PHYTOCHEMICAL AND MICROBIAL SCREENING OF *EUPHORBIA hirta LINN.*

BY

M1KA, AMINA (B.Sc. A.B.U.)

A TMESIS SUBMITTED TO THE POST-GRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF REQUIREMENT FOR THE AWARD OF MASTER OF SCIENCE ORGANIC CHEMISTRY

DEPARTMENT OF CHEMISTRY

AHMADU BELLO UNIVERSITY

ZARIA

JUNE, 1999

DECLARATION

I hereby declare that this thesis contains the report of my research work and has not been presented in any previous application for higher degree. All sources of information used for this write-up and where applicable other writers' views have been duly acknowledged by means of references.

Amina

MIKA, Amina

08-06-99

Date

CERTIFICATION

This thesis titled "Phytochemical and Microbial Screening of *Euphorbia hirta Linn*", meets the regulation governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, Nigeria, and is approved in its contribution to knowledge and literary presentation.

Dr. A. O. Oyewale
Chairman Supervisory Committee — Date: 19/7/99

Dr O. A. Peters
Member Supervisory Committee — Date: 18-8-99

Dr(Mrs) E. Agbaji
Internal Examiner — Date: 19/7/99

Dr. J.M. Nwaedozie
External Examiner — Date: 19/7/99

Dr. J.O. Amupitan
Head of Department — Date: 27.8.99

Professor S.B. Ojo
Dean, Postgraduate School — Date: 18/12/01

DEDICATION

This work is dedicated to God Almighty to whom I owe all my being and success.

ACKNOWLEDGEMENT

My profound gratitude goes to my supervisors Dr. A. O. Oyewale and Dr. Femi Peters for their guidance, encouragement, advises, help with chemicals and practical instrument, and for given me their time. I am also highly indebted to the Head of Chemistry Department, A.B.U. Zaria, Dr. J. O. Amupitan for his numerous assistance to see that this work comes to a successful conclusion.

I am very grateful to all the lecturers of Chemistry Department for their instructions to me in my first and second degrees. Space will not allow me to mention all their names here, but they include:- Dr. J. O. Amupitan, Prof. J. F. Iyun, Assoc. Prof. (Mrs.) E. Agbaji, Prof. J. Y. Olayemi, Dr. E. J. Ekanem, to mention a few. My special gratitude goes to Dr. J. O. Adigun, Mr. C. E. Gimba, Mr. James Kagbu, Dr. N. Y. Lohdip (from University of Jos), Mr. O. T. Audu and Mr. Musa A. Audu for their encouragement, advice, provision of some chemicals and other necessary materials; and even help in some practical aspects of the research work.

Special thanks also goes to all the technical and non-academic staff of the Chemistry Department especially Pr. Silas Ekwueribe, Mr. A. F. Smith, Mr. S. K . Adom, the storekeepers (Malam Salihu Ibrahim and Malam Abubakar Adamu) for their assistance in providing chemicals and working apparatus; and other assistance rendered. Thanks also to Mr. Adamu H. Kachiro, the Departmental Secretary for encouragement. I am also grateful to Mr. M. T. Salawu for encouragement and helping to see that I started this program. The principal of Barewa collge, Zaria in 1994, Alhaji Liman Iyal Adamu and the then staff of Chemistry Department, Barewa College Zaria - Mr. Andrew Nache, Mrs.

Janet S. Dadah and Mr. Billy Zubai are also not left out for the kind understanding and support at the beginning of this programme.

My heart felt gratitude also go to Mr. David Jatau of the Microbiology Department, Ahmadu Bello University, Zaria, for the tremendous assistance he rendered me in carrying out the antimicrobial tests. Thanks also to Mr. M. Musa of the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, for helping to identify the plant used for this research.

Special thanks also to Rev. Fr. (Dr.) Mathew Hassan Kukah, Alhaji Abullahi Ibrahim (of Kaduna State Polytechnic, Zaria), my uncle (Mr. Ishaku Dodo) and his wife (Mrs. Esther Dodo), for their encouragement and financial support.

I also wish to express my appreciation to the management of Kaduna State Polytechnic, Zaria especially the rector (Alhaji Suleiman Aliyu) and the Deputy Rector (Dr. Solomon A. Yabaya) for encouragement and financial support. Thanks also to the Kaduna State Government for sponsoring my secondary school education and first degree without which this program would not have been possible.

My gratitude also goes to the Head of Science Laboratory Technology Department of Kaduna State Polytechnic, Zaria (Mrs. R. Balarabe) and the entire staff of the chemistry section, especially Mr. Ahmed Garba and Mr. Musa A. Audu for the kind understanding and encouragement.

I am grateful to all the post-graduate students of Chemistry Department, A.B.U., Zaria, from 1994 to date, especially those in the organic chemistry section for all their advises, help in some research areas, chemicals and apparatus. These include Miss Bunmi Bolarin, Mrs R. G. Ayo, Mrs. N. F. Amako and Mr. Zakari Ladan. I am also grateful to

Mr. Yakubu Dalhatu for providing me with some materials when I was in dire need of them.

Some beloved friends who were always by my side praying for me in my times of discouragement include Miss Mercy E. Ogah, Mr.Zephaniah M. Mbwance, Barrister and Mrs. Francis Adamu, Mrs. Christiana Andrew, Mrs. Cecilia Gayya, Mrs. VirginaAdamu and her husband (Mr. Emmanuel Adamu), Mrs. Lydia Buba, Mrs. Swanta, the entire Exco Members of Chapel of Grace, Gaskiya, and the entire members of Full Gospel Business Men's Fellowship International-Wusasa Chapter. Your labour of love shall not go unrewarded by the God almighty. My heart-felt gratitude also to my sisters: Mrs Ladi S. Bala, Mrs Rachel Y. Garba and Mrs Ruth I. Yerima for their support and prayers.

From the depth of my heart, I wish to say, 'thank you' to my Parents - Mr. Mika Dodo and Mrs. Paulina Cecilia Duniya, and my step-mother Mrs. Maryamu Dodo, for the immeasurable supports, the greatest of which is their prayers.

Finally, I wish to also express my heart-felt gratitude to my late uncle, Mr. Dauda Dodo, and his wives Mrs. Alisabetu D. Dodo and Mrs. Mary D. Dodo for coming to my aid financially and supporting me with their prayers during my Secondary School and Advanced Level Education.

ABSTRACT

The dried leaves, stems and roots of *Euphorbia hirta Linn* were powdered and extracted successively with petroleum ether (60-80°C), ethyl acetate, chloroform, ethanol and distilled water, in that order. Preliminary phytochemical screening showed that ethano, and water extracts contained virtually the same classes of compounds; the same was true of the petroleum ether and chloroform extracts. Consequently, only three of the solvents (petroleum ether, ethyl acetate and water) were used for subsequent successive extraction.

Phytochemical screening showed that the plant generally contained saponins, tannins, alkaloics and cardiac glycosides. These classes of compounds are found to be generally present ;n the different parts of the plant, when extracted with a particular solvent. Subsequent extraction, therefore, was done with the whole plant (except the tloral parts.)

Thin layer chromatography (T. L.C.) was performed on the crude extracts of petroleum ether, ethyl acetate and water. These fractions, as well as the crude extracts, were :ested on *Staphylococcus aureus, Pseudomonas aerttginosae, Klebsiella pneunu viae, Escherichia coli* and *Salmonella typhi.* The results for the crude extracts showed that water extract had an appreciable effect on *S. aureus* and *Ps. aerttginosae,* but only minimal inhibition on *E. coli,* and no activity on *S. typhi and Kleb.. Pneumoniae.* The ethyl acetate extract was active on *E. coli, S. aureus* and *Ps. Aerttginosae;* showed minimal inhibition on S. *typhi* and not active on *Kleb. Pneumoniae.* The petroleum ether crude extract showed appreciable activity on S. *aureus, Ps. aerttginosae, and Kleb. pneumoniae,* but minimal activity on *E. coli* and no activity on *S. typhi.* This is the first reported detailed microbial screening of the plant. *Euphorbia hirta Linn.*

Cytotoxicity test was performed with the crude petroleum ether, ethyl acetate and water extracts, using brine-shrimps (Artemia salina) and this is the first report of such study. The LC_{50} values obtained from the cytotoxicity test on the extracts showed that, the petroleum ether extract was highly toxic, while the ethyl acetate and water extract were within safety limits.

The petroleum ether extract, initially partitioned into Ethanol-soluble and Ethanol-insoluble fractions, gave six spots and five spots respectively. The R_f values of these T.L.C. spots are 0.066, 0.400, 0.750, 0.875, 0.954 and 0.993 for the Ethanol-soluble fraction; and 0.086, 0.172, 0.920, 0.951 and 0.993 for the Ethanol- insoluble fraction.

The functionality spray test, colour reactions with ammonia gas, blood haemolysis test and their spectral data couple with the antimicrobial test, on the water extract fractions (W) and the ethyl acetate extract fractions (EA), showed that; Fraction W_1 was a saponin and was active on *Ps. aeruginosae* and *S. aureus*; Fraction W_2 did not respond to any of the tests and was not active on any of the microorganisms; Fraction W_3 was a hydrolysable tannin and was not active on any of the microbes; Fractions EA_1,EA_3, EA_4 and EA_5 were condensed tannins and were all active on *E. coli*, *Ps. aeruginosae*, *S. aureus* and *S. typhi*; EA_6 did not respond to any of the functionality test, but was active on *Ps. aeruginosae*, *E. coli* and *S. typhi* and not active on *S. aureus*.

The NMR spectra of the various plant extracts were taken at 60MHz (which is the only one currently assessable) but did not give any useful information which can aid in the structural elucidation of the components of the fractions.

TABLE OF CONTENTS

Chapter Two

Chapter Three

Chapter Five

List of Tables

List of Plate

List of Figures

List of Appendices

CHAPTER ONE

1.0 INTRODUCTION

Phytochemical screening of plant forms an important part of natural product chemistry. Phytochemical screening of plants is carried out for bioactive agents. There are several reasons for the growing research interest in this area of chemistry. One of the reasons is that research in this field is likely to result in the discovery of patentable and industrially exploitable compounds for drug development. Another reason for screening for bioactive agents is that by isolating such an agent it is possible to demonstrate that the reported physiological activity of the plant is real. Phytochemical screening also makes detailed pharmacological and other academic studies possible as it offers information of a particular chemical compound responsible for some said activities in a given plant (Sofowora, 1982).

Steps involved in phytochemical analysis include: extraction of the plant of interest, isolating the constituents, characterization, investigation of the biosynthetic pathway of isolated compounds of interest, and testing for bio-activity (Trease and Evans, 1989).

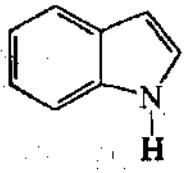
Results from phytochemical analysis have shown that general classes of compounds found in plants which are of medicinal interest include alkaloids, cardiac glycosides, saponins, tannins, anthraquinones, etc. (Sofowora, 1982).

1.1. CLASSES OF COMPOUNDS OF PHARMACOLOGICAL IMPORTANCE.

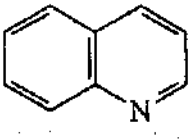
1.1.1. ALKALOIDS

Alkaloids originally meant "alkali-like compounds" and formerly applied to most naturally occurring basic substances. Typical alkaloids contain one or more nitrogen atoms usually in a heterocyclic ring, and have a marked physiological action on man or other animals. Some compounds can, however, be classified under alkaloids not necessarily based on chemical structure but more on pharmacological action (Trease and Evans, 1989b).

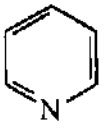
They can be classified based on the types of heterocyclic group they possess. These classes include, indole alkaloids, quinoline alkaloids, pyridine alkaloids, pyrollidine alkaloids, isoquinoline alkaloids etc. (Pelletier, 1970). These heterocyclic groups are represented by structures **(I-VI)** respectively.



Indole **(I)**

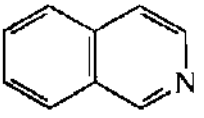


Quinoline **(II)**

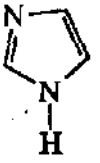


Pyridine **(III)**

Pyrolidine **(IV)**



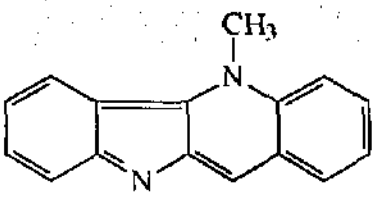
Isoquinoline **(V)**



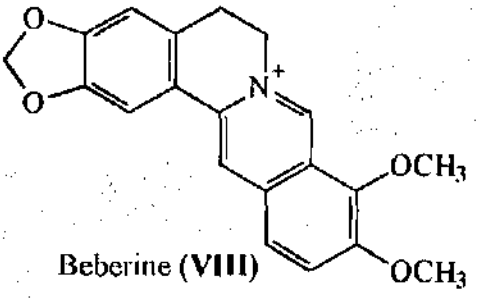
Imidazole **(VI)**

Literature shows that some alkaloids exhibit pharmacological activities. For instance, the indoloquinoline alkaloids, crytolepine **(VII)** showed hypotensive and

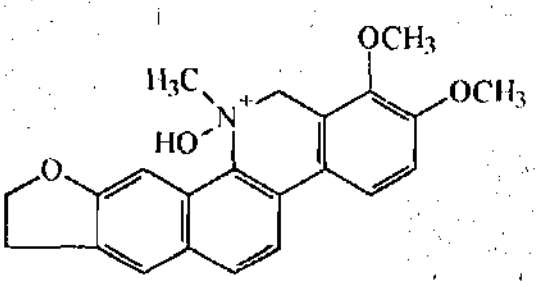
antipyrectic effect on dogs' (Raymond-Hammet, 1937,) and antimicrobial activity against selected gram-positive and gram-negative organisms (Boakye-Yiadom, 1979; Boakye-Yiadom and Heman-Ackha, 1979). Several amide alkaloids have been shown to have antimicrobial, anticonvulsant, antihypertensive, sedative, tranquillizing and insecticidal properties (Adae-Mensah *et. al.*, 1979). Berberine, chelerythrine and canthin–6–one, (**VIII**, **IX** and **X** respectively), have been reported to possess antimicrobial activities (Odebiyi and Sofowora, 1979).



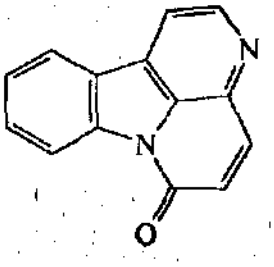
Cryptolepine (**VII**)



Beberine (**VIII**)



Chelerythrine (**IX**)

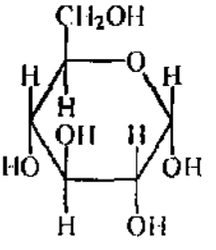


Canthin-6-one (**X**)

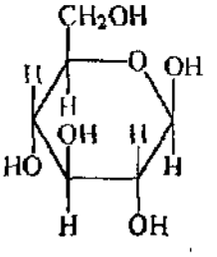
1.1.2. CARDIAC GLYCOSIDES

The term "Glycosides" is a very general one which embraces all the many and varied combinations of sugars and aglycones or genins. The usual linkage between the sugar and aglycone is an oxygen linkage between the reducing group of a sugar and an alcoholic or phenolic hydroxyl group of the aglycone. Since sugars exist in isomeric α–

and β– forms, both types are possible (**XI** and **XII** respectively). Such glycosides are sometimes called O-glycosides. Other glycosides do, however occur in which the linkage is through sulphur (S-glycosides), nitrogen (N-glycosides) or even carbon (C-glycosides). (Trease and Evans, 1989d).

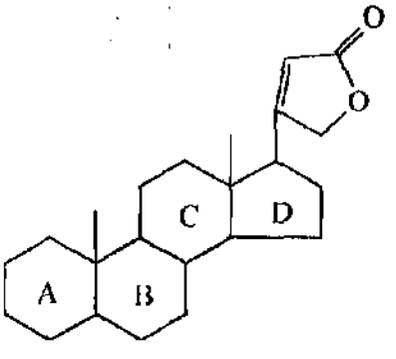


-Glycoside-forming Structure (**XI**)

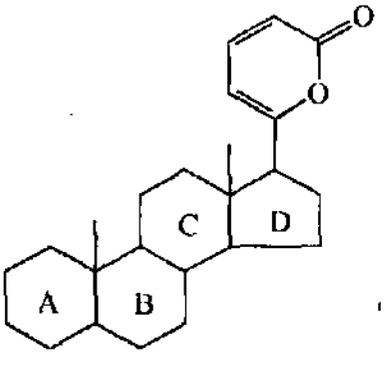


-Glycoside-forming Structure (**XII**)

Two types of aglycones are distinguished based on the presence of a five– or six–membered lactone ring. These types are known respectively as Cardenolides (**XIII**) and Bufanolides (**XIV**). (Trease and Evans, 1989d).



Cardenolide (**XIII**)



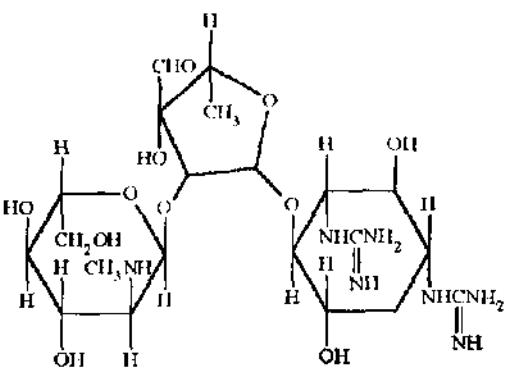
Bufanolide or Bufadienolide (**XIV**

Cardiac glycosides have long been employed as important ingredients for arrow poisons and drugs. They are used therapeutically to strengthen weakened hearts and thus

allow them function more efficiently. The therapeutic efficiency depends both on the structure of the genin and the type and the number of sugar units to which it is attached. (Trease and Evans, 1989d).

Cardiac glycosides have an action on cardiac muscles. A bitter glycoside, extracted from *Pergularia daemia* was found to relax cardiac muscle as well as cause contraction of a cat uterus and antagonize experimentally induced atony in the cat intestine (Oliver, 1959; Dutta and Ghosh, 1947).

Streptomycin (**XV**), which is a good antibiotic, is also a good example of antibiotic glycoside (Trease and Evans, 1989d).

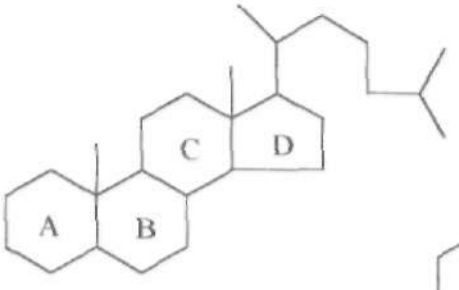


Streptomycin (**XV**)

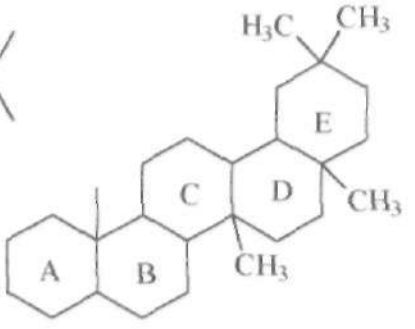
1.1.3 SAPONINS

Saponins are a class of glycosides that have long been used in many parts of the world because of their detergent properties, characterized by their frothing properties. They also have haemolytic properties and when injected into the blood stream, are toxic. When taken orally, saponins are comparatively harmless (Trease and Evans, 1989c).

Two groups are recognized: steroidal and triterpenoid types (**XVI** and **XVII**) – (Trease and Evans, 1972c).



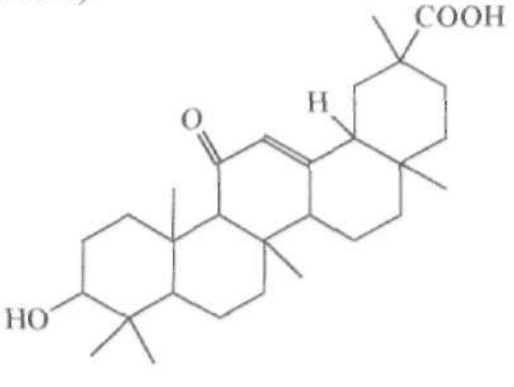
Steroid skeleton (**XVI**)



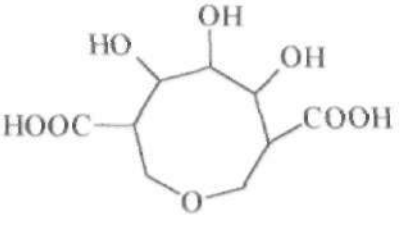
Triterpenoid skeleton (**XVII**)

Some pharmacological activities have been reported which saponins or plants rich in saponins exhibit. Sarsaparilla, which is rich in saponins, is effective in the treatment of syphilis, rheumatism and certain skin diseases (Trease and Evans, 1989c). A drug referred to as *Liquorice** has long been used in pharmacy as a flavoring agent, demulcent and mild expectorant, as well as treatment for rheumatoid arthritis, Addison's disease and various inflammatory conditions (Trease and Evans, 1989c).

[*Liquorice** is a drug consisting of the roots and stolons of various species of Glycyrrhiza. The roots contain 2 to 12 percent of glycyrrhizic acid, a saponin whose genin is glycyrrhetinic acid (**XVIII**) and the sugar is glucouronic acid (**XIX**)] (Trease and Evans, 1989c).



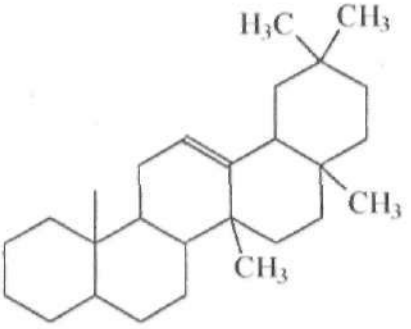
Glycyrrhetinic acid (**XVIII**)



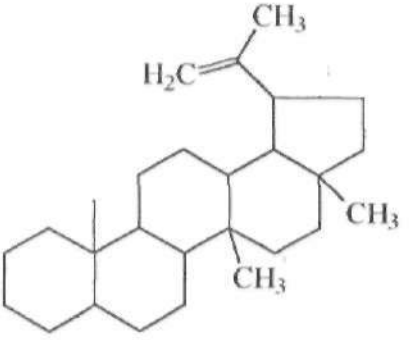
Glucouronic acid (**XIX**)

Senega known as *Senegin* A, B, C and D contains triterpenoid saponins and is used as a stimulant expectorant in chronic bronchitis. It is often prescribed with other expectorants such as ipecacuanha and ammonium carbonate (Trease and Evans, 1989c).

