigricollis.		Envenomenation with LD_{99} of *Echis carinatus.*	
		Survival	% survival	Survival	% Survival
I	0.1	6/15	40	3/15	20
II	0.2	9/15	60	6/15	40
III	0.3	12/15	80	9/15	60
IV	CONTROL	0/15	0	0/15	0

N = 15

The log dose of the extract were taken and the percentage survival plotted against the log dose of the extract. This are shown in figures 3.10 - and 3.11.

From the graphs, the dose that protect fifty percent of mice (effective dose-ED_{50}) f

ED_{50} of extract against venom of *N. nigricollis* = 0.41g/kg

ED_{50} of extract against venom of *E. Carnatus* = 1.45g/kg

Fig. 3.8: Plot of Probit against log dose of venom of *Naja nigricollis* to determine LD_{50} and LD_{99}

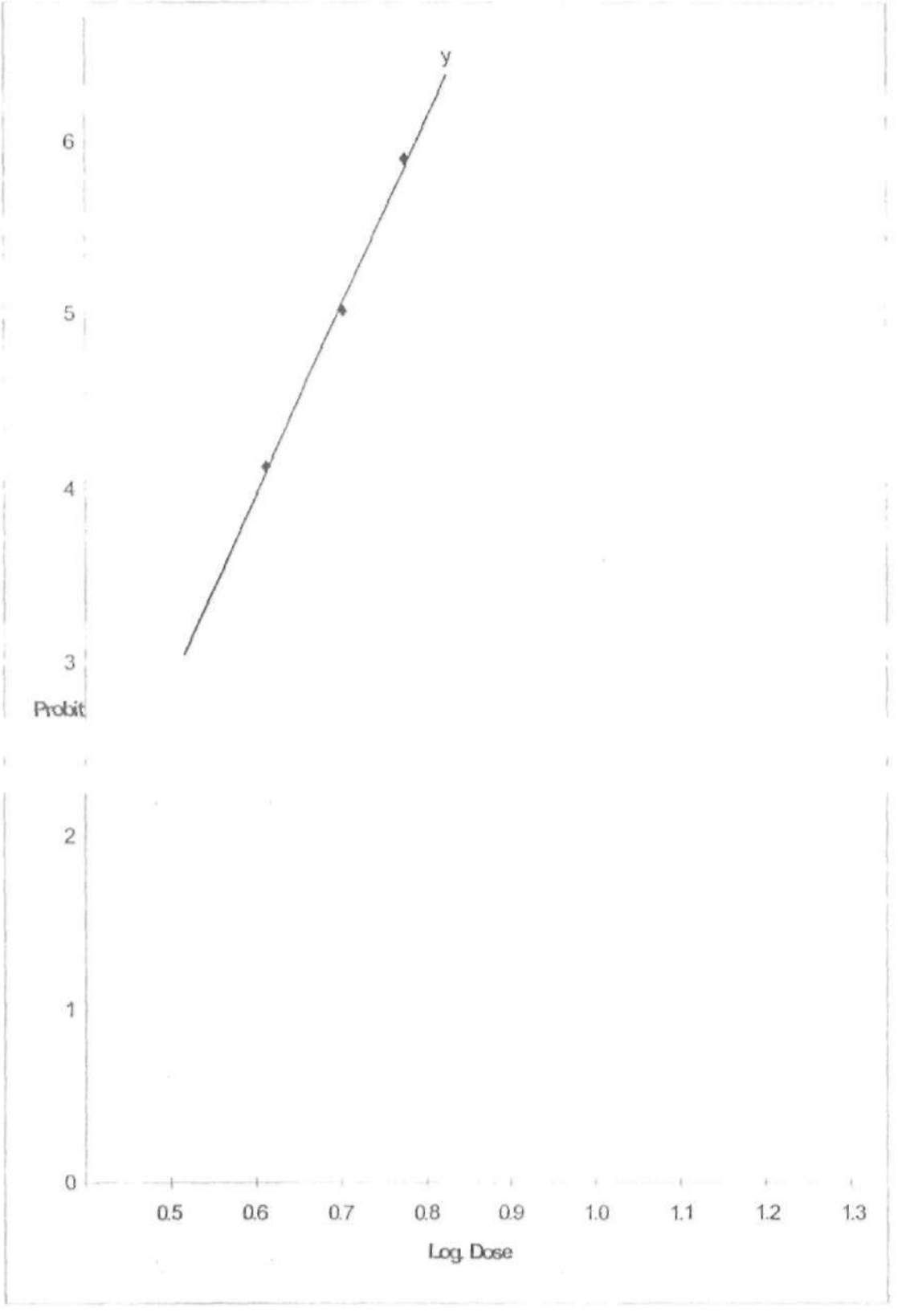


Fig. 3.9: Plot of Probit against log dose of venom of *Echis carinatus* to determine LD_{50} and LD_{99}

