PHYTOCHEMICAL ANALYSIS OF

COCHLOSPERMUM TINCTORIUM A. RICH.

BY

MUSA AKPEMl AUDU B.Sc. (ABU).

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FACULTY OF SCIENCE

AHMADU BELLO UNIVERSITY,

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NOVEMBER, 1998.

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DECLARATION

I hereby declare that this thesis contains the report of my research work and has not been presented in any previous application for a higher degree. All information from other sources have been acknowledged by means of references.



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CERTIFICATION

This thesis entitled "**Phytochemical Analysis of *Cochlospermum tinctorium* A. Rich**" by **MUSA, Akpemi Audu**., meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria - Nigeria, and is approved for its contribution to knowledge and literary presentation.

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To my sisters Amina, Ashiawu, Maimunat, Kelimat, and Bilkis; and my brothers Mutari and Mohammed Kabiru.

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ABU, ZARIA

NOVEMBER, 1998.

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ABSTRACT

The extraction efficiency of continuous and batch extraction methods using four solvent systems of increasing polarities namely petroleum spirit 40 - 60 °C, diethyl ether, 80% acetone and methanol were studied on dried rhizome of *Cochlospermum tinctorium* A. Rich.

Phytochemical screening of extracts obtained by both extraction methods revealed the presence of alkaloids, tannins, saponins, cardiac glycosides, carbohydrates, flavonoids and anthraquinone glycosides which are reported for the first time. Preparative TLC of the ethyl acetate fraction with petroleum spirit (60 - 80 ^{o}C): ethyl acetate (3:2) yield extracts 1 - 6. Extracts 1 - 6 were characterized by R_F value determination and reaction with spray reagents. UV spectra analysis shows extracts 1, 2, 5 and 6 to be flavanone glycosides and 3 and 4 to be flavonol glycosides.

Cytotoxic test using Brine Shrimp Lethality assay was carried out on four crude extracts. The LC_{50} value for n-butanol, 80% acetone and methanol extracts were 437 ± 8 ug/ml, 240 ± 3 ug/ml and 871+5 ug/ml respectively. Meanwhile the ethyl acetate extract was lethal at all concentration level administered.

Antimicrobial sensitivity tests of ethyl acetate, n-butanol, 80% acetone and methanol extract were carried out on *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Esherichia coli.* The zones of inhibition were measured .

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ABBREVIATION

TLC	Thin Layer Chromatography		
MIC	Minimum Inhibitory Concentration	sh	Shoulder
MBC	Minimum Bactericidal Concentration	nm	Nanometer
		cm	Centimeter
UV	Ultraviolet	λ_{max}	Wavelength of maximum absorption
µg/ml	Microgram per millilitres		
°C	Degree Celsius	mm	Millimeter
%	Percent	g	Gramme
cf.	Compare	mg	Milligram
D	Dextrorotatory	ml	Millilitre
L	Levorotatory	l	Litre
α	Alpha	Petrol	Petroleum spirit 60 - 80 °C
β	Beta	Petrol 40 - 60 °C	Light Petroleum Spirit 40 - 60 °C
γ	Gamma		
δ	Delta	NMR	Nuclear Magnetic Resonance
Gal	Galactose	HMBC	Heteronuclear Molecular Bond Correlation
Rha	Rhamnose		
G	Glucose	DEPT	Distortionless Enhancement Polarisation Transfer
EtOAc	Ethylacetate		
MeOH	Methanol	NOESY	Nuclear Overhauser Enhancement Spectroscopy.
BuOH	Butanol		

CHAPTER ONE

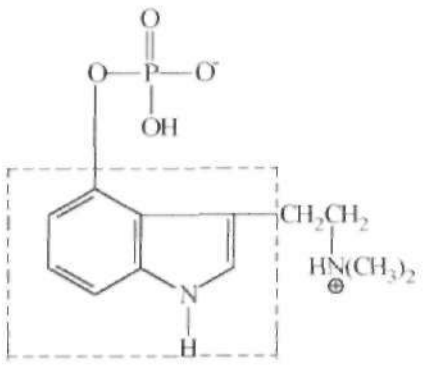
1.0 INTRODUCTION

Phytochemical analysis of a plant may involve the following: extraction of the plant; separation and isolation of the constituents of interest; characterization of the isolated compounds; investigation of the biosynthetic pathways to particular compounds; and quantitative evaluations (Trease and Evans, 1978). However, in the search for new drugs of therapeutic importance, plants are usually subjected to phytochemical screening which involves simple chemical tests to detect the presence of alkaloids, tannins, saponins, anthraquinones, cardenolides etc (Sofowora, 1982). A brief explanation on the nature of classes of natural products present in plants will suffice.