An examination of the plant *Daemia extensa*, from which Seshadri and Vydeeswaran (1971) reported the extraction and characterization of lupeol, β-amyrin (which are saponins represented by structures **XX** and **XXI**), and their acetates, showed antimicrobial activity which was greater with Gram–positive than with Gram–negative organisms. This probably explained the use of the plant in treating wounds. (Sofowora, 1979).



B-Amyrin (**XX**)



Lupeol (**XXI**)

Kalanchoe Cranata (*Andr.*) from which alkaloids and saponins were extracted in the aqueous and alcoholic medium by Dalziel (1956) and Sofowora (1979), is used on abscesses or other swellings, applied to ulcers and burns etc. the leaves are squeezed and applied to septic wounds (Sofowora, 1982).

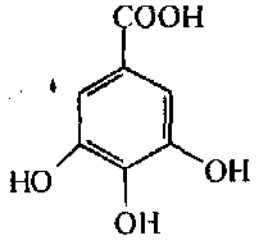
1.1.4. TANNINS

The term Tannins was first applied in 1796 to denote substances present in plant extracts which were able to combine with protein of animal hides, prevent their

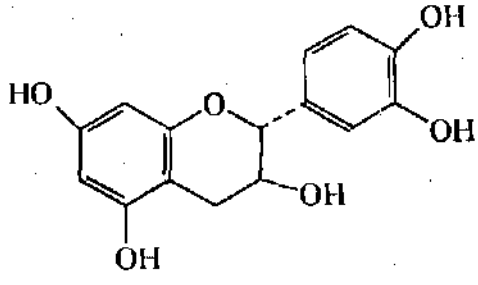
putrefaction, and convert them into leather. This definition excludes simpler phenolic substances, often present with tannins such as gallic acid (**XXII**), catechins (**XXIII**) etc, although these may under certain condition give precipitates with gelatin and be partly retained by hide powder. This class of compounds is usually referred to as "pseudo-tannins".

Two group of tannins namely hydrolysable tannins and non-hydrolysable or condensed tannins, are usually recognized. Examples of hydrolysable tannins include glucogallin, ellagic acid, hexahydroxy diphenoic acid (**XXIV, XXV** and **XXVI**) etc. Examples of condensed tannins are compounds whose structures are more resistant to breakage such as catechin (**XXIII**).

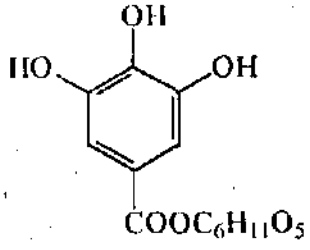
Gallotannins (**XXVII**) and catechins are two types of tannin extracted from the plant, *Acacia milotica Del,* and are used therapeutically to treat diarrhea. (Sofowora, 1982).



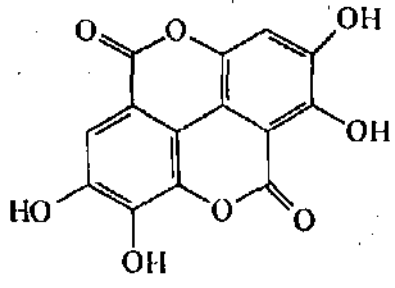
Gallic acid (**XXII**)



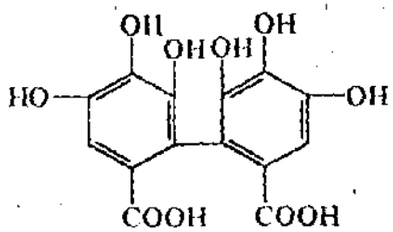
(+)-Catechin (**XXIII**)



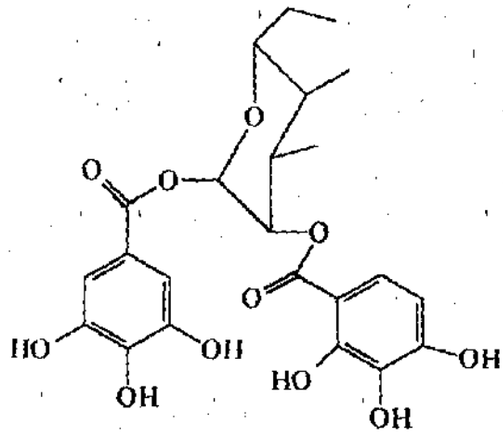
Glucogallin (**XXIV**)



Ellagic acid (**XXV**)



Hexahydroxydiphenoic acid **(XXVI)**



Gallotannin **(XXVII)**

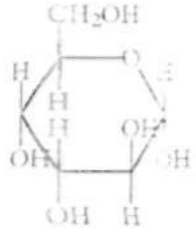
The bark of the plant, *Ximenia americana L.*, which has been found to yield 16-17 percent of tannins is used, in preparation with the branched leaves and roots, for headaches, toothaches, mumps and conjunctivitis in frontal applications. The dry root powder and bark of this same plant mixed with other ingredients are also used for oedema of the face (Sofowora 1982). In Angola the crushed bark is also applied to sores of domestic animals and in West Tropical Africa the pulverized bark and root are used as a dressing for ulcers, craw-craw, ringworm, etc., while the bark is rubbed on the skin during fever (Sofowora, 1982).

1.1.5. ANTHRAQUINONES

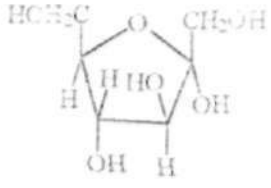
Anthraquinones are phenolic compounds widely distributed in nature, found both in plant and animals.

In nature, they are either in the free or combined states. The combined ones are usually referred to as anthraquinone glycosides and consist of anthraquinone molecules

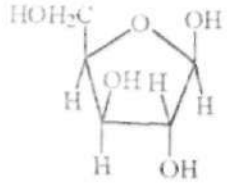
linked to one or more sugar molecules. The sugar molecule could be D-glucose, L-fructose, D-xylose, D-ribose, or L- or D- rhamnose (**XXVIII, XXIX, XXX, XXXI** and **XXXII**). The free anthraquinone is illustrated by structure **XXXIV** while the combined is exemplified by structure **XXXV**.



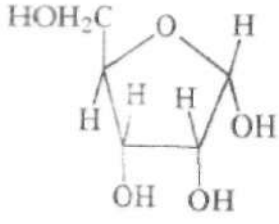
D-Glucose (**XXVIII**)



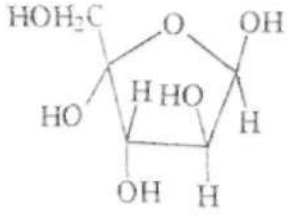
L-Fructose (**XXIX**)



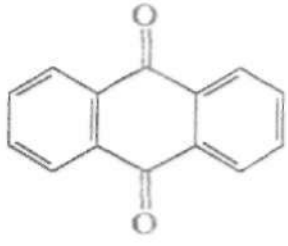
D-Xylose (**XXX**)



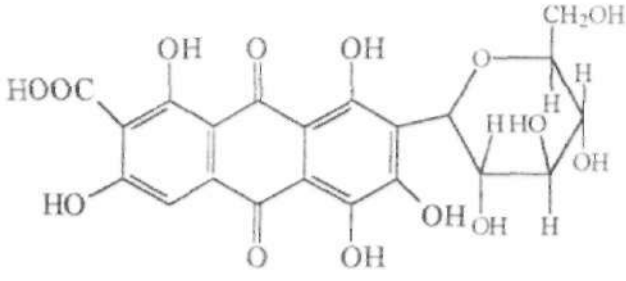
D-Ribose (**XXXI**)



L-Rhamnose (**XXXII**)



Free anthraquinone (**XXXIV**)



Carminic acid, an example of combined anthraquinone (**XXXV**)

Some derivatives of anthraquinones, such as the dihydroxyphenols, are present in some purgatives (Trease and Evans, 1989b).

Some of these classes of compounds extracted from plant samples have been reported to show positive activity on microorganisms such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosae, Klebsiella pneumoniae* and *Salmonella typhi*.

1.2 AIM AND SCOPE OF THIS WORK

Euphorbia hirta Linn has been reported in the literature to be used for curing or arresting many diseases such as dysentery, diarrhea, asthma, etc. Locally in Nigeria, it is used in curing a condition known as "gedigedi" (pile) and dysentery. Discussions with local residence in some parts of Nigeria, revealed that the plant is used in curing wounds on infants-navel after cutting the umbilical cord; a decoction together with some cereals' flour is prepared and taken in order to increase lactation in breast- feeding mother. It is also reported to be used as an anti-venom drug (against scorpion) by simply chewing the whole fresh plant and rubbing it on the point stung by the scorpion. This agrees with the report of Sofowora (1982). The knowledge of its vast medicinal uses and availability as well as ease in procuring the sample aroused the interest in working on this plant, *E. hirta Linn.*

The aim of the work is to carry out phytochemical screening of some extracts of the whole (except the floral part) plant, *E. hirta*. Particular interest will be taken on the water extract, in addition to alcohol, chloroform and ethyl acetate extracts. Screening will include the test for the presence of bioactive agents that include saponins, alkaloids, tannins, phlobatannins and cardiac glycosides; as well as cytotoxicity test and antimicrobial test. Attempt will also be made to isolate components of the plant by thin layer chromatography and their identity studied by spectroscopic analysis.

1.3 JUSTIFICATION OF THE RESEARCH

Based on the numerous uses of this plant and the fact that it is widespread in Nigeria, it was therefore of interest to know more about the plant. Although other research workers have reported some phytochemical work on the plant, its importance warrants further work, which could give more insight on the chemical constituents of the plant and its antimicrobial activity. The medium of application of the extract of the plant locally is water, hence the classes of compounds extractable by this medium will be of particular interest since most work reported on this plant is on other solvents other than water

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. DESCRIPTION OF *EUPHORBIA hirta LINN*

Euphorbia hirta L is one of some 2500 members of the *euphorbiaceae* family. (Hazleton and Heilerman, 1948; El-Naggar *et al*, 1978).

The genera generally consist of herbs which are annual or perennial, or suffrutices, rarely fleshly, but prickly. The leaves are obliquely ovate to lanceolate, rounded on one side, connate on the other at the base and acute at the apex; they are strictly opposite, usually markedly unequal at the base, sometimes up to 5cm long and 2cm broad. Stipules are present. The plants are mostly erect, but sometimes prostrate. The stem, ovary and capsules are hairy. Involucres are borne in dense, rather long pedunculate, axillary and terminal leafless glomerules. They could grow up to 40cm high (Lind and Tallantire, 1971; Kerharo and Adams, 1974; Hutchinson *et al*, 1958; and Sofowora, 1982)–Fig. 3.1.

E. hirta L. is a common weed in places like Senegal, Sierra Leone, Liberia, Cote D'Ivoire and Nigeria. It is widespread in Northern Nigeria. (Hutchinson *et al*, 1958; and Sofowora 1982).

In Nigeria *E. hirta Linn* is referred to by the Hausa speaking people as "Nonon Kurciya" (Dalziel, 1916).

The botanical classification is as follows:

Family: *Euphorbiacea*
Tribe: *Euphorbieae*
Genera: *Euphorbia Linn*
Species: *Euphorbia hirta Linn* (Hutchinson *et al*, 1958).
Synonym: *Euphorbia pilulifera L.* (Sofowora, 1982).

2.2. BRIEF DESCRIPTION OF THE FAMILY *EUPHORBIACEA*

The family *euphorbiacea* is made of trees, shrubs, or herbs, occasionally with milky juice. The leaves are alternate or rarely opposite, simple or digitately compound, sometimes reduced, and mostly stipulate. Flowers mostly reduced, and mostly apetalous. Sepals imbricate or valvate, or in very specialized inflorescences much reduced or absent. Petals absent or rarely present and sometimes united. Stamens 1-1000, free or variously connate. Anthers 2-4 cells, erect or inflexed in bud, opening lengthwise, rarely by pores. Rudimentary ovary often present in the male flowers. Ovary mostly 3-celled; styles free or united at the base; ovules solitary or paired, pendulous from the inner angle of the cells. Fruits are capsules or drupes. Seeds often with a conspicuous caruncles: Endosperm, copious, fleshly; embryo is straight. (Hutchinson, *et al*, 1958). It is a large and heterogeneous family having many tribes. The plants are mainly in the tropics and sub-tropic. (Hutchinson *et al*, 1958).

2.3. BRIEF DESCRIPTION OF THE TRIBE *EUPHORBIEAE*

Male and female flowers are much reduced and enclosed in a common involucre; one stamen. Perianth usually absent or rim-like, rarely cupular. They are plants with milky juice. Ovary with a cup-like, calyx at its base, half surrounded by four involucres formed of free bracts, containing the stamens. The male flower, usually 8 in each involucre, each consisting of a single stamen with a cup-like perienth at its base jointed to the pedicel. Genera include *Anthostema, Dichostemma, Elaeophorbia and Euphorbia*. (Hutchinson *et al*, 1958).

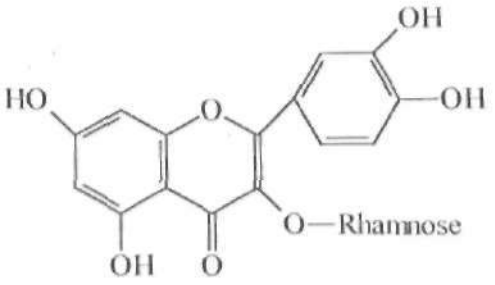
2.4 CONSTITUENTS OF *E. hirta L.*

According to Sofowora (1982), preliminary screening conducted by Kerharo and Adams (1974), Blanc *et al* (1964), Estrada (1959), Power and Browning (1913), Hallet and Parkes (1951), Gupta and Garg (1966), Attallah and Nicholas (1972), Ridet and Chartol (1964), Watt and Breyer-Brandwijk (1962), and Baslas Agarwal (1980), revealed the presence of a gum resin, calcium oxalate crystals, sugars, mucilage, volatile substances, mesityl, palmitic, oleic and linoleic acids. Traces of cetyl alcohol; an essential oil as well as malic and succinic acids and alkaloids were also found to be present. The stems and leaves were reported to contain querecetin 3-rhamnoside (a flavonoid) (**XXXVI**); leucocyanoid (**XXXVII**), quercitol (**XXXVIII**) (and its derivatives) and camphols were also isolated. Taraxerol (**XXXIX**), α,β-amyrin (**XX**) friedelin (**XL**) and β-sitosterol (**XLI**) were also reported to be present in the stem.

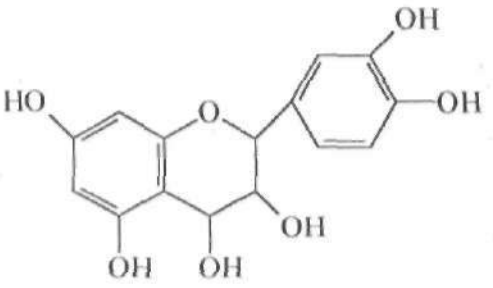
Baslas and Agarwal (1980) also reported the presence of Taraxerone (**XLII**), campesterol (**XLIII**), stimasterol (**XLIV**), l-inositol (**XLV**), cycloartenol (**XLVIII**), ellagic acid (**XXV**) and myricyl alcohol (**XLIX**).

El-Naggar *et al* (1978) and Sofowora (1982) isolated shikimic acid (**XLVII**), l-inositol (**XLV**), glucose, fructose, and sucrose.

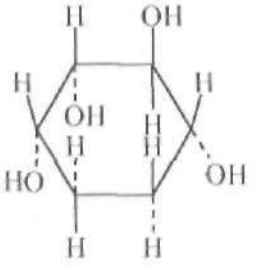
Other constituents reported include jambulol, euphosterol, a new phenolic substance with the formula $C_{28}H_{18}O_{15}$, β-amyrin acetate, 1-hexacosanol, ingenotriacetate, tinyatoxin, 12-deoxy-4β-hydroxyl phorbol-13-dodecanoate-20-acetate, and 12-deoxy-4β-hydroxyl phorbol-13-phenyl-20-acetate.



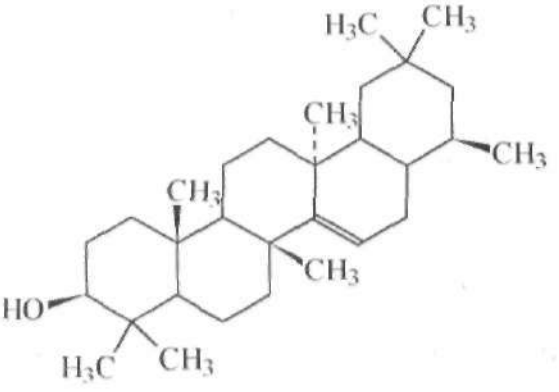
Quercetin-3-rhamnoside (**XXXVI**)



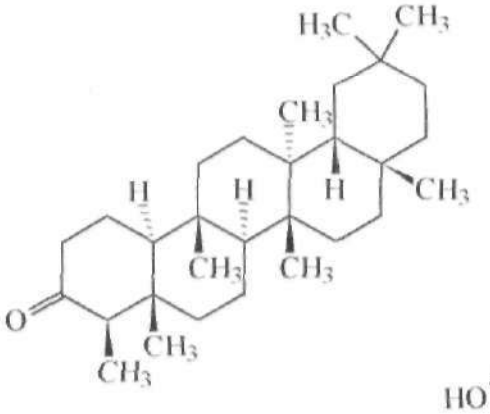
Leucocyanidol (**XXXVII**)



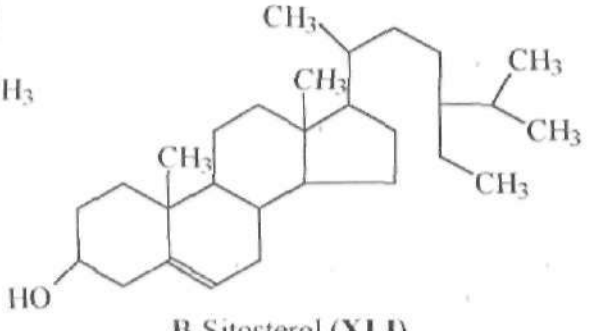
Quercetol (**XXXVIII**)



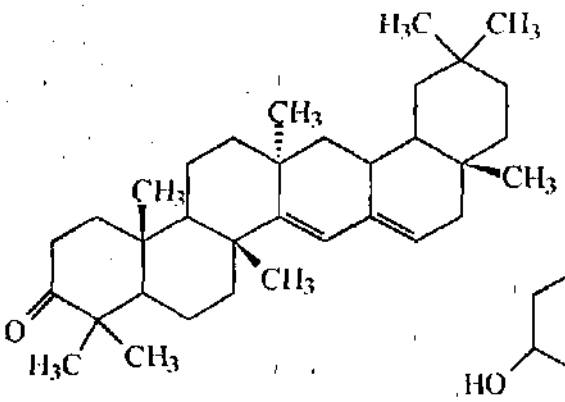
Taraxerol (**XXXIX**)



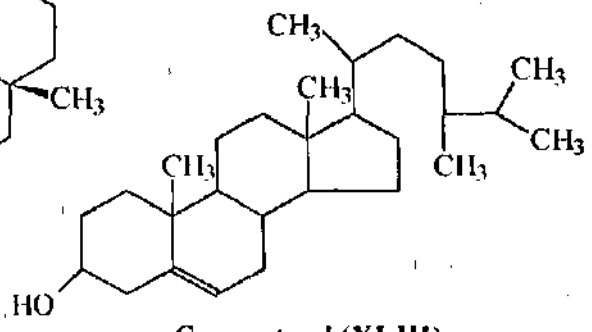
Friedelin (**XL**)



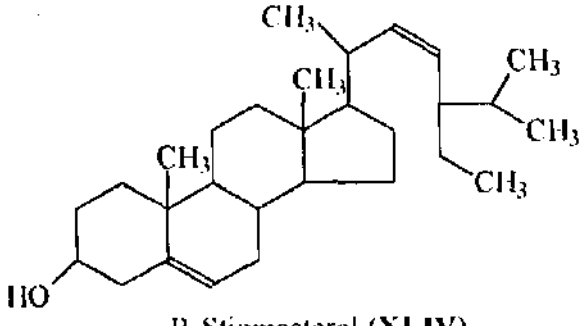
β-Sitosterol (**XLI**)



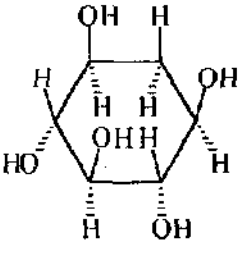
Taraxerone (**XLII**)



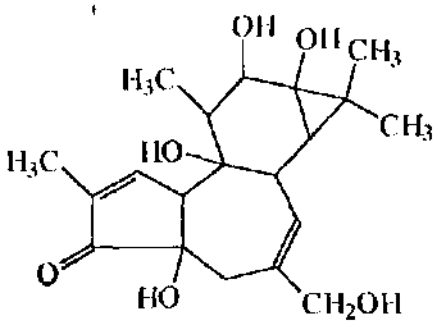
Campesterol (**XLIII**)



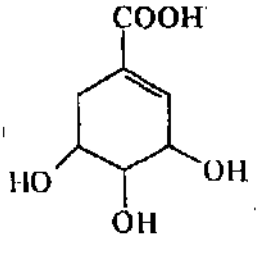
β-Stigmasterol (**XLIV**)



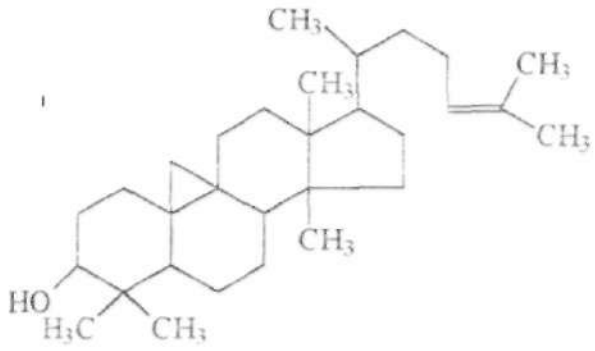
l-Inositol (**XLV**)



Phorbol (**XLVI**)



Shikimic acid (**XLVII**)



Cycloartenol (XLVIII)

$CH_3(CH_2)_{28}CH_2OH$

Myricyl alcohol (XLIX)
(Triacontanol)

2.5. MEDICINAL USES OF *EUPHORBIA hirta LINN*

The plant is reported (Oliver, 1959; Kokwaro, 1976) to be effective in the treatment of asthma, hence, the name 'Australian Asthma Herb'.