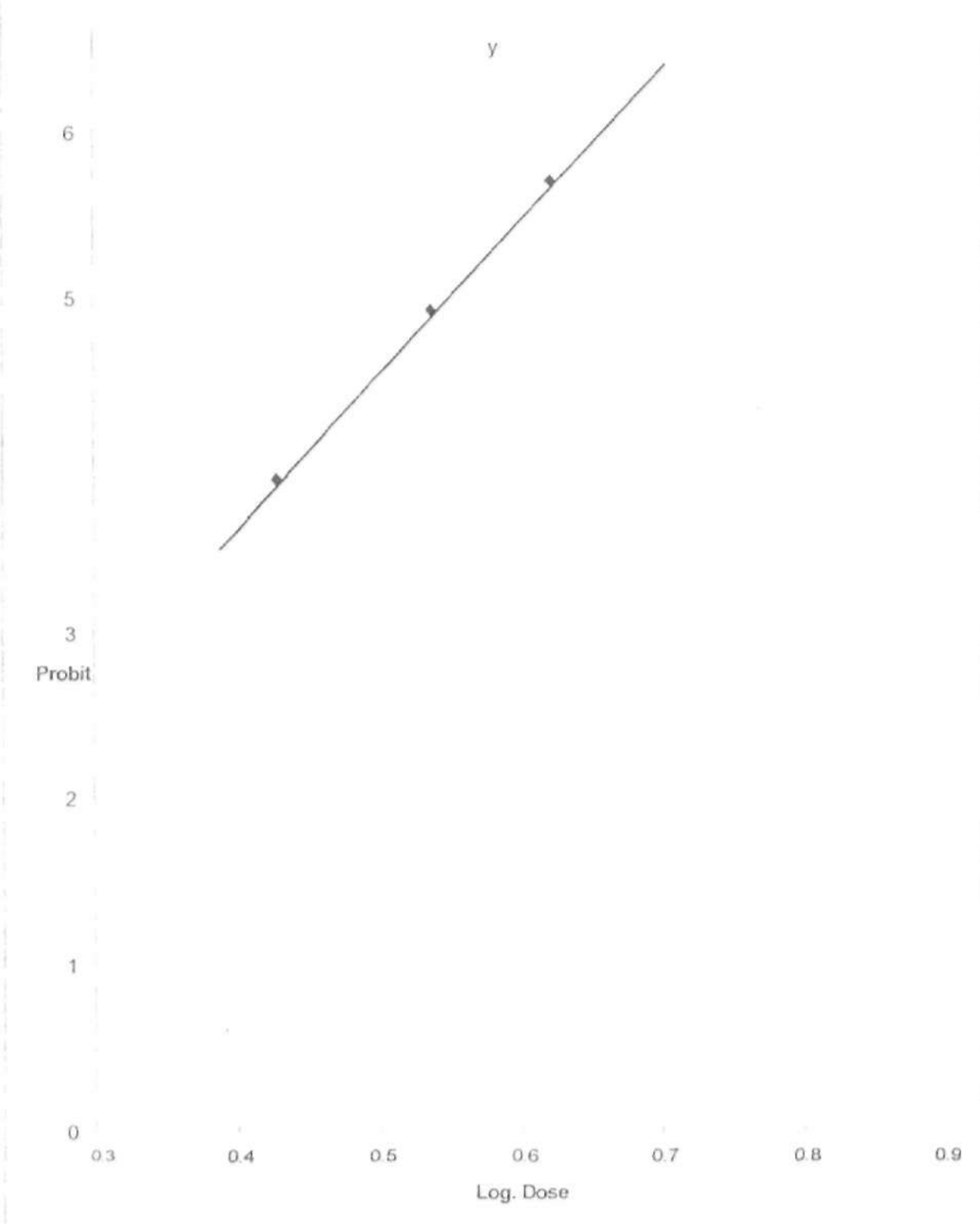


Fig. 3.10: Plot of Percentage survival against log dose of extract after envenomenation with LD_{99} of venom of *Naja nigricollis*