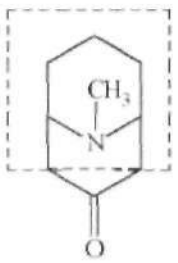
1.1 CLASSES OF NATURAL PRODUCTS

1.1.1 Alkaloids

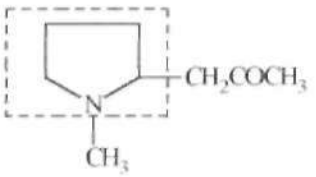
Alkaloids are heterocyclic nitrogenous bases. They have accounted for the active principles of certain plants (e.g. psilocybin is the active principle of Mexican hallucinogenic mushrooms). Alkaloids are classified based on the type of heterocyclic group they possess. Thus indole alkaloids like psilocybin contain an indole or modified indole nucleus and pyrrolidine alkaloids (e.g. hygrine) contain the pyrrolidine ring system. Other examples of this classification include the pyridine, quinoline (cf. quinine, isoquinoline, imidiazole, pyridine - pyrrolidine (cf. micotine) and piperidine - pyrrolidine type alkaloid (Pelletier, 1970). The skeletal nature of alkaloids are shown in figure 1.1.



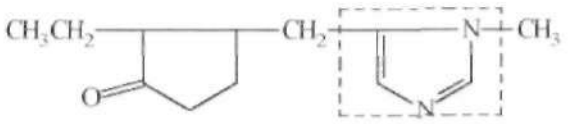
Psilocybin (Indole type)



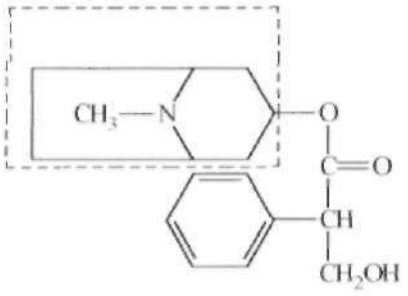
Pseudopelletierine (Pyridine type)



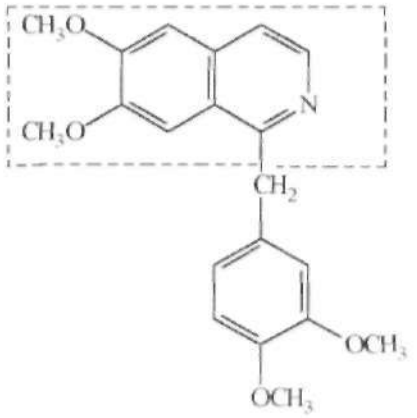
Hygrine (Pyrrolidine type)



Pilocarpine (Imidazole type)



Atropine, Hyoscyamine
(Piperidine-pyrolidine type)



Papaverine (Isoquinoline type)

N.B Dotted lines indicate the parent heterocyclic rings

Figure 1.1 Classification of alkaloids based on heterocyclic ring.

The presence of alkaloids in plant extracts can be established by reaction with chromogenic reagents. Alkaloids respond reddish - brown with Dragendorff and Wagner's reagent, milky/cream/white precipitate with Mayer's reagent. Other reagents include Iodoplatinate, Phosphomolybdic acid, antimony trichloride etc.

1.1.2 **Anthraquinones**.

Anthraquinones are phenolic compounds that are widely distributed in nature. They exist in both plants and animals particularly insects. In nature they are either in free state as typified by anthraquinone (**I**) or in combined state in forms of its derivatives (e.g. **II** and **III**). The combined anthraquinones which are commonly referred to as anthraquinone glycosides are anthraquinones that are linked to one or more sugar molecules as in carminic acid (**VI**). The sugar molecule may be D-glucose, L-fructose, D-xylose, L or D-rhamnose or D-ribose.