In East and West Africa, in addition to the treatment of asthma, it is used in treating respiratory tract inflammations and sometimes combined with bronchial sedatives, like *Grindelia robusta*, in preparations for inhalation; and also to increase lactation in sheep, goat, cattle and breast-feeding women. In Ghana, the plant is pounded and mixed with water for use as an enema for constipation, the leaves are used in sore and wound healing as in East Africa where it is also used for boils.

According to Wong-Ting-Fook (1980) and Kokwaro (1976), in Malagasy (former Madagascar) and Australia, *E. hirta* is used for coughs, chronic bronchitis and other pulmonary disorders.

E. hirta is used for relieving hay-fever and catarrh of the head (Le Strange, 1977).

In Angola, it is widely used against diarrhea and dysentery, especially amoebic dysentery. The Toukouleurs and Wolofs (in North West Africa) are said to use the plant

for colic in infants. It is reported that in Conakry hospital, a decoction of 200-300g of fresh plant daily was found to be successful in amoebic dysentery and good result were reported in acute enteritis and dysentery. In Malay Peninsula, East Africa and Liberia, the latex is said to be used for conjunctivitis and ulcerated cornea, and as an antidote for arrow poison (Sofowora, 1982).

In Malawi, the juice of the plant is used by young girls in the Balaka area for breast development (Hargreaves, 1979).

According to Dalziel (1956), Oliver (1959), Kerharo and Adams (1974), Ayensu (1978), and Wong- Ting-Fook (1980), the plant is used in the Central African Republic as an aphrodisiac and as an anti-helmintic agent.

2.6 PHARMACOLOGY OF *EUPHORBIA hirta LINN.*

Dhar, *et al.* (1968), investigated the ethanol extracts of *E. hirta L.* through a wide biological screening tests. The test done were anti-bacterial, anti-fungal, anti-protozoal, anti-helmintic, anti-viral activities, hypoglycaemic activity, effect on respiration (together with cardiovascular system and nictating membrane), effects on isolated tissues, gross effect and effect on central nervous system, anti-cancer screening, and toxicity test. *Euphorbia hirta L.* was only found to be active in the following areas;

(a) The anti-protozoal test on entamoeba hystolytica strain;

(b) The hypoglycaemic test on albino rats;

(c) The blood pressure test on mongrel dogs or cats;

(d) Contraction of guinea pig ileum; and

(e) The anticancer screening on Friend virus leukaemia in the mouse.

Further fractionation was done on some of the active extracts. However, though *E. hirta L.* showed some activities with the crude extract, these activities could not be confirmed on fractionation. The authors attributed this to some reasons such as:

(i) elimination of some inorganic constituents during fractionation,

(ii) in other cases the activities observed with the crude extract could be due to synergetic effect of two or more constituents which might have got separated in different fractions thereby leading to loss of activity in the fraction, and

(iii) the possibility of some labile constituents getting lost even during this mild chemical treatment.

Hellerman and Hazleton, (1950), carried out some work on the antispasmodic action of *E. pihilifera* (synonym of *E. hirta Linn)* using ileum of guinea pig, mouse ileum, guinea-pig uterus, histamine and egg white. They arrived at the following conclusion:- *E. pilulifera* was effective in relaxing: contractions of isolated mouse ileum, guinea-pig ileum, and guinea- pig uterus produced by acetyl choline, barium chloride and histamine; contraction of guinea-pig tracheal chains by histamine; contractions by egg white antigen in sensitized animals; and normal tracheal musculature but not visceral smooth muscle. *E. pilulifera* was only mildly depressant to the normal muscles.

El-Naggar, *et. al.* (1978) found that choline chloride contracted guinea-pig ileum, while shikimic acid had a relaxation effect.

Holmes(1923) and Dickshit (1934) reported the presence of a toxic principle in the plant, which appeared to be acting directly on the cardiac and respiratory centers.

Belkin, *et. al,* (1952) also reported that *E.pilulifera* produced a weak damage on sarcoma 37 tumours.

Dhar *et. al*, (1968), and Debaille and Petard (1953) also reported that extracts of the plant possess cancer inhibitory activity as well as inhibition of entamoeba histolytica. Similar positive results of the antidysenteric effect have been reported to support the use of this plant in amoebic dysentery (Ridet and Chartol, 1964; Martin, *et. al*, 1964).

Euhorbia hirta preparations for dysentery are available in France under the name 'Socambe' (Osterweil, 1964).

2.7. SOME MICROORGANISMS AND THEIR PATHOGENIC ACTIVITIES.

Some pathogenic microorganisms often use for microbial test of plant extracts include; *Staphylococcus aureus, Escherichia coli. Pseudomonas aeruginosae. Klesiell pneumoniae and Salmonella typhi.*

Staphylococcus aureus is a member of the gram-positive Cocci. It is pathogenic and golden in colour. *S. aureus* cause a wide variety of suppurative diseases in humans including superficial and deep abscesses, wound infections and infection of various internal organs. In addition they cause several toxinoses including food poisoning, toxic epidermal necrolysis and toxic shock syndrome (TSS). It is also the principal cause for bovine mastitis in domestic animals. It is resistant to virtually all-useful antibiotics, including oxacillin and methicillin (which are derivatives of penicillin), except vancomycin. (Novick, *et. al*, 1979).

Escherichia coli is a member of the *Enterobactericeae*. This family causes urinary tract infections, neonatal meningitis and intestinal diseases including diarrhea, dysentery and haemorrhagic colitis. Some of the treatments include oral non-absorbable antibiotics

such as neomycin or gentamycin, together with maintenance of fluid and electrolyte balance. Ampicillin, tetracyclin and trimethoprime-sulfamethoxazole are also used (Falkow, *et. al*, 1987).

Salmonella typhi, as other members of the genus salmonella, are pathogens founds in humans and livestock. It is the most frequently isolated cause, worldwide, of salmonella gastroentritis and bacteremia. Typhoid fever is one of the diseases caused by *S.typhi*, Chloramphenicol is especially effective for treating typhoid. Various β-lactams or trimethoprim-sulfamethoxazoles is also used (Falkow *et. al*).

Klebsiella pneumonia, is the most important human pathogen of the *Klebsiella* group. It causes about 3% of all acute bacterial pneumonia and it is the second most urinary tract pathogen. *Klebsiella* species have been found to also cause chronic inflammatory diseases of the upper respiratory tracts. In acute uncomplicated urinary tract infections, oral sulfonamides, nalidixic acid, ampicillin and tetracyclines are usually effective. In recurrent or chronic infections, sensitivity tests are needed for selection of effective agents. For very serious infections, aminoglycosides, cephalosporins and chloramphenicol are used. Most strains of *Klebsiella* are sensitive to cephalothin (Falkow *et. al*., 1987).

Members of the genus *Pseudomonas* are motile, germ-negative rods capable of growing on simple laboratory media and can be found in most moist environment and occasionally in the normal intestinal or skin flora. Children and young adults suffering from cystic fibrosis invariably become colonized in their respiratory tract by highly mucoid variants. In cystic fibrosis patients, the respiratory tract is colonized by *P. aeruginosa* late in the disease. Patients suffering from haematologic malignancies such as

leukemia, or with neutrepenia from immunosuppressive therapy are also at risk, with bacteremic pneumonia being the most common infection. It could infect facial burns and punctured wounds. In the therapy, most stains of *P. aeruginosa* are resistant to relative high levels of most antibiotics in use. However injecting a combination of tobramycin and an antipseudomonal β-lactam (e.g. azlocillin, piperacillin or ceftazime) treats acute and life-threatening infections. Oral quinolones (e.g. ciprofloxacin) have proved effective in chronic or milder infection, including those of the eyes, urinary tract or bones and joints (Falkow *et. al.* 1987).

CHAPTER THREE

3.0. EXPERIMENTAL

3.1. SAMPLE COLLECTION AND PREPARATION

The sample of *Euphorbia hirta*, was collected by uprooting the whole plant. They were collected within the Queen Amina Hall and around the chemistry Department of Ahmadu Bello University, Zaria, during the rainy season, between the months of August and September. It was properly identified at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria.

The plant was properly cleaned and separated into the different parts- leaves, stems, roots and floral parts. The first three parts were air- dried under the shade, pulverized and the powder sieved using a 1.4mm sieve.

3.2 EXTRACTION

Micro extractions of the leaves, stems, and roots were first of all done separately. 10g, each, of the powdered form was extracted successively using Soxhlet Extractor, with 300ml each of different solvents the following order: petroleum ether (60°-80°) → ethyl acetate → chloroform → ethanol → distilled water. The solvents used were Analar* grades and purified using standard procedure (see appendix IV), until the solvent distilled at constant temperature.

Extraction with petroleum ether was done for 4hrs; ethyl acetate, for 12hrs; chloroform, for 4hrs; ethanol for 8hrs; and water for 24rs. The extracts were concentrated using a Rotary Evaporator. After concentration, each extract was allowed to dry at about 40°c for 2-3 days, then stored in a dessicator for further test.

Results of preliminary phytochemical screening performed did not show any significant difference in the compounds extracted from the different parts for a given solvents, thus subsequent extractions were done using 500g of the powder of the whole plant (except the floral parts), successively, now using petroleum ether, ethyl acetate and water respectively. These three solvents were eventually used because the results of the preliminary screening also showed that ethanol and water extracts virtually contained the same classes of compounds. A similar thing was observed for petroleum ether and chloroform.

3.3. THIN LAYER CHROMATOGRAPHY (T. L. C.)

Thin Layer chromatography (T. L. C.) has been described as one of the most popular and widely used separation techniques. The reasons for these include ease of use, wide application to a great number of different samples, high sensitivity, speed of separation and relatively low cost (Kirchner, 1967).

Microplates (7.5 x 2.5cm) of thickness 0.25mm were initially used for qualitative analytical purpose, to enable the determination of the appropriate solvent mixture required to separate the components of the given extracts. Larger plates (20 x 20cm) of 0.75mm thickness were used for preparatory thin layer chromatography. The supports used were glass plates and the adsorbent silica gel (Kieselgel 60G for the 20 x 20 plates).

3.3.1 Preparation of Plates

(i) Preparation of Thin-Layer Plates (including Microscopic slides)

A slurry was made by stirring silica gel (30g) in distilled water ($65cm^3$) which was allowed to stand for 15 minutes and the contents shaken to give a slurry of a uniform

paste. This was spread as 250µm thickness on a previously arranged ten 10 x 20cm glass plates which were cleaned with propan-2-one. The plates were air dried overnight and activated at 120°C for 30 minutes in the oven and used as soon as they cooled to room temperature.

The microscopic slides (50) were arranged after wetting them with distilled water (to enable them adhere on the plates) on five 20 x 20 cm glass plates which were previously mounted on a Gallenkemp mounting board. These slides were then cleaned with propan-2-one and then spread as 250µm with a slurry made by stirring silica gel (30g) in distilled water (65cm^3). The microscopic slides were dried overnight and activated at a temperature of 120°C for 30 minutes in the oven and stored in the desiccator.

(ii) Preparation of Glass Plates for Preparative Thin-Layer Chromatography

Twenty 20 x 20cm glass plates each of 1.00 mm thickness were prepared. The slurry was made by stirring silica gel (100g) in distilled water (240cm^3). It was allowed to stand for 15 minutes and shaken to obtain uniform paste slurry. This was then spread as 1.00mm thickness on five 20 x 20cm glass plates. They were air-dried and then activated at 120°C for three hours in the oven and used as soon as it cooled down to room temperature.

For spotting the microplates, capillary tubes were used, and for the preparative T. L. C. calibrated micropipets were used.

For developing the chromatograms, various solvent mixtures were tried and the best solvent mixture for good separation was petroleum-ether (60-80°): ethyl acetate (1:1) for

water extract. For the ether extract which was initially fractionated into ethanol soluble (I) and ethanol insoluble (II), benzene:n-heptane:ethanol (5:5:2) and n-hexane:ethyl acetate (3:1) respectively, were used. And for ethyl acetate extract, petroleum ether (60°-80°): ethyl acetate (3:2).

Ascending development method, and in some cases, multiple development was used to enhance proper separation. After each development, the plate was removed, the solvent front quickly marked and the chromatogram exposed to air to allow the solvent to evaporate. The spots/bands were then viewed under UV fluorescent lamp (366nm and 254nm).

The various components were scraped off with a small spatula, dissolved in methanol, filtered and the filtrates concentrated. Finally dried up by allowing the solvent to evaporate, and stored in a dessicator.

3.4. TEST FOR FUNCTIONALITY : SPRAY TESTS FOR ALKALOIDS, CARDIAC GLYCOSIDES AND PHENOLIC COMPOUNDS

For alkaloids, iodine-potassium iodide reagent was used. The reagent was prepared using a mixture of 5% iodine in KI solution:water:2M acetic acid (2:3:5). (Kirchner, 1967). The samples were spotted on micro T.L.C. plates coated with silica gel and sprayed with a manual spray-pump. A reddish brown (or brown) colour was taken as a positive result for alkaloids (Machata, 1960).

1,3- Dinitrobenzene reagent was used for cardiac glycosides. The reagent is made up of solution A (10% 1,3-dinitrobenzene in benzene) and solution B (6g NaOH in 25ml H_2O into which 45ml CH_3OH had been added). After spotting the sample on the T.L.C.

plates, it was sprayed with solution A, heated to 60 °C and then sprayed with solution B. A purple colour rapidly changing to blue which fades rapidly was taken as positive (Touchstone, 1992).

Phenolic compounds (Tannins) were tested using ferric sulphate-potassium ferricyanide reagent. To prepare the reagent, (a) 0.5% ferric sulphate in 1N H_2SO_4, and (b) 0.2% potassium ferricyanide, were prepared. Equal volumes of solutions (a) and (b) were mixed and sprayed on the sample spots. Observation was made for colour change before 10mins and after heating to110°C. (Kirchner, 1967). The fact that hydrolysable tannin solutions turn blue with iron salts and condensed tannins give brownish-green was used for assessment (Haslam, 1966; Trease and Evans, 1989a).

Colour reaction test with ammonia gas, for flavonoids (a class of condensed tannins) was also carried out. The spots were exposed to ammonia gas and viewed under ultraviolet (UV) light. Assessment was done based on the colour observations enumerated in Table 3.1 (Haslam, 1966).

The result are shown in Tables (4.10–4.13).

Table 3.1: Colour Variations of Flavonoids without and with NH_3 Gas

Reagent	Light	Aurone	Flavone	Flavonol	Iso-Flavone	Flavonone	Chalcone
None	Visible	Brown-yellow	Pale-yellow	Yellow	-	-	Yellow
None	UV	Brown-yellow	Dull-brown	Yellow	Weak-Purple	-	Dull-brown
NH_3	UV	Brown-red	Green	Yellow	Weak-Purple	Pale-yellow	Dull-red

3.5 SPCTROSCOPIC ANALYSIS

The infrared (IR) and nuclear magnetic resonance (NMR) spectra of the extracts were taken. ATI Mattson Genesis Series IR–spectrophotometer and EM–360, 60MHz NMR spectrophotometer were used.

The results are shown in Table 4.20 and 4.21 and Figures 4.4-4.12.

3.6 PRELIMINARY PHYTOCHEMICAL SCREENING

Phytochemical Tests

Preliminary phytochemical screening was performed on each of the petroleum ether, ethyl acetate, chloroform, ethanol and water extracts of the leaves, stems and roots of *E. hirta* separately. The tests were performed to screen for the presence of saponins, alkaloids, tannins, phlobatinnins, anthraquinones and cardiac glycosides. The results are shown in Table 4.1.

3.6.1 Test for Saponins

(a) The Frothing Test

For the frothing tests, the method described by Wall *et. al.*(1952 and 1954), was adopted. A small quantity of each extract was shaken with distilled water in a micro test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins.

In order to remove 'false-positive' results, the blood haemolysis test was performed on extracts that frothed in water.

(b) The Blood Haemolysis Test

Small portions of the plant extracts that gave positive frothing results were shaken each in turn, with 5ml of distilled water. To 1ml of this solution was added 1ml of rat blood sample in normal saline and set aside for 3 hours. The solution was then observed for haemolysis.

A positive control test was carried out using 1% solution of saponin. A negative control test was also carried out with 1ml distilled water.

3.6.2 Test for Alkaloid

The extracts were cleaned (to remove non-alkaloidal compounds capable of giving 'false positive' results), using the following procedure.

A small quantity of each extract was dissolved in 1% dilute hydrochloric acid ($5.0cm^3$) on a steam bath and filtered. The filtrate ($1.0cm^3$) was made alkaline with 28% ammonia solution (pH 10) and then extracted with chloroform ($3 \times 5cm^3$). The combined chloroform extracts were concentrated *in vacuo* and then treated with equal volume of 1% hydrochloric acid. This was divided into two equal portions and each was respectively treated with few drops of Mayer's, and a drop of Wagner's reagents. A creamy white (turbidity) or precipitate with either of these reagents was taken as a preliminary evidence for the presence of alkaloids (Trease and Evans, 1989e).

3.6.3 Test for Tannins

A small quantity of each extract was dissolved in distilled water ($10cm^3$) boiled and filtered. To the filtrate was added few drops of 1% Iron (II) chloride solution, a blue-black, green or blue-green precipitate was taken as evidence for the presence of tannins.

3.6.4 Test for Phlobatinnins

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

3.6.5 Test for Anthraquinones

Borntrager's test:- This was used to test the presence of free and combined anthraquinones. Each extract (0.5g) was shaken with benzene ($10cm^3$) and filtered. 10% Ammonia solution ($10cm^3$) was added to the filtrate and shaken. The presence of pink, red or violet colour formed in the lower ammoniacal phase shows the presence of free anthraquinones.

3.6.6 Test for Cardiac Glycosides

Legal Test

A small quantity of each extract was dissolved in pyridine and a few drops of 2% sodium nitroprussicle together with a few drops of 20% sodium hydroxide solution were added. A deep red colour that faded to a brownish yellow showed the presence of Cardenolides in the extracts.

Keller-Kiliani Test

A small quantity of the extract was dissolved in a small quantity of glacial ethanoic acid containing one drop of iron (II) chloride solution. This was then underlayed with tetraoxosulphate (VI) acid. A brown ring obtained at the interface indicated the

presence of a de-oxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the ethanoic acid layer a greenish ring may form just above the ring and spread gradually throughout this layer (Trease and Evans, 1989d).

Lieberman Burchardt Test

A small quantity of each extract was dissolved in ethanoic anhydride ($2cm^3$) and cooled in an ice bath. Tetraoxosulphate (VI) acid ($1cm^3$) was carefully added. A colour change from violet to blue to green indicated the presence of a steroidal nucleus in the cardiac glycosides of the extract (Shoppee, 1964).

Salkowski Test

A small quantity of each extract was dissolved in trichloromethane ($1cm^3$) and concentrated tetraoxosulphate (VI) acid ($1cm^3$) was carefully added to form a lower layer. A reddish-brown colour at the interface that showed the presence of a steroidal ring as the aglycone portion of the cardiac glycoside.

The results for the phytochemical screening on the extracts is summarized in Table 4.1.

3.7 CYTOTOXICITY TEST

The method used for the cytotoxicity test was the Brine Shrimp Lethality Test. The method utilizing the brine shrimp (*Artemia salina*), has been used as a simple bioassay by pharmacognosists and natural product chemists in the detection and isolation of higher plant constituents with a variety of pharmacologic activities. It has the advantages of being rapid, inexpensive and simple.

This test was carried out on 30mg each of the water (EU_1), ethyl acetate (EU_2) and petroleum ether (EU_3) extracts.

3.7.1 Materials for Brine Shrimp Lethality Bio-Assay

(i) Small tank with perforated dividing dam.

(ii) Teat pipettes 0.5cm^3 and 1.0cm^3.

(iii) Test tubes (10mm x 5mm) fifteen per sample plus one control.

(iv) Calibrated pipettes 2.0cm^3 and 1.0cm^3.

3.7.2 Reagents for Brine Shrimp Lethality Bio-Assay

(i) Artemnia saline Leach (brine shrimp eggs)

(ii) Sea water (from Bar beach Lagos)

(iii) Dimethylsulphoxide (DMSO)

3.7.3 Sample Preparation

A stock solution of each of the extracts was prepared to a concentration of 10mg/cm^3 in DMSO. Five sets of three test tubes were labelled A, B, C, D and E for concentrations 1000, 500, 250, 125, and 62.5 µg/cm^3 respectively (i.e. 3 test tubes for each concentration, making a total of 15 test tubes), for each extract, plus one test tube for the control. The concentrations A-E, were made by serial dilution from the stock, as follows: 0.5cm^3 of seawater was placed in each test tube, except the test tube labeled 'A'. 0.5cm^3 of the stock solution was placed in test tubes 'A' and 'B'. 0.5cm^3 of the solution in 'B', after mixing well, was transferred to test tubes labeled 'C'. The same quantity of

solution was transferred from 'C' to 'D' and from 'D' to 'E'. From test tube 'E', 0.5cm^3 solution was discarded. Ten shrimps were added into all the test tubes including the control, which contained seawater only. Each solution was made up to 5.0cm^3.

3.7.4 Hatching the Shrimps

The instrument used for hatching the brine shrimp eggs was a tank with perforated dividing dam, which divided the tank into two unequal compartments. 30mg of the eggs was weighed and sprinkled into the larger compartment, which was covered while the smaller compartment was illuminated under the fluorescent lamp; so as to attract larvae through the perforations. The egg was kept under the light for 48 hours. after which the phototropic nauphi (shrimp larvae) were ready for use.

3.7.5 Bioassay

Ten shrimps were collected from the lighted side with teat pipettes and placed in each test tube and made up to 5.0cm^3 with sea water immediately after adding the shrimps giving a total of thirty shrimps per dilution.

The test tubes were maintained under illumination for twenty-four hours and the survivor shrimp larvae were counted macroscopically in the test tubes against lighted background. From the survivors the number of deaths at each dose and control were recorded.