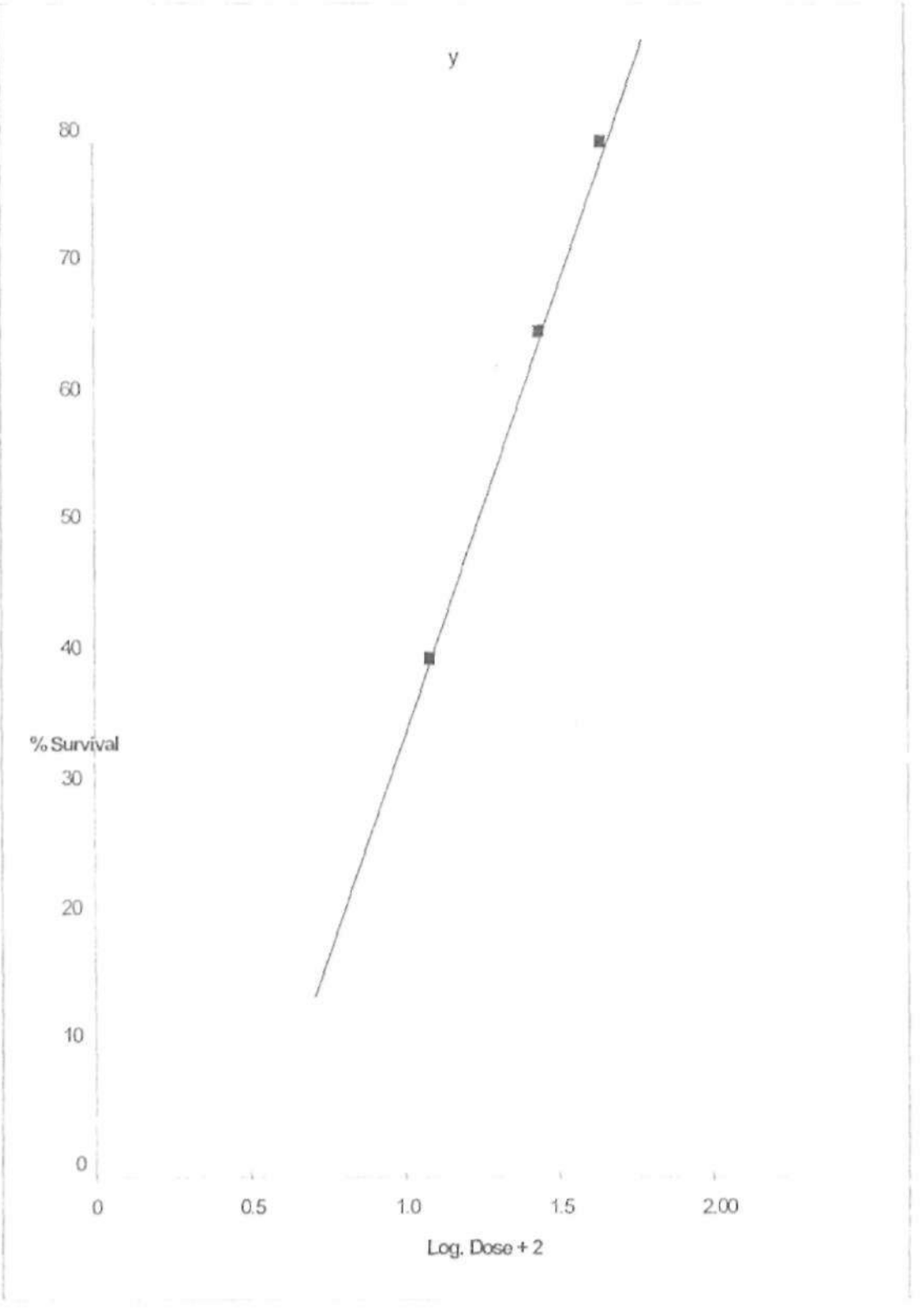
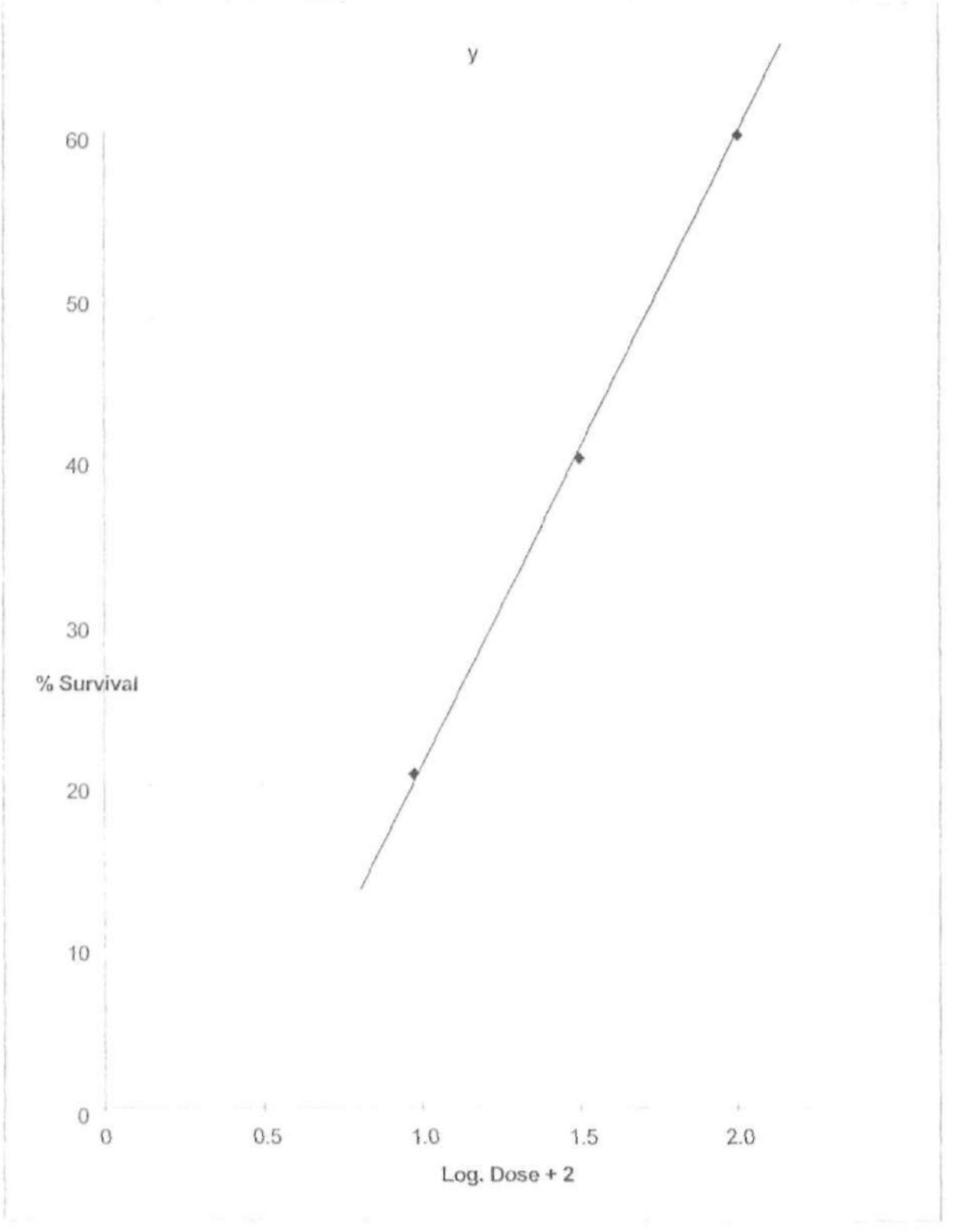