The derivatives of anthraquinones present in purgative drugs may be dihydroxyphenols such as chrysophanol (**IV**), trihydroxy phenols such as emodin (**V**) or tetrahydroxy phenols such as carminic acid (**VI**). Other groups are often present, e.g. methyl in chrysophanol, hydroxmethyl in aloe - emodin and carboxyl in rhein and carminic acid.

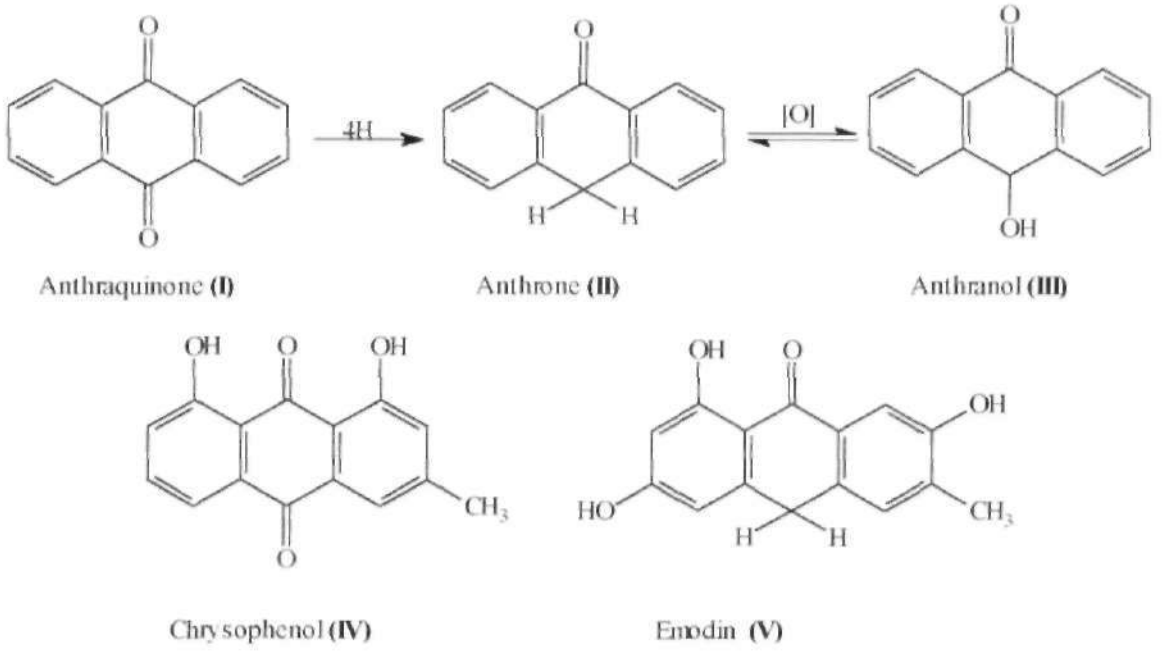
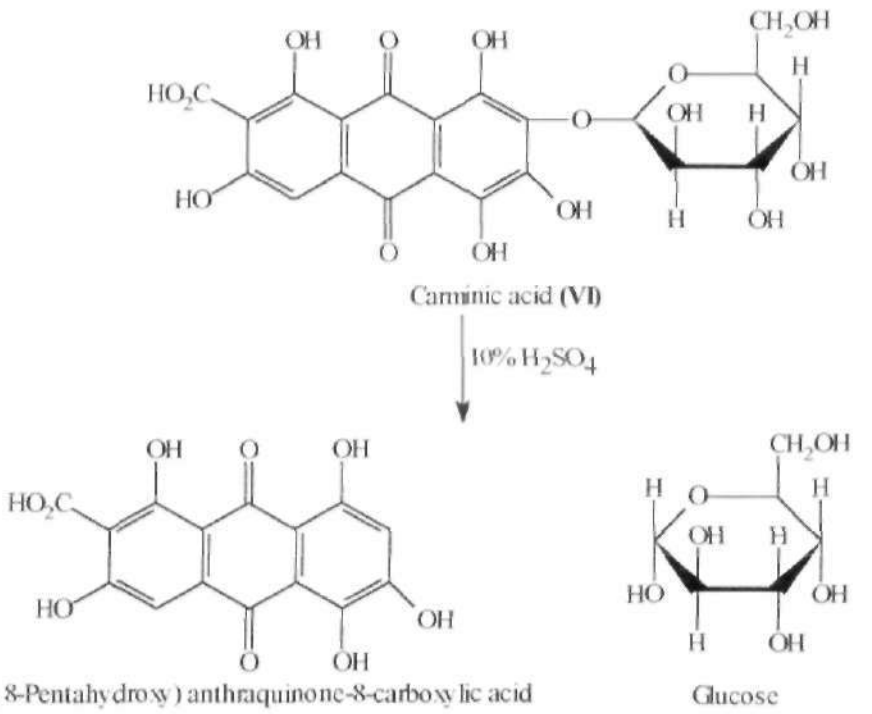