3.7.6 Determination of LC_{50}

This at 95% confidence interval were determined for the extracts based on the recorded percentage death using the method of Linear regression analysis described by Saunder and Fleming (1971).

Results of the brine shrimp lethality test are given in Tables 4.2, 4.3 and 4.4.

3.8 ANTIMICROBIAL TEST USING THE AGAR DIFFUSION METHOD

The test is Antibacterial Test and the method used is the Dilution Testing. Dilution susceptibility testing methods are used to determine the minimal concentration, usually expressed in units of micrograms per milliliter, of an antimicrobial agent required to inhibit or kill a microorganism. Procedure for the determination of antimicrobial inhibitory activity is carried out by either agar- or broth- based methods, the former method was employed for this study.

The antimicrobial agents were tested at $\log_2$ (twofold) serial dilutions, and the lowest concentration that inhibited visible growth of an organism, recorded as the MIC. (It is important to note that the actual antimicrobial concentration required to inhibit growth is between the highest tested twofold dilution that inhibited growth and the next-lowest dilution at which growth was observed).

3.8.1 Preparation of Innoculum

Five microorganisms: *Staphylococcus aureus, Pseudomonas aeruginosae, Klebsiella pneumoniae, Salmonella typhi and Escherichia coli.* were sub-cultured to nutrient Agar Slants using a wire loop (done asceptically), and incubated for 24hours at a temperature of 37°C. This served as the store. These were in-turn sub-cultured to nutrient broth and incubated again for 24hours. Growth of microorganisms in the broth is indicated by turbidity. Before innoculating the plates, the broth culture was further diluted (one drop of broth culture to four drops of normal saline- 9% NaCl solution).

3.8.2 Preparation and Storage of Media

Petridishes were washed and sterilized in autoclave (at about 120°C) for 15minutes and allowed to equilibrate to 48° – 50°C, before use. They were labeled indicating the concentrations, test organisms and type of extracts. Mueller-Hinton agar is the recommended medium for most commonly encountered bacteria. The Mueller-Hinton agar (initially prepared by dissolving 38g MHA in 1litre distilled water), was melted in boiling water in an autoclave and poured to a depth of 3 to 4mm (i.e. 20 to25 ml of agar per plate). This was allowed to cool for about 1hour, the plates were not completely closed. It hardened on cooling. The plates were then kept closed and upside down in a fridge (4°C).

3.8.3 Dilution of Antimicrobial Agents

The dilution of the extracts was a twofold serial dilution. Solutions of the extracts were prepared (1000µg\ml) in appropriate solvents – distilled water for water extract, DMSO for petroleum ether and ethyl acetate extracts.

The extracts were sterilized by using a membrane filter (0.2µm). Six serial dilutions were done in sterile bijou bottles labeled 1 to 7. The first contained 1ml of the neat (1000µg\ml); 1ml of normal saline was put into bottles 2 to 7. 1ml of the neat extract was transferred to the 2nd bottle making 2ml, 1 ml out of this was transferred to the 3rd bottle and the process repeated to the 6th bottle from which 1 ml was discarded. The 7th bottle was left as control containing only normal saline. This was done for each of the extracts.

3.8.4 Innoculation of the Plates and Application of the Antimicrobial Agents

To innoculate the plate, a drop of the adjusted sub-cultured nutrient broth was applied to the surface of the MHA in a plate and evened to cover the surface of the MHA with the microbes, using a bent L-shaped glass rod. One microbe was innoculated to 3 plates for the three extracts making a total of 15 plates for the five microbes. After about 30 minutes, seven holes (wells), were punched on each of the plates using the bottom of Pasteur pipettes of diameter 0.5cm, to contain the 7 dilutions of the diluents. The wells were labeled corresponding to the series of dilutions. Each well was filled up (5-6 drops) with the corresponding diluents. These were allowed to stand until innocula have been completely absorbed by the media. Then they were inverted in an incubator at 37°C and allowed for about 18 hours before checking for activity.

Before testing with the T.L.C. fractions of the extracts, the crude extract starting with (100mg\ml) of the extracts were tested on *Staphylococcus aureus, Pseudomonas aeruginosae, Klebsiella pneumoniae, Salmonella typhi and Escherichia coli.*

After isolating the ethyl acetate and water extracts into various fractions using prep. T.L.C., the fractions were tested for microbial activities. Concentrations of the fractions prepared for the antimicrobial test were prepared based on calculation of approximate quantity of each of these fractions that could be contained in 100mg of the crude extract, since for the antimicrobial test initially done with the crude extracts, the highest concentration was 100mg\ml.

The microorganisms used for this second antimicrobial test were based on the result obtained from the antimicrobial test done with the crude extracts. For fractions obtained from ethyl acetate extract, *Staphylococcus aureus, Pseudomonas aeruginosae, Salmonella typhi and Escherichia coli* were used (Table 4.15). For the water extract fractions, *Staphylococcus aureus, Pseudomonas aeruginosae and Escherichia coli* were used (Table 4.16).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Preliminary Phytochemical Screening

The phytochemical screening was carried out to test for the presence of saponins, alkaloids, tannins, phlobatannins, anthraquinones, and cardiac glycosides. The result is summarized in Table 4.1. The results of the phytochemical screening performed on *E. hirta* shows that, the water extracts of the leaves contain saponins, tannins and cardiac glycosides, the stems contain saponins, alkaloids and tannins and the roots contained saponins and tannins. In the ethanol extracts, the leaves contain saponins, tannins and cardiac glycosides; the stems contain saponins, tannins and cardiac glycosides; while the roots also contain saponins, tannins and cardiac glycosides. The ethyl acetate extracts of the leaves, stems and roots all contain tannins and cardiac glycosides. The petroleum ether and chloroform extracts contain only cardiac glycosides.

Generally, from the result, the plant can be said to contain alkaloids, cardiac glycosides, saponins and tannins. These classes of compounds have been reported to possess so many pharmacological properties including antimicrobial activities.

Table 4.1: Results of Phytochemical Screening of the Leaves, Stems and Roots of *Euphorbia hirta L.*

EXTRACTS		TESTS									
Part of Plant	Solvent	Saponin		Alkaloid		Tannin	Phloba-tannin	Anthra-quinone	Cardiac glycoside		Cardenolides Steroidal ring	
		Fr.	B.H	M.	W.				Legal	K/K	Sal	Lieb
Leaves	Water	+	+	-	-	+	-	-	+	-	-	+
	Ethanol	+	+	-	-	+	-	-	-	+	+	-
	Ethylacetate	-	-	-	-	+	-	-	+	+	-	+
	Pet. ether	-	-	-	-	-	-	-	-	+	+	+
	Chloroform	-	-	-	-	-	-	-	-	+	+	+
Stems	Water	+	+	+	+	+	-	-	-	-	+	-
	Ethanol	-	+	-	-	+	-	-	-	+	+	-
	Ethylacetate	-	-	-	-	+	-	-	+	+	+	+
	Pet. ether	-	-	-	-	-	-	-	-	+	+	+
	Chloroform	-	-	-	-	-	-	-	-	+	+	+
Roots	Water	+	+	-	-	+	-	-	-	-	-	-
	Ethanol	+	+	-	-	+	-	-	+	+	+	-
	Ethylacetate	-	-	-	-	+	-	-	+	+	+	-
	Pet. ether	-	-	-	-	-	-	-	-	+	+	+
	Chloroform	-	-	-	-	-	-	-	-	+	+	+

NOTE: A positive result is indicated by (+), while a negative result is indicated by (-).

Fr. = Frothing Test; B.H. = Blood Haemolysis Test; K/K = Keller-killiani Test;

Sal = Salkowski Test and Lieb. = Libermamm's Test.

4.2 Brine-Shrimps (Toxicity) Test

The toxicity test was performed on 30 Brine-Shrimps (Artemia saline leach) with each of the crude extracts of the solvents used. Since the result of the preliminary phytochemical screening shows that the petroleum ether and chloroform extracts; and the water and ethanol extracts virtually contained the same classes of compounds, only petroleum ether, water and ethyl acetate were used for the toxicity tests on *Euphorbia hirta*. The results are shown in Tables 4.2, 4.3 and 4.4 for the water, ethyl acetate and petroleum ether (60 – 80°) respectively.

Table 4.2: Result of Brine-Shrimps Test for Crude Water Extract of *E. hirta*

Concentration (µg/ml)	Number of Deaths Out of 30	Percentage Death Response
1000	13	43.33
500	7	23.33
250	4	13.33
125	3	10.00
62.5	1	3.33

Table 4.3: Result of Brine-Shrimps Test For Crude Ethyl Acetate Extract of *E. hirta*

Concentration (µg/ml)	Number of Deaths Out of 30	Percentage Death Response
1000	12	40
500	9	30
250	6	20
125	2	6.7
62.5	2	6.7

Table 4.4: Result of Brine-Shrimps Test for Crude Petroleum Ether (60-80°) Extract of *E. hirta*

Concentration (µg/ml)	Number of Deaths Out of 30	Percentage Death Response
1000	30	100
500	24	80
250	18	60
125	9	30
62.5	6	20

The death responses for the three extracts reduce with decrease in the in concentrations of the extracts. Of the three extracts (water, ethyl acetate and petroleum ether) extracts, the number of deaths out of 30 brine shrimps reduced as the concentrations of the extracts reduced from 1000 µg/ml to 62.5 µg/ml. For ethyl acetate and petroleum ether extracts, the drop in number of death response of the brine-shrimps was almost linear with respect to decrease in concentrations from 1000µg/ml → 500µg/ml → 250µg/ml → 125µg/ml respectively. The drops were 3, 3 and 4 for ethyl acetate and 6, 6 and 7 for petroleum ether respectively. Further reduction in concentrations had less effect for the ethyl acetate. For the water extract, the death response reduced with increase in dilutions, but showed no uniform pattern in drop of death response – 6, 3, 1 and 2 from 1000µg/ml → 500µg/ml → 250µg/ml → 125µg/ml → 62.5µg/ml respectively.

The probits of these quantal responses were calculated and shown in Table 4.5. These were plotted against log_{10}Dose, (Figure 4.1, 4.2, and 4.3). The best straight line was drawn through the points, and the log_{10}Dose on this line which corresponds to 50 per cent mortality in a group, i.e to a Probit 5, was read off. The antilog of this probit is called the median lethal dose (M.L.D) and gives a quantitative measure of the toxicity of the drug (Leonard and Robert, 1971).

[Note that the dose, which kills 50 per cent of a group of test animals, is also referred to as LD_{50} or LC_{50} (Von Oetingen, 1956)],

The probits (of the % death response) corresponding to the different log_{10}Dose for water, ethyl acetate and petroleum ether extracts are shown in Table 4.5 below:

Table 4.5: Log_{10}Dose with Their Corresponding Probits For Water, Ethyl Acetate and Petroleum Ether Extracts of *E. hirta*.

DOSE (µg/ml)	Log_{10}DOSE (X*)	PROBITS (Y*)		
		Water	Ethyl Acetate	Petroleum ether
1000	3.000	4.82	4.75	8.09
500	2.699	4.26	4.48	5.84
250	2.398	3.87	4.16	5.25
125	2.092	3.72	3.45	4.48
62.5	1.796	3.12	3.45	4.16

* See Appendix 1 for the calculation.

Another method of determining the M.L.D value is by using the regression line equation as shown in Appendix I. The calculations follow the methods suggested by Saunders and Fleming, 1971.

The M.L.D. or LC_{50} values obtained from the calculations for the three extracts are as shown in Table 4.6.

Table 4.6: Calculated LC_{50} Values for the Brine-Shrimp Test with the Crude Extracts of *E. hirta*.

EXTRACT	LC_{50} (µg /ml)
Water	1412.54 ± 4.68
Ethyl Acetate	1513.56 ± 16.23
Petroleum ether	162.18 ± 63.1

The Brine-Shrimp lethality test has been used to estimate LC_{50} for comparison of potencies of drugs. (Meyer *et al*, 1982).

From the plots of probits against log_{10}Dose from Table 4.5, as shown in Figures 2, 3, 4 and the calculation done in Appendix I which are summarized in Table 4.6, the LC_{50} values for water, ethyl acetate and petroleum ether extracts are 1412.5 ± 4.68, 1513.56 ± 16.23, and 162.18 ± 63.1µg/ml, respectively. This implies that petroleum ether extract is the most potent and the ethyl acetate extract is the least potent of the three extracts. The cytotoxicity (LC_{50}) values for the water and ethyl acetate extracts can be said to be non significant in terms of activity, since the LC_{50} values are >1000µg/ml. Extracts are said

to display toxicity in the brine-shrimp assay when LC_{50} is <1000µg/ml, as such only petroleum ether crude extract showed significant toxicity since the LC_{50} <1000. This cytotoxicity test results is an indication that *E. hirta* possess some pharmacologically active principles, the most toxic of which are present in the petroleum ether fractions. Note, however, that significant activity in the alcohol and petroleum ether extracts of *E. hirta* have been reported (El-Naggar *et al*, 1978; Dhar *et al*, 1968; Hellerman and Hazleton, 1950).

From the LC_{50} values of water, ethyl acetate and petroleum ether extracts, the high toxicity of petroleum ether extract makes it unsafe for use as a drug, while water and ethyl acetate extracts of *E. hirta* can be used with a substantial margin of safety (Sofowora, 1982).

The high toxicity of petroleum ether extract compared to the others could be attributed to the fact that, as shown by the result of the phytochemical screening it contains, mostly, cardiac glycosides. Cardiac glycosides are poisonous and could therefore, account for the high toxicity of *E. hirta*.

4.3 Purification by Preparatory Thin Layer Chromatography.

Preparatory Thin Layer Chromatography was used to purify the petroleum ether, ethyl acetate and water extracts.

The petroleum ether extract was first of all partitioned into two fractions – Ethanol soluble and Ethanol insoluble. The ethanol insoluble fraction gave five spots, while the ethanol soluble fraction gave six spots on T.L.C. using n-hexane:ethyl acetate

(3:1), and Benzene:n-heptane:ethanol (5:5:2) respectively as developing solvents. The R_f values are shown in Tables 4.7 and 4.8 respectively. The ethyl acetate extract gave six spots while the water extracts gave three spots. See Tables 4.9 and 4.10.

Table 4.7: R_f Values for Ethanol Insoluble Fraction of Petroleum ether Extract of *E. hirta*

FRACTION	R_f VALUES
1	0.086
2	0.172
3	0.920
4	0.951
5	0.993

Table 4.8: R_f Values for Ethanol –Soluble Fraction of Petroleum ether Extract of *E. hirta*.

FRACTION	R_f VALUES
1	0.066
2	0.400
3	0.750
4	0.875
5	0.954
6	0.993

Table 4.9: R_f Values for Ethyl Acetate Extract of *E. hirta*.

FRACTION	R_f VALUES
1	0.23
2	0.46
3	0.75
4	0.85
5	0.96
6	0.99

Table 4.10: R_f Values for Water Extract of *E. hirta*.

FRACTION	R_f VALUES
1	0.06
2	0.50
3	0.79

The number of fractions obtained for the two petroleum ether fractions: Ethanol – soluble and Ethanol–insoluble, are in agreement with the number of spots reported by Baslas and Agarwal (1979). They reported six spots for the Ethanol–soluble fraction corresponding to the following R_f values 0.08, 0.19, 0.23, 0.36, 0.55 and 0.69, which were identified as β-sitosterol, 24-methylene cycloartenol, cycloartenol, β-amyrin, 1-hexacosanol and euphorbol hexacozonoate respectively. While for the Ethanol-insoluble fraction they reported the R_f values as 0.31, 0.36, 0.54, 0.79 and 0.87, corresponding to tinyatoxin, β-amyrin acetate, 12-deoxy-4β-hydroxyphorbol-13-phenylaceto-20-acetate, 12-deoxy-4β-hydroxyphorbol-13-dodecanoate-20-acetate and ingenol triacetate respectively.

The irreproducibility of the exact R_f values of these spots could be due to so many reasons. Touchstone (1992), indicated several factors that could be responsible for irreproducibility of exact R_f values, among which include the atmosphere in the developing chamber (effect of chamber saturation), effect of depth of mobile phase, nature of the sorbent, effect of sample size, etc.

Due to the unavailability of authentic samples of the different types of cardiac glycosides, alkaloids, tannins and saponins for comparison of the R_f values this parameter could not be used to deduce the compounds.

4.4 Tests For Functionality of Extract Fractions.

The spray tests results are shown in Tables 4.11 to 4.16, the colour reaction with NH_3, Tables 4.17 and 4.18

Table 4.11: Result for Iodine-Potassium Iodide Spray for Alkaloids for Ethyl Acetate Extract Fractions of *E. hirta*.

FRACTION	ORIGINAL COLOUR	COLOUR AFTER SPRAY
1	Dark green	Grey
2	Green	Green
3	Faint green	Faint green
4	Faint green	Faint green
5	Yellowish green	Green
6	Green	Green

Table 4.12: Result for Iodine-Potassium Iodide Spray for Alkaloids for Water Fractions of *E. hirta.*

FRACTION	ORIGINAL COLOUR	COLOUR AFTER SPRAY
1	Reddish brown	Grey which turned brown
2	Brown	Brown
3	Brown	Brown

Table 4.13: Result for 1,3-Dinitrobenzene Spray Test for Cardiac Glucosides for Ethyl Acetate Extract Fractions of *E. hirta.*

FRACTION	ORIGINAL COLOUR	COLOUR AFTER SPRAYING WITH 'A'	COLOUR AFTER HEATING AND SPRAYING WITH 'B'
1	Dark green	Brown	Greenish brown
2	Green	Green	Green
3	Faint green	Faint green	Brownish green
4	Faint green	Faint green	Brown
5	Yellowish green	Yellowish green	Green
6	Green	Green	Brownish green

Table 4.14: Result for 1, 3-Dinitrobezene Spray Test for Cardiac Glucosides for Water Extract Fractions of *E. hirta*.

FRACTION	ORIGINAL COLOUR	COLOUR AFTER SPRAYING WITH 'A'	COLOUR AFTER HEATING AND SPRAYING WITH 'B'
1	Reddish brown	Brown	Brown
2	Brown	Brown	Brown
3	Brown	Brown	Purple

Table 4.15: Result for Ferric Sulphate-Potassium Ferricyanide Spray Test for Phenolic Compounds (Tannins) For Ethyl Acetate Extract Fractions of E. hirta.

FRACTION	ORIGINAL COLOUR	COLOUR BEFORE 10 MINUTES	COLOUR AFTER HEATING TO 110^0
1	Dark green	Brown	Brownish green
2	Green	No change	No change
3	Faint green	No change	Faint brown
4	Faint green	No change	Brown
5	Yellowish green	No change	No change
6	Green	No change	No change

Table 4.16: Result for Ferric Sulphate–Potassium Ferricyanide Spray Test for Phenolic Compounds (Tannins) for Water Extract Fractions of *E. hirta*.

FRACTION	ORIGINAL COLOUR	COLOUR BEFORE 10 MINUTES	COLOUR AFTER HEATING TO 110^0
1	Reddish brown	No change	No change
2	Brown	Blue	Green
3	Brown	Greenish yellow	Blue

NB: For Tables 4.11 and 4.12, change to brown colour was taken as a positive result; Tables 4.13 and 4.14. purple colour rapidly changing to blue was taken as positive and Tables 4.15 and 4.16, blue colour was taken as positive for hydrolysable tannins, green brown (or green changing to brown) was taken as positive.

Table 4.17: Result for Colour Reaction Test for Flavonoids on Exposure to Ammonia Gas - For Ethyl Acetate Extract Fractions of *E. hirta*.

REAGENT	LIGHT	FRACTIONS AND COLOUR CHANGES					
		1	2	3	4	5	6
None	UV (254nm)	Dark green	Green	Yellowish green	Yellowish green	Yellowish green	Green
NH_3	UV (254nm)	Green	Green	Yellowish green	Yellowish green	Yellowish green	Green

Table 4.18: Result for Colour Reaction Test For Flavonoids on Exposure to Ammonia Gas-for Water Extract Fractions of *E. hirta*.

REAGENT	LIGHT	FRACTIONS AND COLOUR CHANGES		
		1	2	3
None	UV(254nm)	Brown	Brown	Brown
NH_3	UV(254nm)	Dark green	Yellowish green	Yellowish green

UV = Ultraviolet light

Note that the assessment for this test is given in Table 3.1

Table 4.19: Result for Blood Haemolysis Test for Water Extract Fractions.

FRACTION	RESPONSE TO BLOOD HAEMOLYSIS TEST
W_1	+
W_2	-
W_3	-

NB: (+) indicates positive response, while(-) indicates negative response.

The spray test result for cardiac glycosides in Tables 4.13 and 4.14 did not show any apparent positive response for any of the fractions of water and ethyl extracts of *E. hirta*. Since cardiac glycosides were indicated in the preliminary phytochemical screening of the crude extracts, the negative response of any the fractions could be due to some factors such as:- The possibility of getting lost during the isolation and treatment; and/or the positive response in the crude extract could be due to synergistic effect of two

or more constituents which might got concentrated in the fractions (Dhar *et al*, 1968). The activity of cardiac glycosides depends both on the structure of the genin (or aglycon) and the type and number of sugar units attached to it (Trease and Evans, 1983), it therefore implies that any factor or reaction that will alter the structure will affect the activity of such glycosides. Such reactions could include interaction (hydrogen bonding) between the –O- or -OH group of the glycosides and the –OH groups of the silica gel (silic acid). Another reaction could be hydrolysis of the glycosides in the course of separation and recovery from the sorbent using CH_3OH. There is also the possibility of acetylation by ethyl acetate during the course.

From Tables 4.11 and 4.12 for the spray test for alkaloids, only W_1, (fraction 1 for water extract) seemed to have responded positively (from brown to grey and back to brown). Change to brown is considered positive for alkaloids, but this fraction was brown originally, so a conclusion cannot be made. However, W_1 responded positively to the blood haemolysis test for saponins which revealed that it is a saponin. The result however, for the ethyl acetate fractions agrees with the result of the preliminary phytochemical screening of the crude which showed that ethyl acetate crude extract did not contain alkaloids.

From Tables 4.15 and 4.16 based on the blue colouration for hydrolysable tannins and the green-brown for condensed tannins as well as Tables 4.17 and 4.18 for the colour reaction with NH_3 gas, W_3 is a hydrolysable tannin, while EA_1, EA_3, EA_4 and EA_5 are likely to be condensed tannins (EA_4 and EA_5 being flavonoids).