Fig. 3.11: Plot of Percentage survival against log dose of extract after envenomenation with LD_{99} of venom of *Echis carinatus*



3.3 DISCUSSION

3.3.1 Phytochemical Studies of Extracts of *Olax manni.*

The extraction procedures of ciulei (1981) was adapted. Highest yield was given by methanol (4.2% due to high penetration of methanol into plant tissues (cannel 1998)

The chemical constituent of plants are known to be influence by season, age and geographical location (Watt and Breyer Brandwijk 1962) variation also occur in distribution of constituent in different organs of the same plant.

In this work, the three various extracts, petroleum ether, methanol and water extracts were screened. Photochemical products seen are similar to those reported in other plants in the same genus and family. e.g. tannins as in *Ximenia americana* (Olacaceae), fatty acids in *Heisteria pervifolia* (Olacaceae) and saponins in *Olax subscorpoidea* (Olacaceae) (Irivine 1961, Mabry 1963).

The absence of alkaloid and alkaloidal salts is indicated by the negative test with Dragendorff's, Mayer's and Wagner's reagents. Presence of saponins and free carboxylic acid indicated confirms the acidity observed with the methanol and aqueous extracts.

The bicarbonate test indicated the presence of fatty acids. Eluent xxii (fraction 260 –263) from the methanol region gave almost a single spot (major) on thin layer chromatography. This fraction was worked-up to afford a

colourless crystal coded 'C' (mp – 139 - 140^{0}C) which on repeated recrystalization gave c_1 (mp 139 – 140).