Figure 1.2 Chemical structure of some anthraquinones.

It is noteworthy to point out that carminic acid (**VI**) is found in *Cochineal* an ancient insect colouring matter, and emodin (**V**) is perhaps the most ubiquitous anthraquinone.

Borntrager's test is commonly used to detect the presence of free anthraquinone. For the detection of combined anthraquinone a modified Borntrager's test is employed which involve hydrolysis with dilute acid to free the anthraquinone from the bonded sugar (Trease and Evans, 1978).

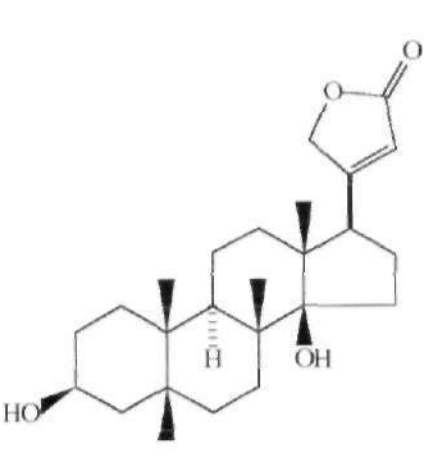


1.1.3 **Cardiac glycosides.**

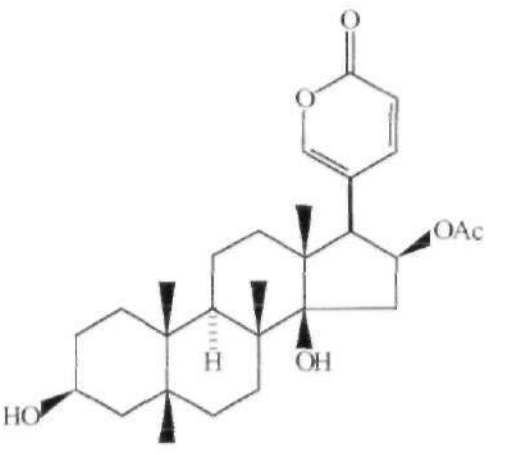
Cardiac glycosides are substances which exerts a powerful stimulant action on heart muscle. They are found predominantly in certain plants, notably those of the order *Apocynaceae*. Plants of the digitalis family have provided drugs used in cardiac therapy to strengthen the action of the heart though cautious administration is necessary, since excessive doses causes cardiac arrest.

Cardiac glycosides are also called cardenolides. Upon total hydrolysis, cardiotonic glycosides give an aglycone or genin (or a dehydration product) and one or more sugars. The aglycone are C_{23} or C_{24} diol or triol with an unsaturated lactone side - chain, and are of

two types. The commoner cardenolides, typified by digitoxigenin (**IX**), possess an α , β - unsaturated γ - lactone ring, and are found chiefly in plants of the foxglove, lily and buttercup families. The bufadienolides or scilladienolides, exemplified by bufotalin (**X**), have a δ - lactone ring containing a conjugated diene system, and occur both in plants of the squill family and as conjugates with suberylarginine, in the skin secretions of poisonous toads.