EA_2, EA_6, and W_2 did not respond positively to these spray tests.

4.5 Spectral Data

The nuclear magnetic resonance (n.m.r) and infrared spectra of ethyl acetate and water extracts were taken. However, the n.m.r machine used was 60 megahertz and the spectra could not give reliable informations to be reported, so only the infrared data is reported here (Tables 4.20 and 4.21). The infrared spectra are in Figures 4.4-4.12

Table 4.20: Infrared Spectral Data for Ethyl Acetate Fractions of *E. hirta*.

FRACTION	INFRARED (cm^{-1})
EA_1	3375, 1726.68, 1556.15, 1412.70, 1091.99
EA_2	3385.70, 1553.61, 1412.66, 1098.31
EA_3	3400, 1725.32, 1582.38, 1411.46, 1098.06
EA_4	3425, 2924.61, 2853.70, 2340.60, 1565.38, 1407.31, 1096.62
EA_5	3416.09, 2925, 2358.64, 1730.18, 1096.12
EA_6	3150, 2359.42, 1584, 1403.79, 1098.06

Table 4.21: Infrared Spectral Data for Water Fractions of *E. hirta*.

FRACTION	INFRARED (cm^{-1})
W_1	3200, 2924.34, 1711.13, 1588.04, 1412.84, 1091.9
W_2	3425.68, 3170.74, 1565.27, 1409.14, 1104.18, 1022.47
W_3	3429.79, 1104.44, 1022.35

The infrared data of W_1 shows a wide band at 3200cm $^{-1}$ which could be said to be due to O-H bond stretching, a C=O stretching band at 1711cm $^{-1}$ and also one due to C–O stretching at about 1091cm $^{-1}$. The blood haemolysis test shows that W_1 is a saponin and since these functional groups are also present in saponins, it could be deduced that W_1 is a saponin.

W_3, EA_1, EA_3 and EA_5 also show O-H, C=O and C–O stretching, W_3 at 3429.79cm $^{-1}$, 1104.44 and 1022.3, EA_1 at 3375, 1726.68 and 1091.79; EA_3 at 3400, 1725.32 and 1098.06; EA_5 at 3416.09, 1730.18 and 1096.12. The test for functionality and the preliminary phytochemical screening showed that these fractions are not cardiac glycosides, saponins, or alkaloids. But they proved positive for tannins from the results on Tables 4.15, 4.16, 4.17 and 4.18. W_3 is a hydrolysable tannin, EA_1, EA_3, EA_4 and EA_5 are condensed tannins (EA_4 and EA_5 flavonoids).

The infrared spectra for EA_2, EA_6 and W_2 show the O-H and C-O bands, but no C=O stretching implying they may be alcohols. They did not respond positively to any of the spray tests. However, some or all could be the genins of the unisolated glycosides detected in the crude extracts.

4.6 Antimicrobial Tests

The antimicrobial tests were performed with crude petroleum ether, water and ethyl acetate extracts, using minimum inhibition Method and with Thin Layer Chromatography fractions of the ethyl acetate and water extracts. The bacteria used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudemonas aeruginosae*, *Klebsiella pneumonia* and *Salmonella typhi*. The results are shown in Table 4.22 below.

Table 4.22: Result of Antimicrobial Test with the Crude Petroleum ether, Water and Ethyl Acetate Extracts of *E. hirta*.

EXTRACT	MICROORGANISM	ZONES OF INHIBITION (mm) FOR VARIOUS CONCENTRATION (µg/ml)					STANDARD
		1000	500	250	125	62.5	
Water	E. coli	M	-	-	-	-	-
	S. aureus	17	12	-	-	-	-
	Ps. aeruginosae	10	8	-	-	-	-
	S. typhi	-	-	-	-	-	-
	Kleb.	-	-	-	-	-	-
Ethyl acetate	E. coli	10	M	-	-	-	-
	S. aureus	10	M	-	-	-	-
	Ps. aeruginosae	12	7	-	-	-	-
	S. typhi	M	-	-	-	-	-
	Kleb.	-	-	-	-	-	-
Petroleum ether	E. coli	M	-	-	-	-	-
	S. aureus	17	11	10	-	-	-
	Ps. aeruginosae	10	-	-	-	-	-
	S. typhi	-	-	-	-	-	-
	Kleb.	10	-	-	-	-	-

NB: (-) indicates 'No inhibition'

M indicates 'Minimum inhibition'.

Table 4.23: Result of Antimicrobial Test with the Various of Ethyl Acetate Extract of *E. hirta*

FRACTION	ZONES OF INHIBITION (mm) OF VARIOUS			
	E. coli	Ps. aeruginosae	S. aureus	S. typhi
EA_1	10	15	17	10
EA_2	12	10	14	9
EA_3	M	17	12	M
EA_4	M	12	12	10
EA_5	11	13	18	M
EA_6	M	15	-	M

NB: Concentrations of the various fractions used were as estimate in 1g of the crude extract, since the maximum concentration of the crude used was 1000ug/ml

(-) indicates 'No inhibition'.

M indicates 'Minimum inhibition'

Table 4.24: Results of Antimicrobial Test with Various Fractions of Water Extract of *E. hirta*.

FRACTION	ZONES OF INHIBITION (mm) OF VARIOUS MICROORGANISMS		
	E. coli	Ps. aeruginosae	S. aureus
W_1	-	10	M
W_2	-	-	-
W_3	-	-	-

The results of the antimicrobial tests performed on the crude extracts of ethyl acetate and water showed that water extract has appreciable effect on *Staphylococcus aureus* and *Pseudomonas aeruginosae*, but minimal inhibition on the growth of *Escherichia coli* and no effect (at least at the given concentrations) on *Salmonella typhi* and *Klebsiella pneumoniae*. [Any extract with zone of inhibition, i.e diameter, 6mm or more was considered active, (Boda, 1997)]. Ethyl acetate crude extract was active on *E. coli*, *S. aureus* and *Ps. aeruginosae*, but showed minimum inhibition on *S. typhi*, and the petroleum ether crude extract showed appreciable activity on *S. aurens*, *Ps. aeruginosae*, and *Kleb. pneumoniae*, but minimum activity on *E. Coli*.

S. typhi was resistant to water and petroleum ether extracts. *Kleb. pneumoniae* was resistant to water and ethyl acetate extracts.

The fact that the plant extracts were found to inhibit the growth of these bacteria could account for their use in curing some of the diseases, which have been reported to be caused by these bacteria. *Staphylococcus aureus* is reported to cause wound infection, diarrhea, conjunctivities and vomiting caused by food poisoning; *E. coli* also causes wound infection (especially abdominal wound infection), urinary tract infection and diarrhea, *Pseudomonas aeruginosae* infects the respiratory tracts of cystic fibrosis patients, causes burns and wounds infection. *S. typhi* causes gastro entritis and typhoid fever; and *kleb. pneumoniae* causes common urinary tract diseases, acute diarrhea in children. (Novick *et al*, 1979, Falkow *et al*, 1987; Bailey, 1987).

The above mentioned diseases are some of the diseases against which *Euphorbia hirta L.* has been reported to be used medicinally (Wiley, 1982).

When the antimicrobial tests were repeated on the fractions from the water and ethyl acetate extracts, the activity by the ethyl acetate extract was reproducible on the four isolates on which it was initially active, i.e *E. coli*, *Ps. aeruginosae*, *S. aureus* and *S. typhi*. For the water extract fractions the activity on *Ps. aeruginosae* and *S. aureus* were reproducible, while that on *S. typhi* was not reproducible. The irreproducibility of the activity of the water extract fractions on *S. typhi* could be attributed to the following reasons. The elimination of inorganic constituents during fractionation; The activity of the crude extract could be due to synergistic effect of two or more constituents which might have got concentrated in the fractions. Finally, the possibility of some labile constituents getting lost even during the chemical treatment (Dhar *et al*, 1968).

CHAPTER FIVE

5.0 CONCLUSION

Euphorbia hirta Linn is a plant reported to possess many medicinal uses. The preliminary phytochemical screening revealed that the plant contains saponins, tannins, alkaloids and cardiac glycosides. These classes of compounds were reported to have been extracted using alcohol and petroleum ether. This work revealed for the first time that saponins, tannins, alkaloids and cardiac glycosids can also be extractable from *E. hirta* using water, a cheap and the common solvent, which apparently is the solvent used in administering local drugs from plant; also ethyl acetate can be used to extract tannins and cardiac glycosides.

The LC_{50} values performed on the crude petroleum ether, ethyl acetate and water extracts revealed that petroleum ether extract was lethal at all concentrations, while ethyl acetate and water extracts were within a safe range. This is the first reported determination of the LC_{50} of the plant extracts.

The T. L. C. result of petroleum ether extract initially partitioned into ethanol soluble and ethanol insoluble revealed six spots and five spots respectively, as earlier on reported by Baslas and Agarwal (1979).

Functionality test, which were further performed on the T. L .C fractions of water and ethyl acetate extracts, couple with infrared analyses revealed that the first water extract fraction was saponin which was active on *Pseudomonas aeruginosae* and *Staphylococcus aureus*. The second water fraction did not respond to any of the functionality test and was not active on any of the microorganism. The third water fraction was a hydrolysable tannin which was not active on any of the microbes. The first, third, fourth and fifth ethyl

acetate fractions were condensed tannins (the fourth and fifth being flavonoids) and were active on *Escherichia coli*, *Pseudomonas aeruginosae*, *Staphylococcus aureus*, and *Salmonella typhi*. The sixth fraction of ethyl acetate extract did not respond to any of the functionality tests, but was active on *Ps. aeruginosae*, *E. coli* and *S. typhi*. The activity of the water and ethylacetate plant extracts on *Escherichia coli*, *Pseudomonas aeruginosae*, *Staphylococcus aureus*, and *Salmonella typhi* is reported for the first time.

This result shows that water and ethyl acetate can also be use, just as the reported alcohol and petroleum ether, to extract the bioactive compounds from *E. hirta*. The extract can be use to cure wound infection, diarrhea and conjunctivitis caused by *S. aureus* and *E. coli*, urinary tract infections also caused by *E. coli*. As well as for respiratory tract infections, burns and wound infections caused by *Ps. Aeruginosae*, gastroenteritis and typhoid fever caused by *S. typhi*, and urinary tract infection and acute diarrhea in children caused by *Kleb. pneumoniae*. This justifies the claim in the literature and by the locals that *E. hirta* is use in remedying these heath conditions.

5.1. RECOMMENDATION

Proper structural elucidation using a powerful NMR (higher than 60MHz) and mass spectroscopic analysis needs to be carried out on the water and ethyl acetate extracts fractions. Such study will ascertain the structure of bioactive compounds isolated by these solvents, especially that of water, which is the common medium for plants drug consumption.

Further antimicrobial sensitivity tests needs to be carried out to also ascertain the minimum inhibition concentrations (MIC), for the various plant fractions.

REFERENCES.

1. Awosika, D.F. (1986). 'Preliminary antimicrobial screening of *Onion segatum*'. Clinical Pharmacy and Herbal Medicine, **9**, 28-31.

2. Bailey, L.H. (1987). 'Manual of Cultivated plants'. Macmillan, New York.

3. Barry, A. L. and Thornsberry, C. (1990). 'Susceptibility test: Diffusion procedures'. In: Balows, A. Hausler, W.J., Herrmann, K.L., Ienburg, H. D., and Shadomy, H. J. Manual of clinical Microbiology American Society For Microbiology, Library of Congress Cataloguing in Publication Date. pp 1117-1124 .

4. Baslas, R. K. (1982). 'Phytochemical studies of the plants of genera Euphorbia', Part 11; Herba Hungarica, **21(1)**, 115-126. In: Horticultural abstract, (1985), 53.

5. Baslas, R. K. and Agarwal, R. (1980). 'Isolation and characterization of different constituents of *Euphorbia hirta Linn*'. Current Science, **49(8)**, 311-312.

6. Baslas R. K. and Agarwal, R. (1980b.). Chemical investigation of some anticancer plants of Euphorbia genus. Ind. J. Chem., **19B(8)**, 717-718.

7. Belkin, M., Fitzgerald, D. B. and Cogan, G. W. (1952). Tumour damaging capacity of plant materials-plants used as cathatic. . J. Nat. Cancer, **13**, 139-155. In: Sofowora, A. (1982). Medicinal plants and Traditional Medicine in Africa.

8. Blanc, P. and De Saqui-sannes, G. (1972). Plant phytoter., **6**, 106.

9. Boakye-Yiadom, K. (1979). The antimicrobial activity of some West African medicinal drugs, Quart. J. Crude Drug Research, **2**, 78-80. In: Sofowora, A. (1982). Medicinal Plants and Traditional Medicine in Africa.

10. Boakye-Yiadom, K. and Heman-Ackah, S. M. (1979). Cryptolepine hydrochloride effect on *Staphylococcus aureus*. J. Pharm. Sci., **68**, 1510-1514.

11. Boda, M. (1997). Screening of Local Medicinal plants on *Trichomu gallinae*. An unpublished M.Sc. Thesis, Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

12. Braude, A. I. (1982). Microbiology. Saunders Company. pp. 276, 315, 318, 345, 349, 365 and 370.

13. Budzikiewicz, J. Djerassi, C. and Williams, D. (1964). Structure Elucidation of Natural Products by Mass Spectroscopy, **2**, 124.

14. Claus, P. E., Tyler, V. E., and Brady, L. R. (1970). Pharmacognosy. Henry Kimpton, London, p.121.

15. Dalziel, J.M. (1916). A Hausa Botanical Vocabulary. T. Fisher Unwin, London, p.66.

16. Dalziel, J. M. (1955). The Useful Plants of West Tropical Africa. Crown Agents, London, pp.340 and 433.

17. Debaile, G., and Petard, P. (1953). "Notes preliminaires sur les plants antidysenteriques du Soudan et du Haute-Volta"; Bull. Med. AOF, **10**, 11-14.

18. Dhar, M. L., Dhar, M. M., Dhawan,B. N., Mehrotra, B. N., and Ray, C. (1968). Screening of Indian plants for biological activity. Ind. J. Expt. Bio., **6**, 232-236.

19. Dickshit, R. A.O. (1934). Proceedings of Ind. Sci. Congress. pp.349. In: Sofowora, A. (1982). Medicinal plant and Traditional Medicine in Africa. pp . 208 –213.

20. Eddy, N. B. and Leimbach, D. (1953). J. Pharmac. Exp Ther., **107**, 385.

21. El- Naggar, I., Beal, J. L., Parks, L. M., Salaman, K. N. and Patil, P. (1978). A note on isolation and identification of two phamacologically active constituents of *Euphorbia pilulifera*. Lioydia, **4(1)**, 73.

22. Elujoba, A. A. (1989). Positive trends in pharmacognosy. Pharmacy World Journal, **6(1)**, 5-6.

23. Evans, F. J. Schmidt, R. J. (1976). Phytochemistry, **15**, 333-335.

24. Evans, F. J. and Schmidt, R. J. (1978). Phytochemistry, **17**, 1436-1437.

25. Falkow, S., Small, P., Isberg, R., Hayes, S. F. and Corwin, D., (1987). A molecular strategy for the study of bacterial invasion. Rev. Infect. Dis., **9(suppl. 5)**, S450. In: Davis, B. D; Duibecco, R., Eisen, H. D, Dulbecco, R., Eisen, H. N., and Ginsberg, H. S. (1990). Microbiology. Harper and Row Publishers inc. Philadelphia, Pensylvania, U.S.A., pp. 568-598.

26. Gupta, D. R. and Garg, S. K. (1965). A chemical examination of *Euphorbia hirta Linn.* Bull. Chem. Soc.(Japan), **39**, 2532-2534.

27. Harbone,J.B.(1973). Phytochemical methods. Chapman and Hall Ltd., London.

28. Hargreaves, B. J. (1979). Harmful and helpful *Euphobiaceae* in Malawi. 3rd OAU/STRC International African Symposium on African Medicinal Plants and Traditional Pharmacopoeia, Abidjan.

29. Haslam, E. C. (1966). Chemistry of Vegetable Tannins. Academic Press. London and New York, pp.20, 94-100 and 102-105.

30. Hazleton, L. W. and Hellerman, R. C. (1984) Studies on the pharmacology of *Euphorbia pilulifera*. J. Amer. Pharm. Ass. (Sc. Ed.), **37**, 491-497.

31. Hellerman, R. C. and Hazleton, L. W. (1950). The antispasmodic action of *Euphorbia pilulifera*. J. Amer. Pharm. Ass., **39**, 142-146.

32. Holmes, E. M. (1923). *Euphorbia pilulifera*. J. Pharm. Pharmac., **10**, 162-163.

33. Hui, W. H. and Sung, M. L. (1968). Aust.J.Chem., **21**, 2137.

34. Hutchinson, J. and Dalziel, J. M. (1954). Flora of West Tropical Africa. Crown Agents, **1(1)**, 133-135.

35. Iwangwu, M. A. (1987). Dosage standardization in traditional medicinal practice. Clinical and Herbal Medicine, **3(3)**, 21-23.

36. Jawetz, E., Melnick, J. L., Adelberg, E. A, Brooks, G. F., Butel, J. S. and Ornston, L. N. (1989). Medicinal Microbiology (ed). Appliton and Lange, pp.187-191, 204-213.

37. Kerharo, J. and Adams, J.G. (1974). La pharmacopee senegalaise traditionelle. Vigot Freres, Paris. In: Sofowora, A. (1982). Medicinal Plants And Traditional Medicine in Africa. John Wiley & Sons Ltd. pp. 208-213.

38. Kinghorn, A. D. and Evans, F. J. (1975). J. Pharm. Pharmac., **27**, 329-333.

39. Kirchner, J. G. (1967). Thin Layer Chromatography. John Wiley & Sons. New York. London. Sydney.

40. Lind, E. M. and Tallantire, A. C. (1971). Some Common flowering Plants of Uganda, rev. ed. Oxford University Press, Nairobi. p.182.

41. Logie, C. G., Grue, M. R. and Liddell, J. R. (1994). Proton NMR spectroscopy of pyrrolizidine alkaloids. Phytochemistry, **37**, 43-45.

42. Machata, G. (1960). Mikrochim. Acta 79. In: Kirchner, J. G. (1967). Thin Layer Chromatography. John Wiley & Sons. New York London. Sydney. p.164.

43. Meyer, B. N., Ferigni, N. R., Putnam, J. E., Jacobsen, L. B., Nicohols, D. E. and Mclaughli, I. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica, **45**, 31-35.

44. Nnaemeka, I. N. (1991). Screening of some Nigeria Medicinal plants for anti-bacteria activities. Unpublished Thesis; Ahmadu Bello University, Zaria, Nigeria

45. Novick, R., Edelman, I., Schwesinger, M., Gruss, A., Swanson, E. and Pattee, P. A. (1979). Genetic translocation in *Staphylococcus aureus*. Proc. Natl. Acad. Sci., U. S. A., **76**, 400. In: Davis, B. D., Dulbecco, R., Eisen, H. N. and Gingsberg, H. S. (1990). Microbiology. Harper and Row Publishers. Philadelphia. Pensylvania. U. S. A. pp. 539-550.

46. Oliver, B. (1959). Medicinal Plants in Nigeria. College of Acts, Science And Technology: Ibadan. In: Sofowora, A. (1982). Medicinal Plants and Traditional Medicine in Africa. John Wiley & Sons Ltd. pp. 208-213.

47. Palacious, P., Joshi, K. C., Sing, P. and Tanaja, S. (1983). Some pharmacologically active substances from Medicinal plants. Planta Medica, 49 (1 and 2), 127-128 and 199.

48. Panscorbo, S. and Hammer, R. H. (1972). J. Pharm. Sci., **61**, 954.

49. Persing, G. J. and Quimby, M. W. (1967). Nigeria plants III: Phytochemical screening for alkaloids, saponins and tannins. J. pharm. Sci., **56(11)**, 1512-1515.

50. Raymond, H. (1937). Several physiological properties of an alkaloid from *Cryptolepis sanguinolenta* schlecter. C.R. Soc. Biol, **126**, 768-770.

51. Ridet, J. and Chartol, A. (1964) Les properties; antidysenteriques de l' *Euphorbia hirta*. Med. Trop. Fr., **24(2)**, 119-143. In: Sofowora A. (1982). Medicinal and Traditional Medicine in Africa. John Wiley & Sons. pp 280-213.

52. Rishi, A.K., George, A. and Kapoor, R. (1979). Planta Medica, **35**, 195.

53. Sahm, D. F. and Washington (II), J.A. (1990). Antibacterial susceptibility tests: Dilution Methods. In: Balows, A., Hausler (Jr), W. J., Herrmann, K.L., Isenburg, H. D. and Shadomy, H. J. Microbiology. An American Society for Microbiology, Library of Congress Cataloguing in Publication Data. pp. 1105-1115.

54. Saunders, L. and Fleming, R. (1971). Mathematics and Statistics:(For use in Biological and Pharmaceutical Science).(ed) The Pharmaceutical Press, London. pp. 199-207, 225-277 and 286.

55. Seshadri, T. R. and Vydeeswaran, S. (1971). Chemical constituents of *Daemia extensa* roots. Curr. Sci. (India), **49**, 594-595.

56. Shriner, R.L. and Fuson, R.C. (1979). The Systematic Identification of Organic Compounds. John Wiley and Sons. pp. 279.

57. Silverstein, R.M., Bassler, G.C. and Morrill, T. C. (1970). Spectrometric Identification of Organic Compounds (ed.). Von Reinhold Company.

58. Sofowora, A. (1982) Medicinal Plants and Traditional Medicine in Africa. John Wiley and Sons Ltd. p. 209.

59. Touchstone, J. C. (1992). Practice of Thin Layer Chromatography. John Wiley & Sons inc. New York/Chichester/Brisbane/Singapore. p. 178.

60. Trease, G. E. and Evans, W.C. (1989). Pharmacognosy(ed.) Bailliere tindall-Eastbourn. pp. (386-393) [a], (395-398) [b], (480-499) [c], (500-517) [d] and (544-547) [e].

61. Tyler, V. E., Brady, L. R. and Robbers, J. E. (1976). Pharmacognosy (ed.) Lea and Febiger. p. 73.

62. Vogel, A. I. (1978). A Textbook of Practical Organic Chemistry. B. S. Furniss. London: Longman. pp. 161-173.

63. Von-Oetinger, W.F. (1956). Handbook of Toxicology, **1**, 1-4.

64. Wong-Ting-Fook, W.T.H. (1980). The Medicinal Plants of Mauritius. ENDA Publication, No. 10, Dakar. In: Sofowora, A. (1982). Medicinal Plants and Traditional Medicine in Africa. John Wiley & Sons. pp.208-213.