On TLC, C_1 was found to be a pure compound and was thus subjected to other analytical procedures, UV, IR, ^{13}C ^1H – NMR and ^{13}CNMR, DEPT. From the various chemical test carried out on C_1 (turns blue black with ferric chloride, reacts with bicarbonate) and the spectral data C_1 was deduced to have the structure **XXVII**

The two absorption peaks in the UV spectrum implies the presence of two chromophoric groups. This are the aromatic ring and the carbonyl functional group from the carboxylic moiety (see structure **XXVII**).

The IR spectrum, absorption peaks at 732 cm^{-1} shows a *ortho* disubstituted aromatic ring. The C=C stretching is indicated by the absorption at 1453 cm^{-1} and C-H str at 3148 cm^{-1}. C=O group in the carbonxylic is indicated by the absorption at 1729cm^{-1} and 3424 cm^{-1} indicates OH due to intermolecular hydrogen bonding.

The ^{13}C -NMR indicates the presence of 12 C - atoms. Out of this three C -atoms are quaternary and shows signal at 151.12, 161.99 and 169.93 ppm. The aryl and the alkene C - atoms all show signals between 102 - 170 ppm which is the normal range for this C - atoms. The methoxy C atom show absorption of 20.93.

In the proton NMR, (^{1}H-NMR,) the protons on the methoxy group shows a broad singlet at 2.54 ppm. The protons on the benzene ring and the

alkene side chain are indicated by the various signals doublets, triplets and all fall within their normal range, 6.0 - 8.5.

Suggested structure, **XXVII** also conforms with the ^{1}H-^{1}H two dimensional and C-H two dimensional - NMR spectra.

The number of protonated carbon atoms in structure XXVII conforms with the Carbon -13 DEPT Spectrum.

3.3.2 ANTI SNAKEVENOM STUDIES

The methanolic extract of the leaves of ***Olax manni*** was found to posses antivenin activity on both venoms of *E carinatus* and *N nigricollis* Swiss albino mice preteated with extract followed by venom challenge (LD_{99}) were found to survive more than the mandatory 24 hours survival period (as suggested by Theakson and Reid 1983) while animal that do not received any treatment with the methanolic extract died before 24 hours. This suggest an *in vivo* antivenin effect which is dose dependent (Table 3-6). A ntivenin effect was more pronounced with venom of *Naja nigiricollis* than the venom of *Echis carinatus* (Table 3-1 and 3-3). The antivenin effect may be due to the chemical constituent in the plant. The phytochemical screening of the methanolic extract revealed the presence of tannins, steroids and other phenolic compounds (Table 3.2) it is known that the virulencey of snake venom is due to the protein and enzyme components of the venoms (TU) 1987). Plant constituents, that these may have beneficial effects on snake envenomentation.

Tannins and other phenolic compounds are known to precipitate proteins and affect enzyme activity. The of tannins in plants may have some effect on venoms.

The *in vitro* activity of tannins against venoms has been demostrated by Takashi and Kunio 1987. This effect may be possible *in vivo*. However, the venoms of Elapidae have high molecular weight (TU1987). This may help to explain why the methanolic extract is more protective against the venom of *N nigricollis* than the venom of *E carinatus* as tanins efficiently affect low molecular weight than high molecular weight proteins (Takashi and Kunio 1987).

Steroids have inflammatory activity (foye, 1976). Oyedapo et al has also reported the anti inflammatory effect of the methanolic extract of *Olax subcorpoidea* (a plant of same genus with *Olax manni*) on bovine red blood cell. The presence of steroids in methanolic extract of *Olax manni* may be beneficial in snake envenomenation. Inflammatory responses are one of the immunological effect of snake venoms which may be neutalised by steroids. In addition, Steroids are a component of treatment of snake envenomenation (Warrel. 1993).

Plant are known to poteniate the immune system (immuno stimulants) . this effect is utilised in snake envenomenation)walter 1997). Although this effect by *Olax manni* may not be ruled out. There is needs to subtantiate this effect.

Another theory advanced to prove mechanism of action of extracts is competitive antagonism at receptor sites (Haruna 1995). Some specific receptors have been identified where some attacks and cause toxic action.

So it has been postulated that some constituent of the extract blocks these receptors and prevent snake venoms access to these receptors to cause toxic and lethal action.

The use of the plants in snakebites may provide an alternative source of cheap and reliable treatment of snake envenomenation.

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