Digitoxigenin (**IX**)



Bufotalin (**X**)

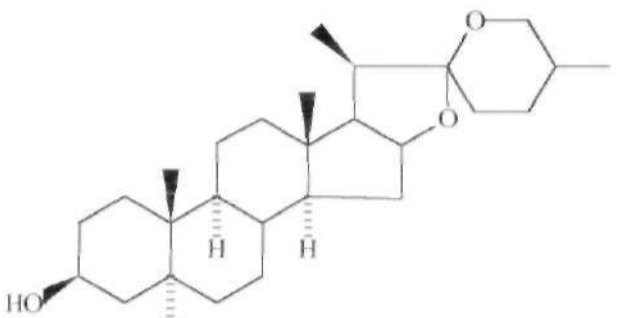
Both types of aglycones invariably posses a 14β-hydroxyl group and a C-3 hydroxyl group, and have the normal steriod configuration at C-8, C-9, C-10, C-13 and C-17; members of both series vary in their configuration at C-3 and C-5, and in the presence or absence of oxygen functions and unsaturations at other positions. The sugar moieties of the glycosides usually contain several hexose units, and are always linked to the steroid nucleus at C-3 (Templeton, 1969A)

The presence of cardiac glycosides in plant extracts can be determined by Legal test, Kedde test indicates cardenolide which possesses a lactone ring. *Digitalis*-like cardenolide which possesses deoxy sugar respond to Keller - kiliani test. Cardenolides which possess a terpenoid or steriodal nucleus can be detected by Liebermann - Burchardt test. Salkowski test gives the same response as Liebermann - Burchardt test (Trease and Evans, 1978; Sofowora, 1982).

1.1.4 **Saponins.**

The saponins are a group of plant glycosides characterised by their ability to foam in water. Their aqueous solutions cause haemolysis (destruction of the red blood cells) when injested intravenously into animals, though they are relatively harmless when taken orally, and they are also toxic to fish. Saponins often occur along with cardenolides in nature; thus various species of *Digitalis* contain digitonin, gitonin and tigonin.

The saponin, aglycones or sapogenin, are C_{27} alcohols with a unique spiroketal side-chain, and may contain additional hydroxyl groups, carbonyl and double bonds. A typical saponin is typified by tigogenin (**XI**) (Templeton, 1969B).



Tigogenin (**XI**)

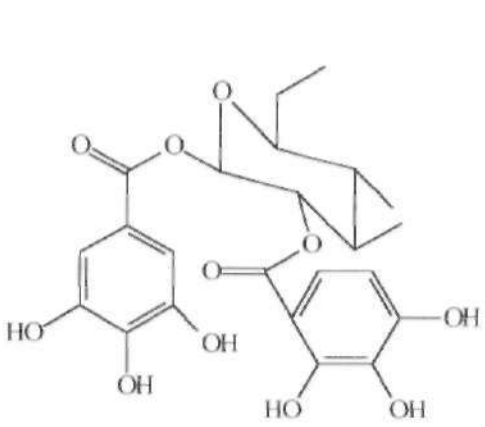
1.1.5 **Tannins**

Vegetable tannins are generally defined as "water soluble" phenolic compounds having molecular weights between 500 and 3000. Besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and proteins (Bate - Smith and Swain 1962; Haslam, 1979A). They are divided into two classes, the hydrolysable and non-hydrolysable tannins or condensed tannins.

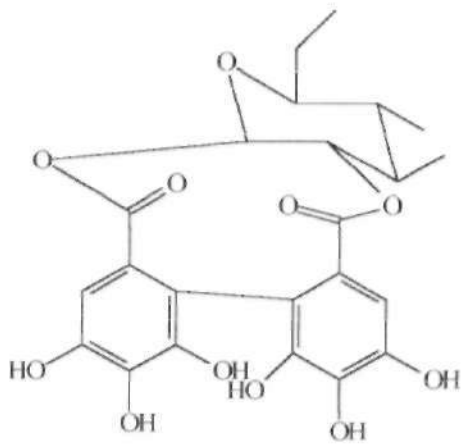
Hydrolysable tannins: The hydrolysable tannins are complex polyphenols which may be degraded into simpler fragments under hydrolytic condition. Hydrolysable tannins are subdivided on the basis of the phenolic acid(s) liberated on hydrolysis. Those which yield

only gallic acid (**XII**) upon hydrolysis are known as gallotannin (**XIII**) and those which give hexahydroxy diphenic acid (**XIV**) normally as its dilactone ellagic acid (**XV**) amongst the acidic product are known as ellagitannins (**XVI**).