65. Ye, Y., Qin, G., and XU, R. (1994). Alkaloids from *Stemona tuberosa*. Phytochemistry, **37(4)**, 1201-1203, 1205-1208

66. Zechmeister, K., Brandl, E., Hoppe, W., Hecker, E., Opferkuoh, H. J. and Adolf, W. (1970). Tetrahedron Letters, **47**, 4075.

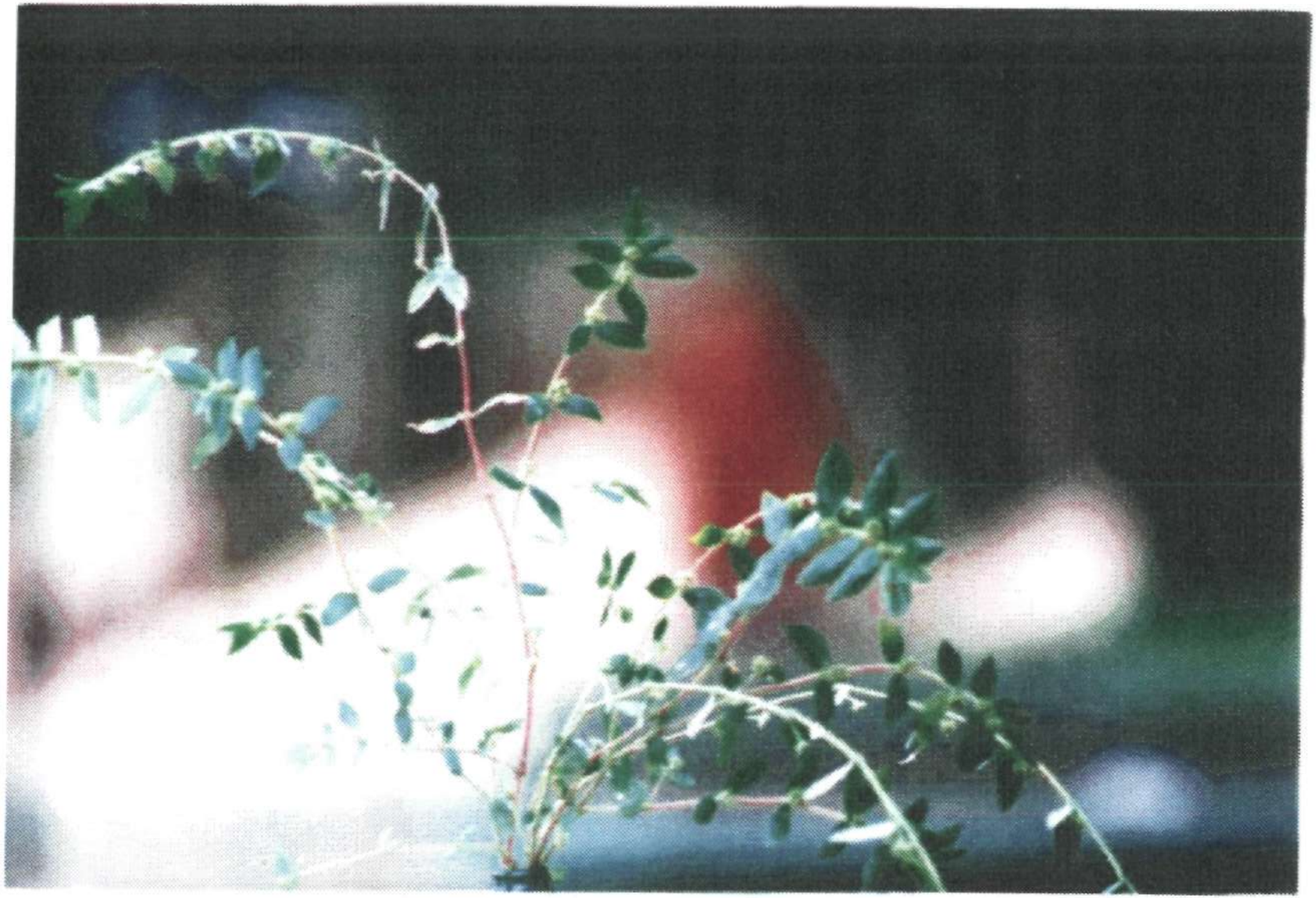


Figure 3.1: Picture of the Plant Euphorbia hirta Linn

Figure 4.1: Plot Probits Vs Log.Dose for Water Extract

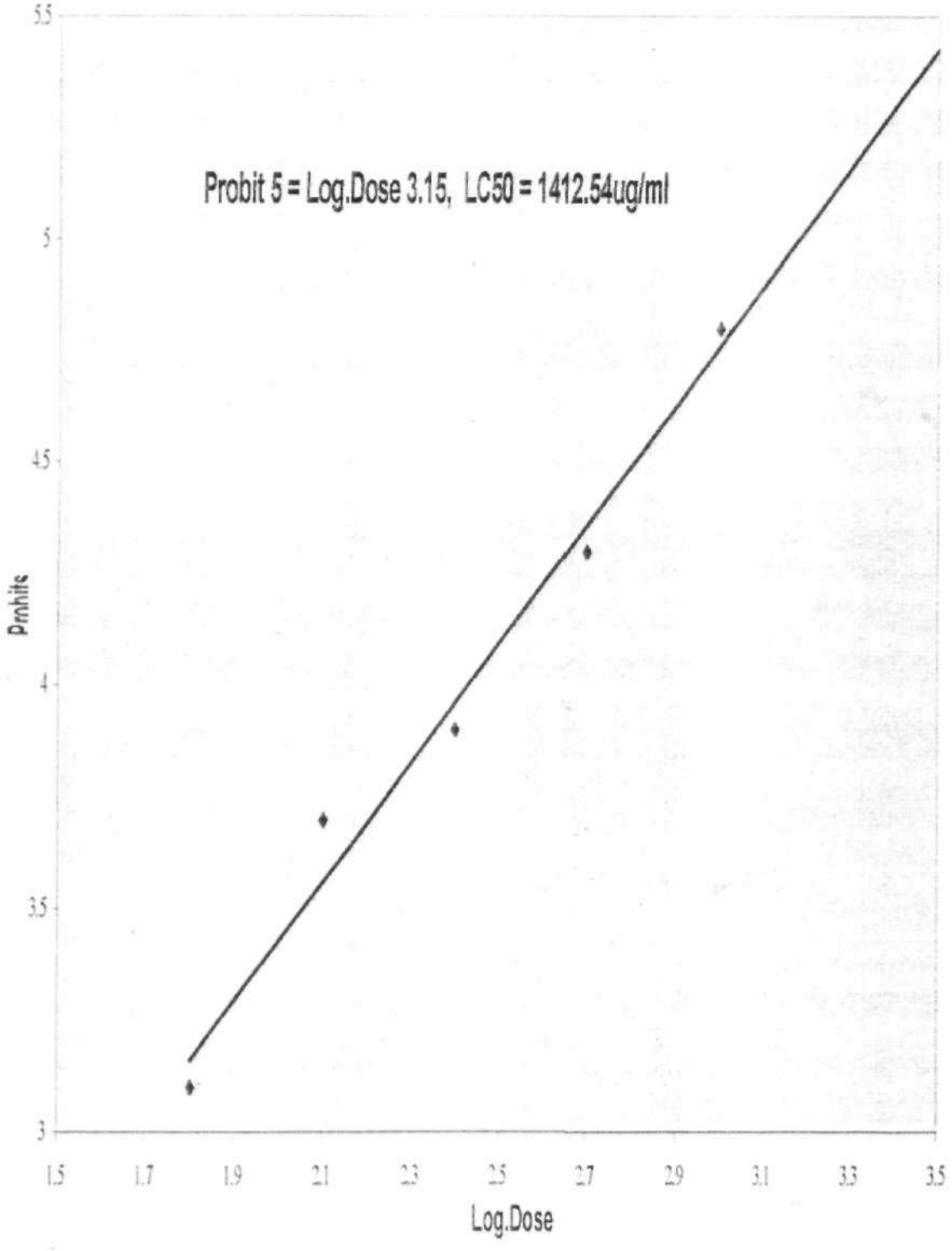


Figure 4.2: Plot Probits Vs Log Dose for Ethyl Acetate Extract

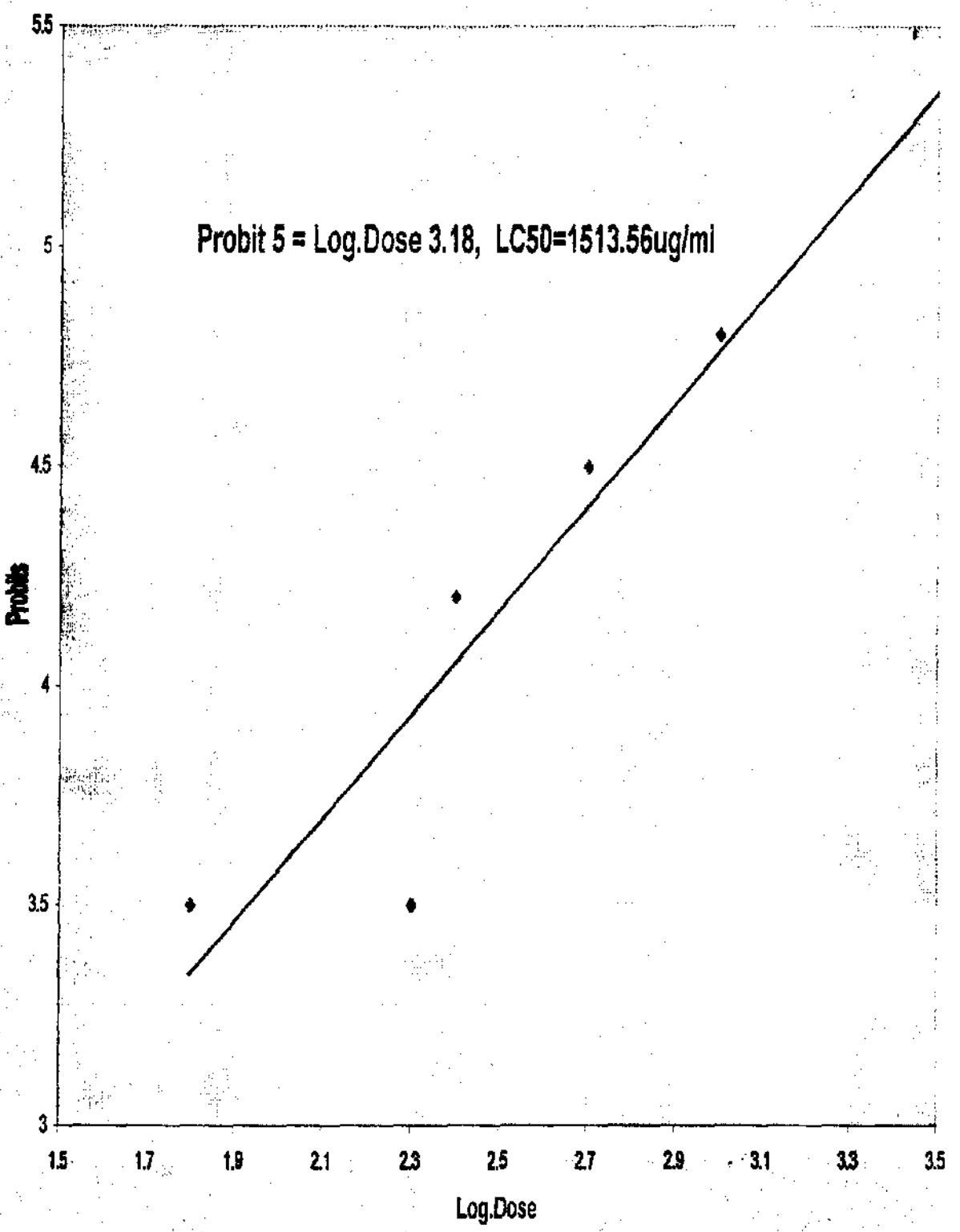


Figure 4.3: Plot Probits Vs Log.Dose for Petroleum Ether Extract

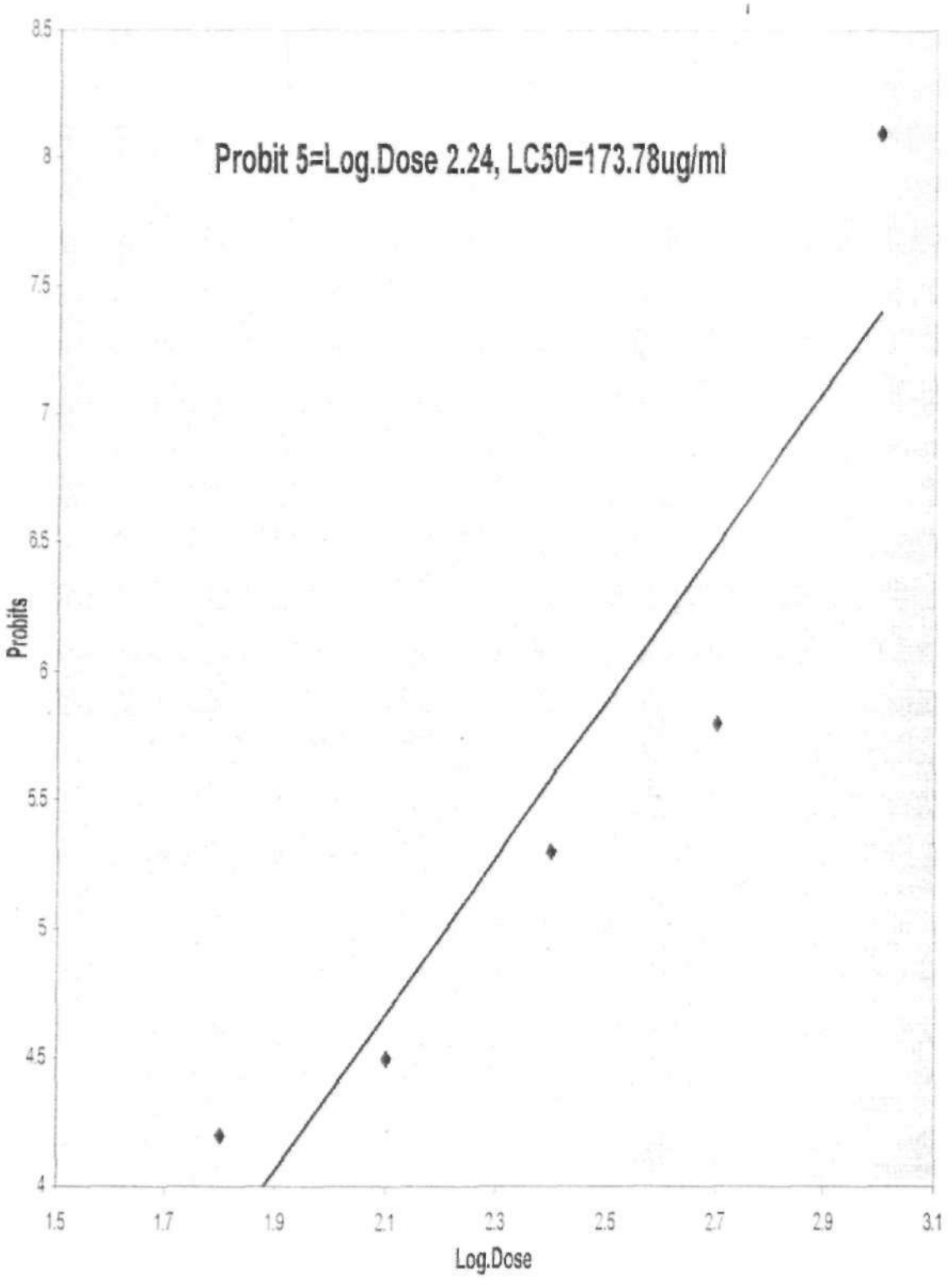
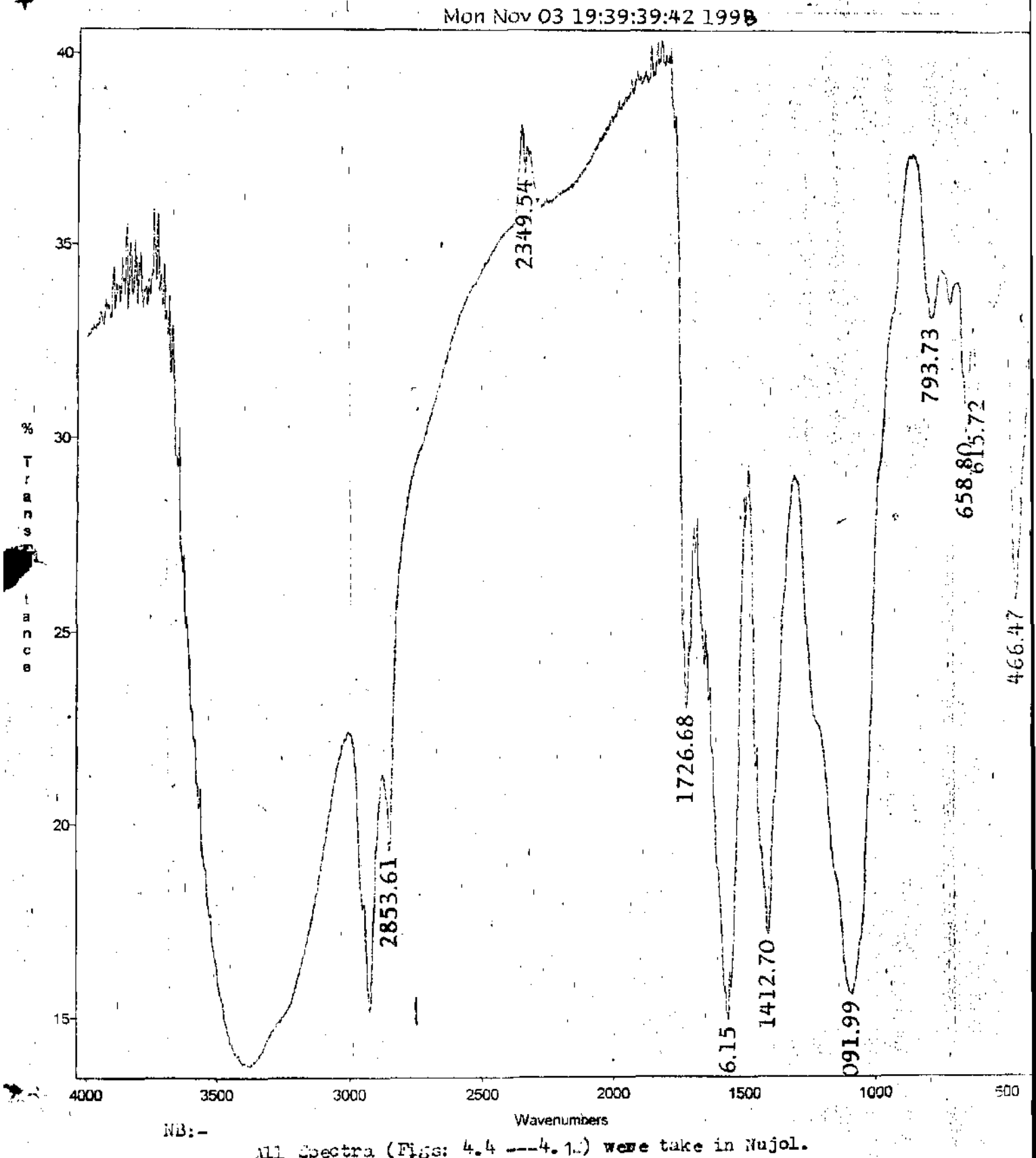


FIGURE 4.4: Infrared Spectrum of Ethyl Acetate Extract Fraction EA/1



NB:- All Spectra (Figs: 4.4 ---4.1.) were take in Nujol.

FIGURE 4.3: Infrared Spectrum of Ethyl Acetate Extract Fraction EA2

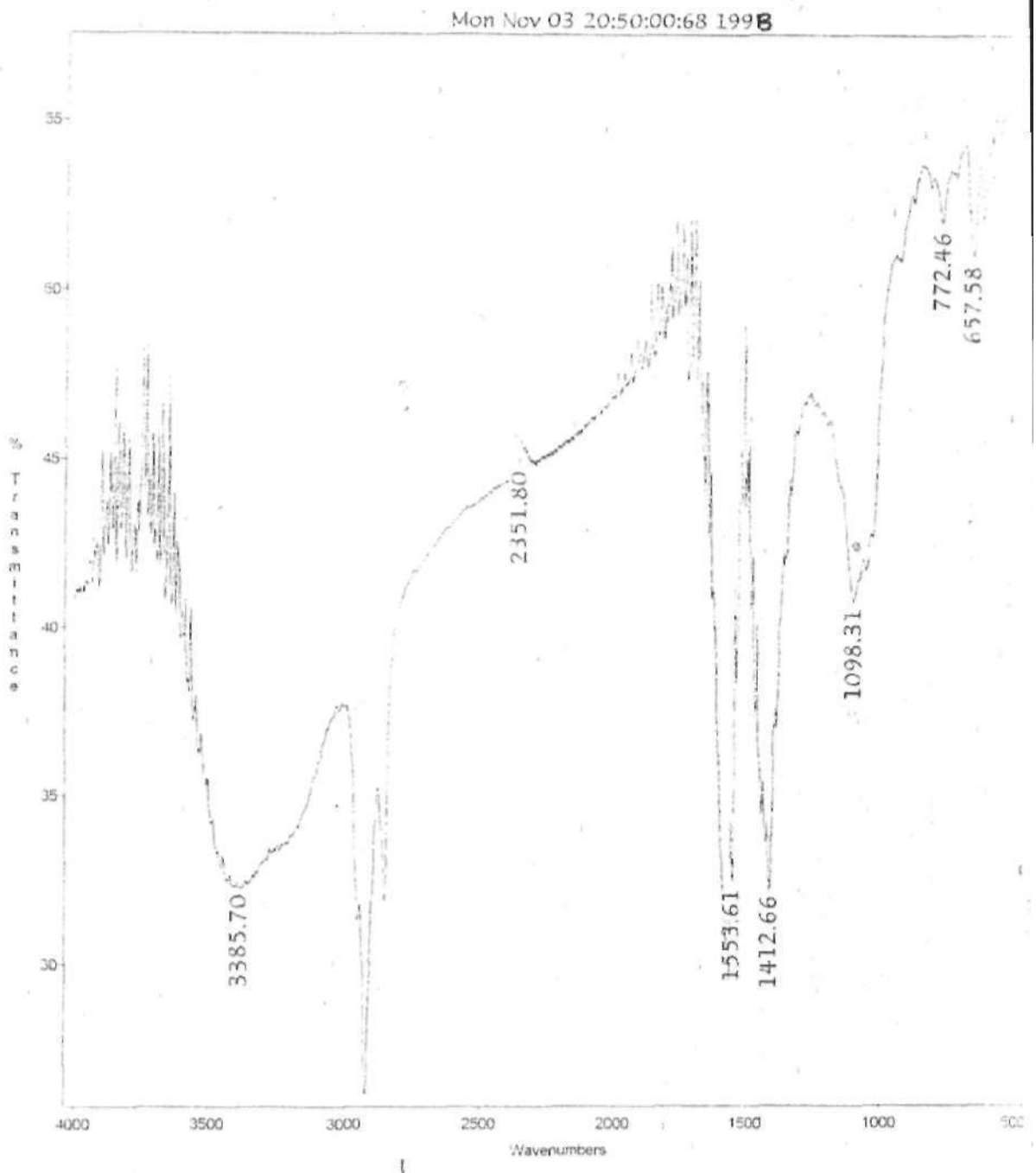


FIGURE 4.6: Infrared Spectrum of Ethyl Acetate Extract Fraction EA 3

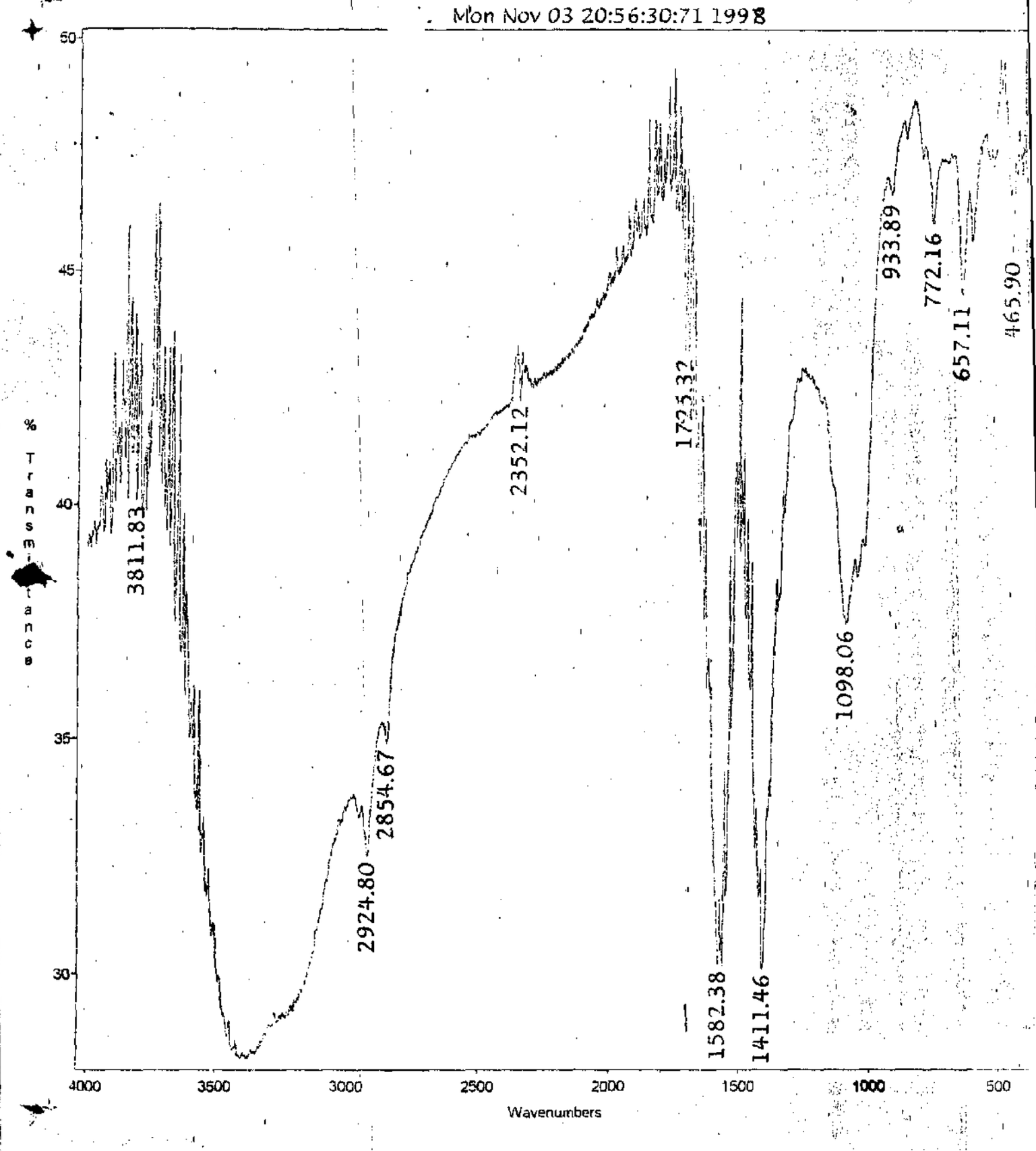


FIGURE 4.7 Infrared Spectrum of Ethyl Acetate Extract Fraction EA 4

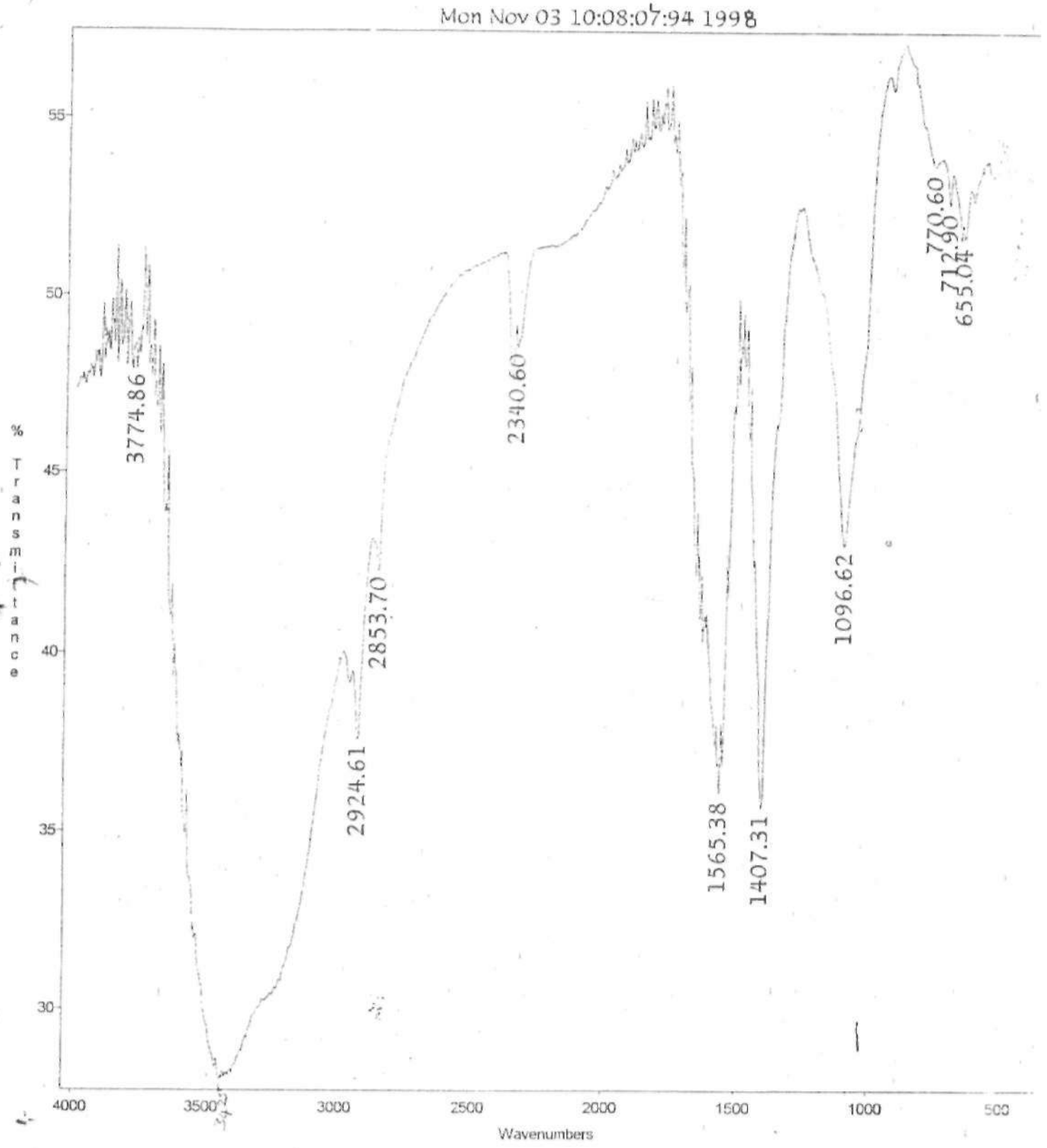


FIGURE 4.8: Infrared Spectrum of Ethyl Acetate Extract Fraction EA 5

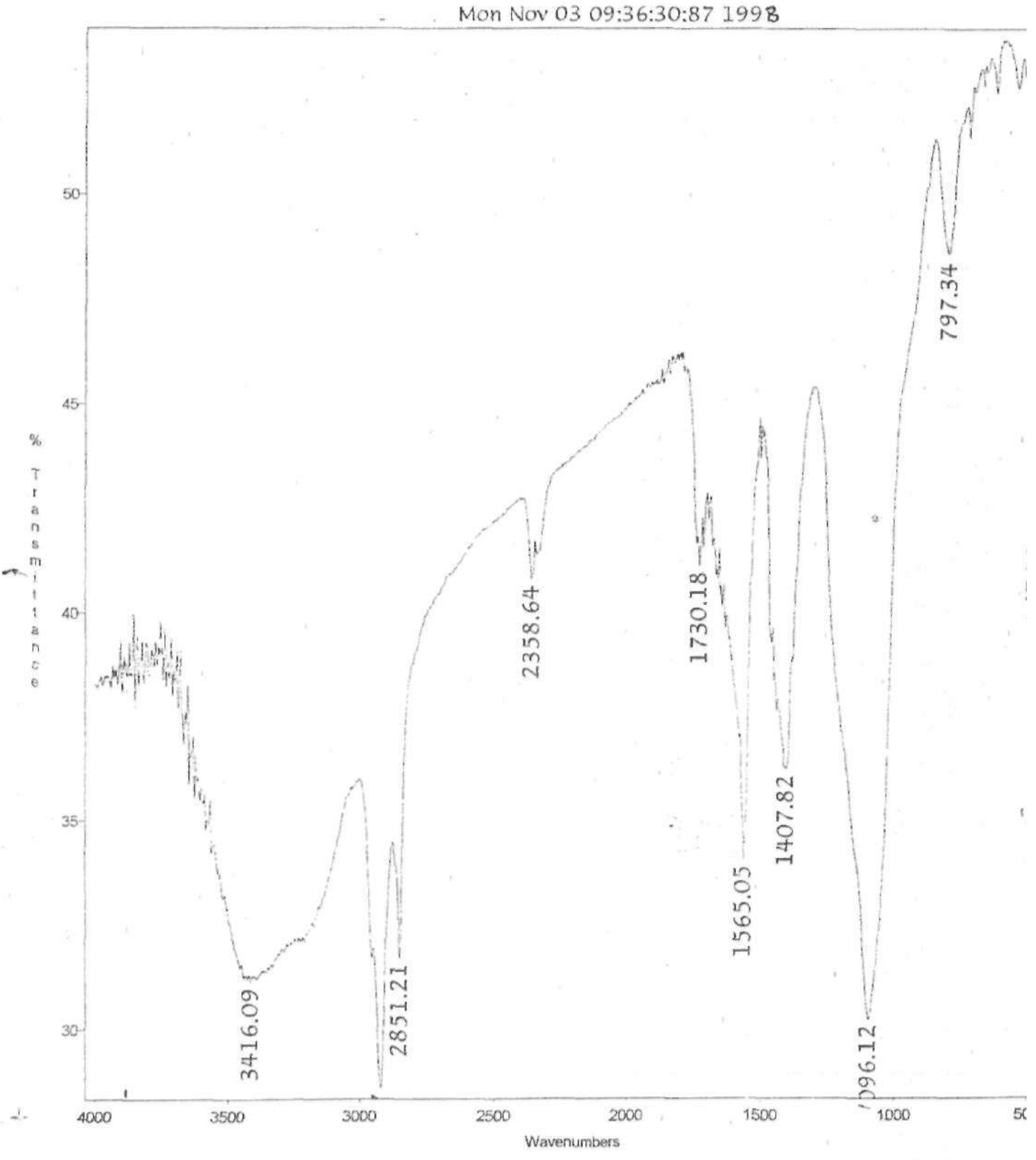


FIGURE 4.9: Infrared Spectrum of Ethyl Acetate Extract Fraction EA 6

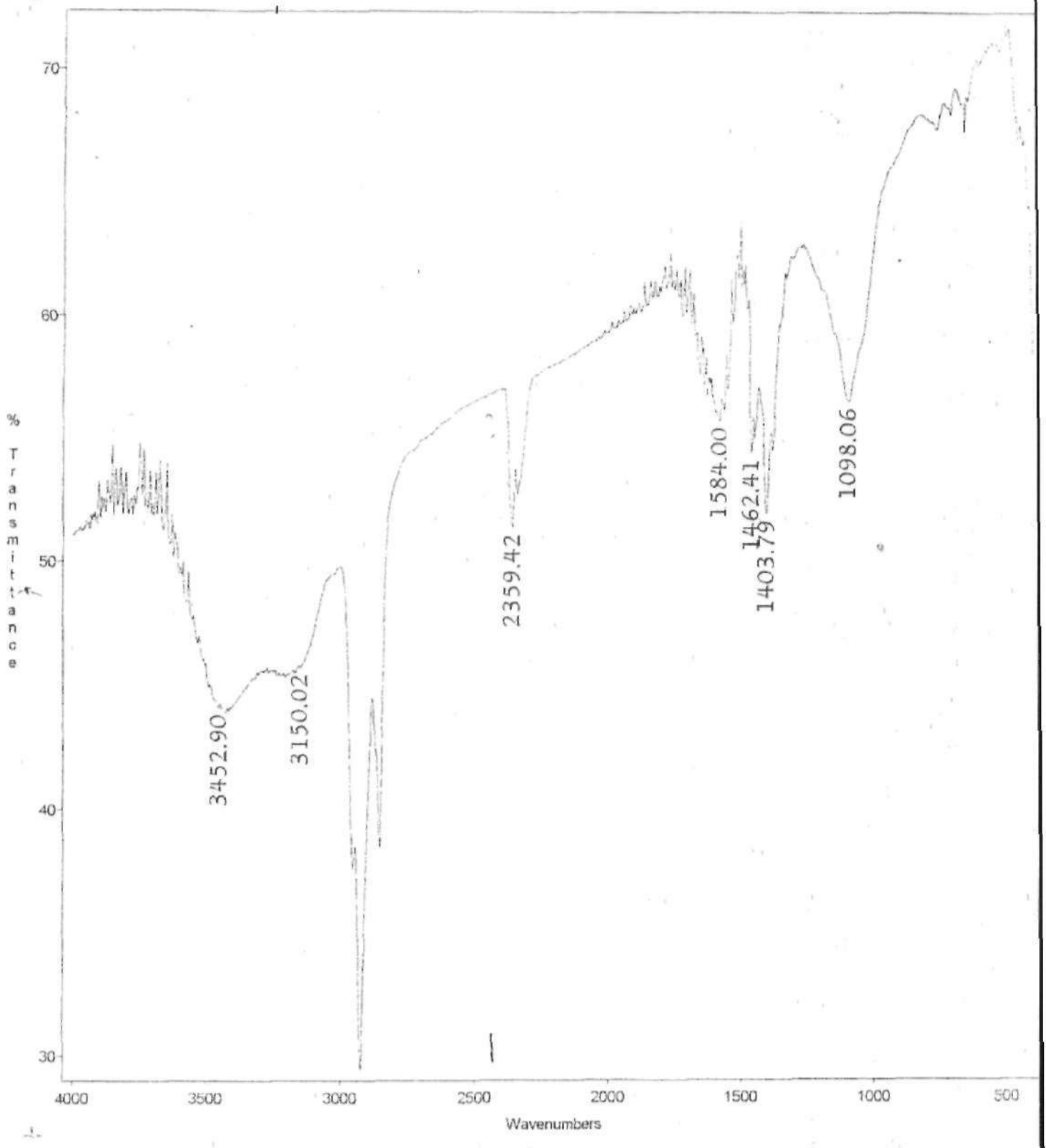


FIGURE 4.10: Infrared Spectrum of Water Extract Fraction 7.1

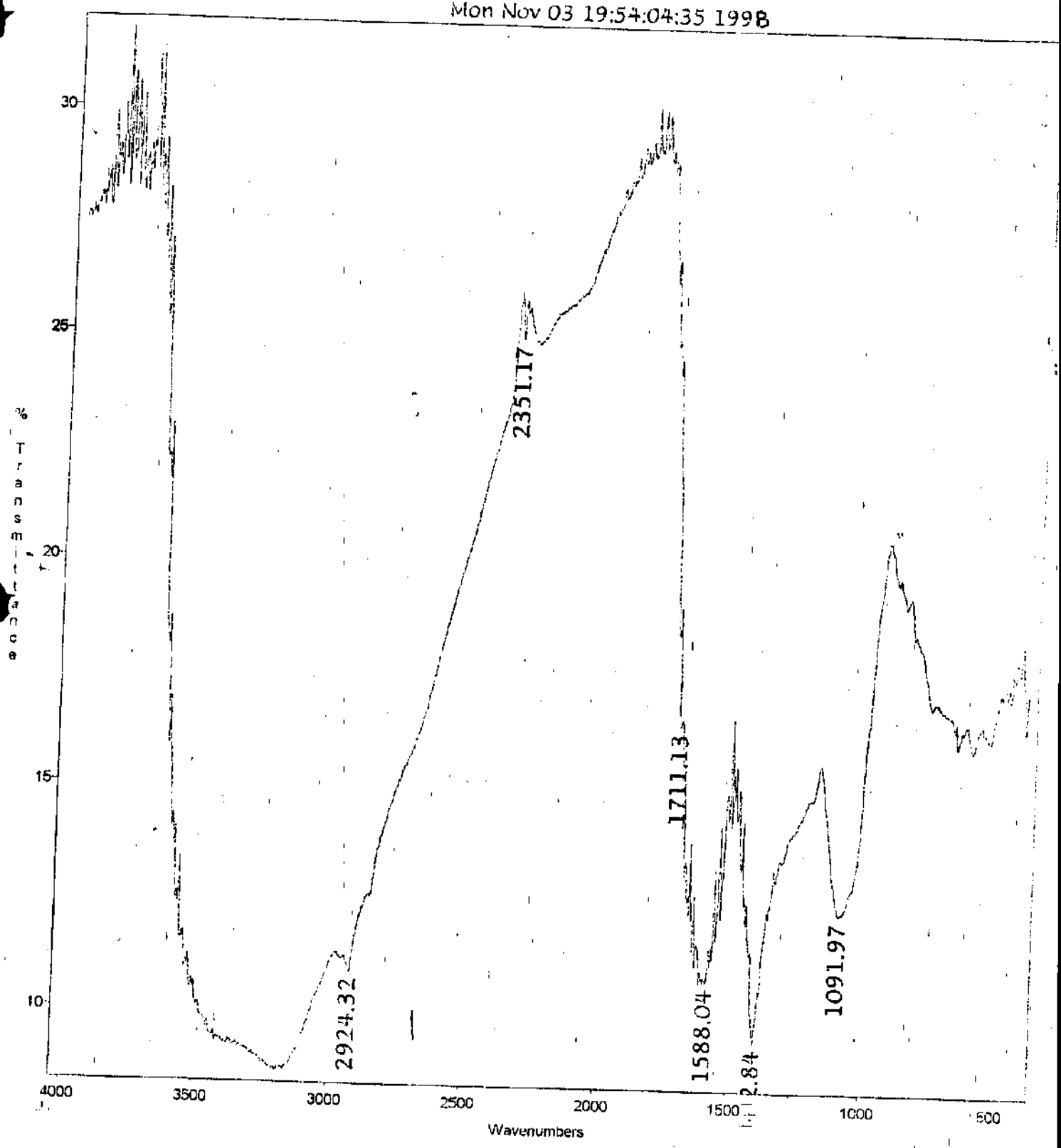


FIGURE 4.11: Infrared Spectrum of Water Extract Fraction W 2

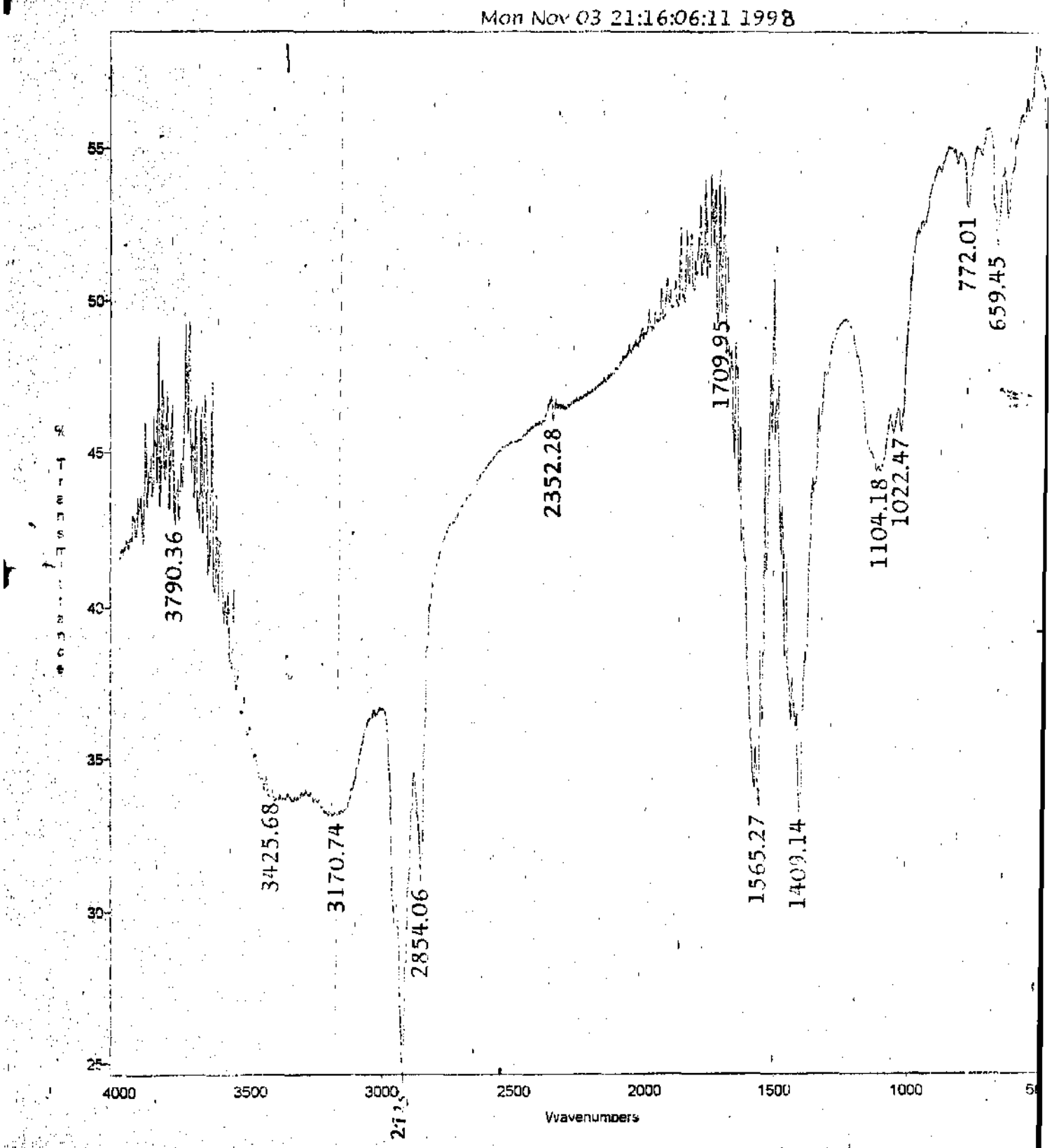
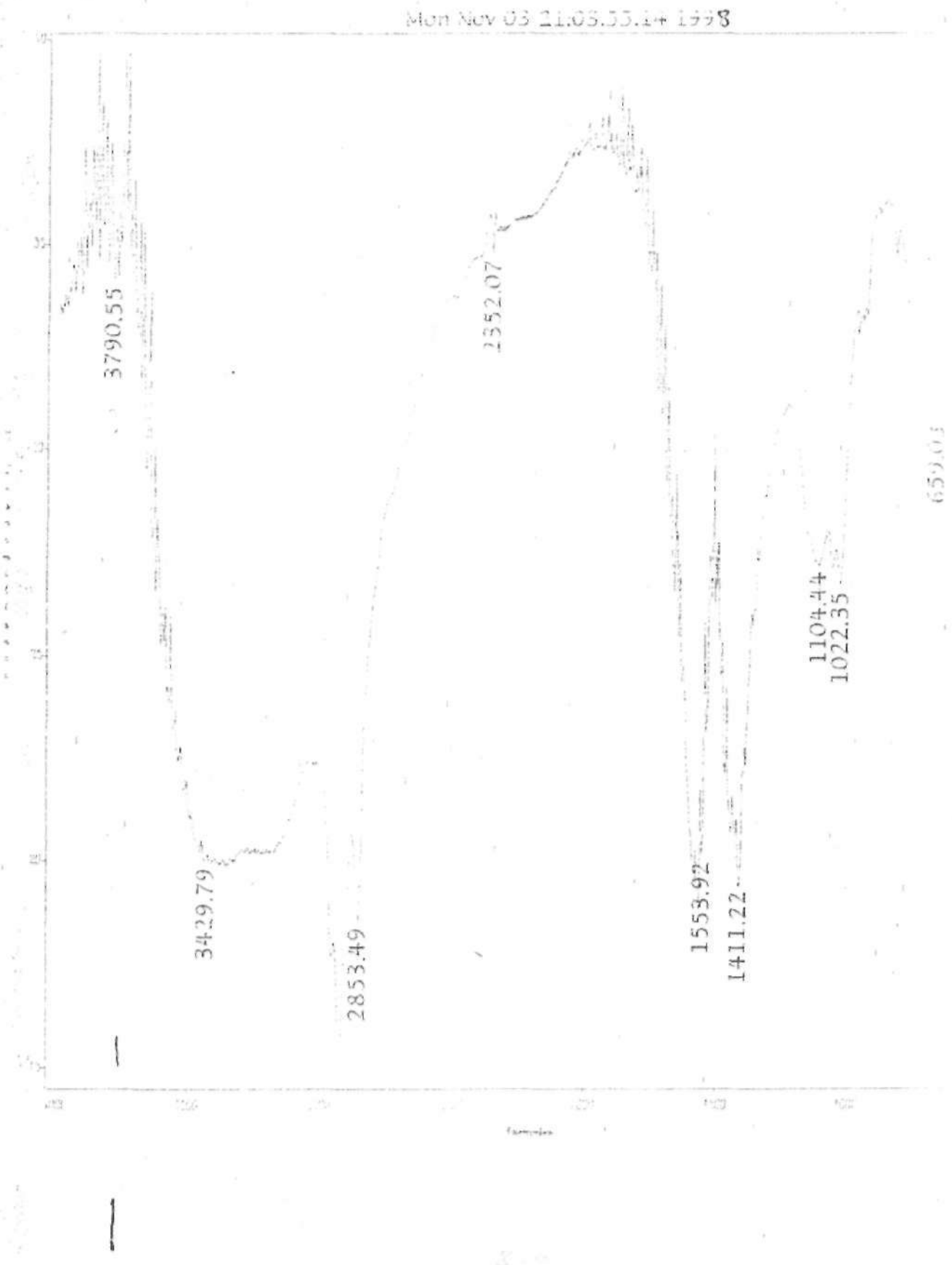


FIGURE 4.12: Infrared Spectrum of Water Extract Fraction W 3



nujol Tue May 20 08:51:08:63 1998

APPENDIX I

In order to get the most reliable value of LC_{50}, the equation of the probit, log dose regression is calculated.

The regression line equation is given as

$$(Y - \bar{Y}) = b(X - \bar{X})$$

Where $\bar{y}$ and $\bar{x}$ are the mean values for the probits and log.dose respectively, and 'b' (regression coefficient) is calculated by the following equation

$$b = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sum(X^2) - \sum(X)^2/N}$$

If a scatter diagram is drawn in which the variate 'y' is independent of 'x', the slope of the regression line will not be significant, and the line may be parallel to the x-axis; the variations in 'y' are only random variations about their mean $\bar{y}$. If 'b'=0, the two variates are independent.

However, before carrying out a regression calculation, a test is made to determine whether there is a significant linear correlation between the two sets of figures. This can be done by calculating the correlation coefficient, 'r'. This quantity is symmetrical with respect to both 'x' and 'y' and has a magnitude of 1 when there is a perfect linear correlation between the two variables. If 'x' and 'y' are independent, then 'r'=0. The correlation coefficient is defined by the equation-

$$r = \frac{\sum\{(X-\bar{X})(Y-\bar{Y})\}}{\sqrt{\{\sum(X-\bar{X})^2\sum(Y-\bar{Y})^2\}}}$$

$$r = \frac{\sum(XY)-\sum(X)\sum(Y)/N}{\sqrt{\{(\sum(X^2)-\sum(X)^2/N)(\sum(Y^2)-\sum(Y)^2/N\}}}$$

If the magnitude of 'r' calculated from a set of data is greater than the theoretical value for a chosen probability level, then there is a significant correlation between 'x' and 'y'; if the magnitude of 'r' is less than the theoretical value there is no significant correlation between 'x' and 'y', it is not worth proceeding with the regression analysis.

Some theoretical values of 'r' for P'=0.05 are given in the table in Appendix II.

Variance About Regression

The variance V_y of a set of points about their regression line is the sum of the squared vertical deviations from the line divided by the number of degree of freedom in the calculation, usually N-2, where 'N' is the number of points. The final equation for calculation of V_y is –

$$V_y = (\sum dy^2 - b^2\sum dx^2)/\phi$$

A formula by means of which the variance V_x of a value of 'X' can be determined from an observed reading of 'Y' using the regression line equation is-

$$V_x = \frac{V_y}{b^2}[1+\frac{1}{N}+\frac{(Y-\bar{Y})^2}{b^2}\bullet\frac{1}{\sum\{(X-\bar{X})^2\}}]$$

From this formula, limits of error can be calculated as $\pm t\sqrt{V_x}$. The value of ϕ used to obtain 't' is N-2.

Appendix 1A

Calculation of LC_{50} values of water extract EU_1

X	X^2	Y	Y^2	XY
3.000	9.000	4.82	23.232	14.46
2.699	7.285	4.26	17.893	11.42
2.398	5.750	3.87	14.977	9.28
2.092	4.397	3.72	13.838	7.78
1.796	3.226	3.12	9.734	5.60

$$\sum(X) = 11.985 \sum(X^2) = 29.658 \sum(Y) = 19.79 \sum(Y^2) = 79.674 \sum(XY) = 48.54$$

$$number.of.\deg rees.of.freedom, ... N = 5, ... \bar{X} = 2.40, ... \bar{Y} = 3.95$$

(i) Correlation Coefficient, 'r':

$$r = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sqrt{\{(\sum(X^2) - \sum(X)^2/N)(\sum(Y^2) - \sum(Y)^2/N\}}}$$

$$\sum(X)\sum(Y)/N = 11.99 \times \frac{19.79}{5} = 47.38$$

$$\sum(X^2) - \sum {(X)^2}/{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$\sum(Y^2) - \sum \frac{(Y)^2}{N} = 79.67 - \frac{19.76^2}{5} = 1.58$$

$$r = \frac{48.54 - 47.38}{\sqrt{0.91 \times 1.58}}$$

$$r = 0.97$$

(ii) The regression coefficient, 'b':- The equation is given by

$$b = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sum(X^2) - \sum(X)^2/N}$$

$$b = \frac{48.54 - 47.38}{0.91}$$

$$b = 1.27$$

(iii) Regression line equation - is given by

$$(Y - \bar{Y}) = b(X - \bar{X})$$

Therefore Y-3.95 = 1.27(X-2.40)

Y = 1.27X + 0.90

$\Rightarrow$ The regression line equation for EU_1 is Y = 1.27X + 0.90

(iv) The variance V_y of Y about the regression line is given by

$$V_y = (\sum dy^2 - b^2 \sum dx^2)/\phi$$

$$\sum dy^2 = \sum(y^2) - \sum\frac{(y)^2}{N} = 79.67 - \frac{19.76^2}{5} = 1.58$$

$$\sum dx^2 = \sum(x^2) - \sum\frac{(x)^2}{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$V_y = \frac{1}{3}(1.58 - 1.27^2 \times 0.91) = \frac{1}{3}(1.58 - 1.47) = 0.037$$

The variance V_x of a value of X predicted from the regression line when Y=5 is given by

$$V_X = \frac{V_y}{b^2}\left[1 + \frac{1}{N} + \frac{(Y-\bar{Y})^2}{b^2} \bullet \frac{1}{\sum\{(X-\bar{X})^2\}}\right]$$

$$\sum(X-\bar{X})^2 = \sum(X^2) - \sum(X)^2/N = 0.91$$

$$V_X = \frac{0.037}{(1.27)^2}\left[1 + \frac{1}{5} + \frac{(5-3.95)^2}{(1.27)^2} \bullet \frac{1}{0.91}\right]$$

$$V_X = 0.023(1.2 + 0.75) = 0.045$$

$$\therefore \sqrt{V_X} = 0.212$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and $P = 0.95$; these limits are $\pm 3.18 \times 0.21 = \pm 0.67$.

Calculation of the Value of 'X' from the Regression Line Equation

The regression line equation for EU_1 is $Y = 1.27X + 0.90$,

The value of X calculated from the regression line equation for $Y = 5$,

$5 = 1.27X + 0.90$

$X = 4.1/1.27 = 3.15$

Since the limit of error for 'X' is 0.67

Then $X = 3.15 \pm 0.67$

Antilog of $X = 1412 \pm 4.68$

Therefore LC_{50} $(EU_1) = 1412 \pm 4.68 \mu g/ml$

Appendix 1B

Calculation of LC_{50} values of ethyl acetate extract EU_2

X	X^2	Y	Y^2	XY
3.000	9.000	4.75	22.56	14.26
2.699	7.285	4.48	20.07	12.09
2.398	5.750	4.16	17.31	9.98
2.092	4.397	3.45	11.90	7.22
1.796	3.226	3.45	11.90	6.20

$$\sum(X) = 11.985 \sum(X^2) = 29.658 \sum(Y) = 20.29 \sum(Y^2) = 83.74 \sum(XY) = 49.75$$

$$number.of.\deg rees.of.freedom,...N = 5,...\bar{X} = 2.40,...\bar{Y} = 4.06$$

(i) Correlation Coefficient, 'r':

$$r = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sqrt{\{(\sum(X^2) - \sum(X)^2/N)(\sum(Y^2) - \sum(Y)^2/N\}}}$$

$$\sum(X)\sum(Y)/N = 11.99 \times \frac{20.29}{5} = 48.66$$

$$\sum(X^2) - \sum^{(X)^2}\!/_{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$\sum(Y^2) - \sum\frac{(Y)^2}{N} = 83.74 - \frac{20.29^2}{5} = 1.40$$

$$r = \frac{49.75 - 48.66}{\sqrt{0.91 \times 1.40}}$$

$$r = 0.96$$

(ii) The regression coefficient, 'b':- The equation is given by

$$b = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sum(X^2) - \sum(X)^2/N}$$

$$b = \frac{49.75 - 48.66}{0.91}$$

$$b = 1.20$$

(iii) Regression line equation:- is given by

$$(Y - \bar{Y}) = b(X - \bar{X})$$

Therefore Y- 4.06 = 1.20(X-2.40)

Y = 1.20X + 1.18

$\Rightarrow$ The regression line equation for EU_2 is Y = 1.20X + 1.18

(v) The variance V_y of Y about the regression line is given by

$$V_y = (\sum dy^2 - b^2 \sum dx^2)/\phi$$

$$\sum dy^2 = \sum(y^2) - \sum\frac{(y)^2}{N} = 83.74 - \frac{20.29^2}{5} = 1.40$$

$$\sum dx^2 = \sum(x^2) - \sum\frac{(x)^2}{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$V_y = \frac{1}{3}(1.40 - 1.20^2 \times 0.91) = 0.09$$

The variance V_x of a value of X predicted from the regression line when Y=5 is given by

$$V_X = \frac{V_y}{b^2}[1 + \frac{1}{N} + \frac{(Y - \bar{Y})^2}{b^2} \bullet \frac{1}{\sum\{(X - \bar{X})^2\}}$$

$$\sum(X - \bar{X})^2 = \sum(X^2) - \sum(X)^2 / N = 0.91$$

$$V_X = \frac{0.09}{(1.2)^2}[1 + \frac{1}{5} + \frac{(0.94)^2}{(1.20)^2} \bullet \frac{1}{0.91}]$$

$$V_X = 0.078(1.2 + 0.67) = 0.146$$

$$\therefore \sqrt{V_X} = 0.38$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and P = 0.95; these limits are ± 3.18 x 0.38 = ± 1.21.

Calculation of the Value of 'X' from the Regression Line Equation

The regression line equation for EU_2 is Y = 1.20X + 1.18,

The value of X calculated from the regression line equation for Y = 5,

5 = 1.20X + 1.18

X = 3.82/1.20 = 3.18

Since the limit of error for 'X' is 1.21

Then X=3.18±1.21

Antilog of X = 1513.56 ± 16.23

Therefore LC_{50} (EU_2) = 1513 ± 16.23µg/ml

Appendix 1C

Calculation of LC_{50} values of petroleum ether extract EU_3

X	X^2	Y	Y^2	XY
3.000	9.000	8.09	65.448	24.27
2.699	7.283	5.84	34.110	15.76
2.398	5.750	5.25	27.560	12.59
2.092	4.397	4.48	20.070	9.37
1.796	3.226	4.16	17.306	7.47

$$\sum(X) = 11.99 \sum(X^2) = 29.658 \sum(Y) = 27.82 \sum(Y^2) = 164.424 \sum(XY) = 69.46$$

$$number\ of\ .\deg rees\ of\ .freedom, \ldots N = 5, \ldots \bar{X} = 2.40, \ldots \bar{Y} = 5.56$$

(i) Correlation Coefficient, 'r':

$$r = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sqrt{\{(\sum(X^2) - \sum(X)^2/N)(\sum(Y^2) - \sum(Y)^2/N\}}}$$

$$\sum(X)\sum(Y)/N = 11.99 \times \frac{27.82}{5} = 66.71$$

$$\sum(X^2) - \sum {(X)^2}/{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$\sum(Y^2) - \sum \frac{(Y)^2}{N} = 164.42 - \frac{27.82^2}{5} = 9.63$$

$$r = \frac{69.46 - 66.71}{\sqrt{0.91 \times 9.63}}$$

$$r = 0.93$$

(ii) The regression coefficient, 'b' - The equation is given by

$$b = \frac{\sum(XY) - \sum(X)\sum(Y)/N}{\sum(X^2) - \sum(X)^2/N}$$

$$b = \frac{69.46 - 66.71}{0.91}$$

$$b = 3.02$$

(iv) Regression line equation:- is given by

$$(Y - \bar{Y}) = b(X - \bar{X})$$

Therefore Y- 5.56 = 3.0(X-2.40)

Y = 3.0X - 1.64

⇒ **The regression line equation for EU_3 is Y = 3.0X - 1.64**

(iii) The variance V_y of Y about the regression line is given by

$$V_y = (\sum dy^2 - b^2 \sum dx^2)/\phi$$

$$\sum dy^2 = \sum(y^2) - \sum\frac{(y)^2}{N} = 164.42 - \frac{27.82^2}{5} = 9.63$$

$$\sum dx^2 = \sum(x^2) - \sum\frac{(x)^2}{N} = 29.66 - \frac{11.99^2}{5} = 0.91$$

$$V_y = \frac{1}{3}(9.63 - 3.02^2 \times 0.91) = \frac{1}{3}(9.63 - 2.75) = 2.29$$

The variance V_x of a value of X predicted from the regression line when Y=5 is given by

$$V_X = \frac{V_y}{b^2}[1 + \frac{1}{N} + \frac{(Y-\bar{Y})^2}{b^2} \bullet \frac{1}{\sum\{(X-\bar{X})^2\}}$$

$$\sum(X-\bar{X})^2 = \sum(X^2) - \sum(X)^2 / N = 0.91$$

$$V_X = \frac{2.29}{(3.02)^2}[1 + \frac{1}{5} + \frac{(5-5.45)^2}{(3.02)^2} \bullet \frac{1}{0.91}]$$

$$V_X = 0.25(1.2 + 0.02) \approx 0.305$$

$$\therefore \sqrt{V_X} = 0.55$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and $P = 0.95$; these limits are $\pm 3.18 \times 0.55 = \pm 1.75$.

Calculation of the Value of 'X' from the Regression Line Equation

The regression line equation for EU_3 is $Y = 3.0X - 1.64$,

The value of X calculated from the regression line equation for $Y = 5$,

$5 = 3.0X - 1.64$

$X = 6.64/3.0 = 2.21$

Since the limit of error for 'X' is 1.75

Then $X = 2.21 \pm 1.75$

Antilog of $X = 162.18 \pm 63$

Therefore LC_{50} (EU_3) = $162.18 \pm 63 \mu g/ml$

Appendix IIA

Probit corresponding to percentages

Percentages	Probits									
	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Appendix IIB

Theoretical values of the correlation coefficient P'=0.05

Degree of freedom ϕ	2	3	5	7	10	15	20	30	50	100
Correlation coefficient r	0.95	0.88	0.75	0.67	0.58	0.48	0.42	0.35	0.27	0.20

Degree of freedom ϕ=N-3

Appendix IIC

Values of 't'

Degree of freedom ϕ	Value of 't'		Degree of freedom ϕ	Value of 't'	
	P=0.95 P'=0.05	P=0.99 P'=0.01		P=0.95 P'=0.05	P=0.99 P'=0.01
1	12.71	63.7	11	2.20	3.11
2	4.30	9.92	12	2.18	3.05
3	3.18	5.84	15	2.13	2.95
4	2.78	4.60	20	2.09	2.85
5	2.57	4.03	25	2.06	2.79
6	2.45	3.71	30	2.04	2.75
7	2.37	3.50	40	2.02	2.70
8	2.31	3.36	60	2.00	2.66
9	2.26	3.25	120	1.98	2.62
10	2.23	3.17	∞	1.96	2.58

Appendix III

PURIFICATION OF SOLVENTS

(a) Purification of Ethyl acetate

Water and water-soluble impurities were removed by adding 100ml of 5% sodium carbonate and saturated sodium chloride solution into 500ml ethyl acetate in a 1000ml flask. This was shaken to wash and then the aqueous layer drained out using a separating funnel. The ethyl acetate was finally dried up by pouring dry potassium carbonate (initially dried at 300°C for 15minutes) and left overnight. It was then distilled to a constant temperature of 76-78°C.

(b) Purification of Chloroform

500ml of chloroform was washed using 250ml of distilled water by shaking in a separating funnel. Chloroform being denser than water was removed and further dried over calcium chloride for 24hours. The chloroform was decanted from the calcium chloride and distilled to a constant temperature of 58-60°C.

(c) Purification of Methanol

0.5g of Iodine and 5g of magnesium turnings were first added into 50-70ml of methanol and refluxed until the colour of iodine disappeared. Chloroform was then added to make up 1000ml and refluxed for 3hours.

(d) Purification of Ethanol

The same purification procedure was used as that of methanol.

(e) Purification of Petroleum Ether(60-80°C)

Petroleum ether was purified by distillation and the distillate from 60-80°C collected (Vogel, 1978).