Condensed Tannins: These are vegetable tannins which are not readily broken down by acid, alkali, and enzymatic (e.g. tannase) hydrolysis. Sugars do not contribute to their overall structure. They are typified by substances such as (+)-catechins (**XVII**) (flavan -3-ols) (Haslam, 1979B). Condensed tannins are sometimes referred to as flavonoid aglycones. Ferric salts such as ferric chloride are used for the detection of tannins. Gallotannins and ellagitannins which are hydrolysable tannins give blue - black precipitates while condensed tannins blue - green, green and brownish - green precipitates with ferric chloride. Tannins irrespective of class form white precipitates with gelatin - salt reagent (Persinos and Quimby, 1967; Trease and Evans, 1978B).

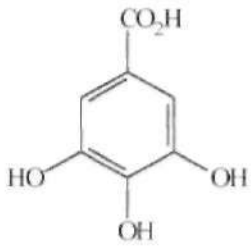


Gallotannin (**XII**)



Ellagitannin (**XVI**)

H^+ or Tannase

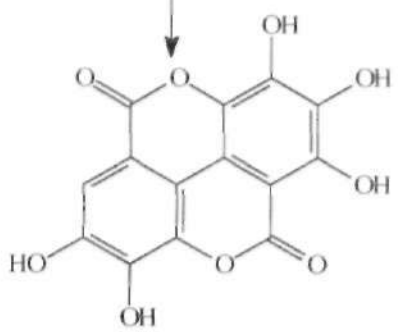


Gallic acid (**XIII**)

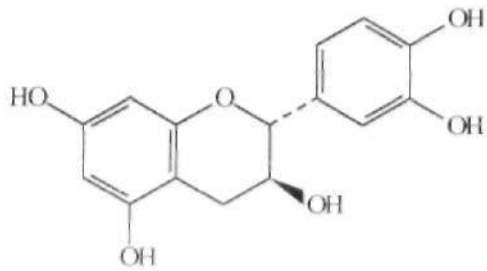
H^+ or Tannase



Hexahydroxydiphenic acid (**XIV**)



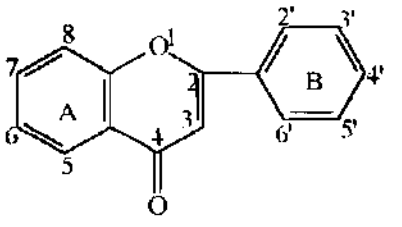
Ellagic acid (**XV**)



(+)-Catechin (**XVII**)

1.1.6 **Flavonoids**

Flavonoids are a group of organic compounds in which parent skeleton of the group is flavan (2-phenylchroman) in which the heterocyclic ring is fully reduced. Flavonoids are represented by a general structure based on flavone (**XVIII**) which consist of two benzene rings (A) and (B) joined together by a three carbon link which is formed into a γ-pyrone ring. The various classes of flavonoid compounds differ one from another only by the state of oxidation of this three carbon link.



Flavone (XVIII)

The classes of flavonoids are flavones e.g. 7-hydroxy flavone (**XIX**), flavonols e.g. 3-methoxy flavone (**XX**), flavonones e.g. naringenin (5,7,4′ - trihydroxy flavanone) (**XXI**), isoflavones e.g. 7,4′-dihydroxy-5-methoxyisoflavone (**XXII**), chalcones e.g. 2′ - hydroxychalcone (**XXIII**), aurones e.g. 6-hydroxyaurone (**XXIV**), flavan -3-ol e.g. (+)-catechin (**XVII**), anthocyanins e.g. pelargonidin (**XXV**), dihydroflavonols, e.g. (2*S*:3*S*)-7-hydroxydihydroflavonol (**XXVI**), dihydrochalcones e.g. 2′-*O*-glucosidephloridzin (**XXVII**) and flavan-3,4-diols (proanthocyanidins) e.g. (2R, 3S, 4R mollisacacidin (**XVIII**). Their structures are shown in fig. 1.3.

In most cases the flavonoid compounds exist as glycosides, that is one or more of their hydroxyl group is joined by a semi-acetal link to a sugar. The sugar-free compounds are referred to as aglycones. The sugars which have been found in flavonoid glycosides include simple hexose and pentoses (monosides), di- and tri-saccharides (biosides and triosides) always combined through oxygen at the C - 1 position usually by a β- linkage. In many cases, more than one phenolic hydroxyl group in the flavonoid molecule may be involved, giving rise to dimonosides and so on. Examples of sugars present in flavonoid

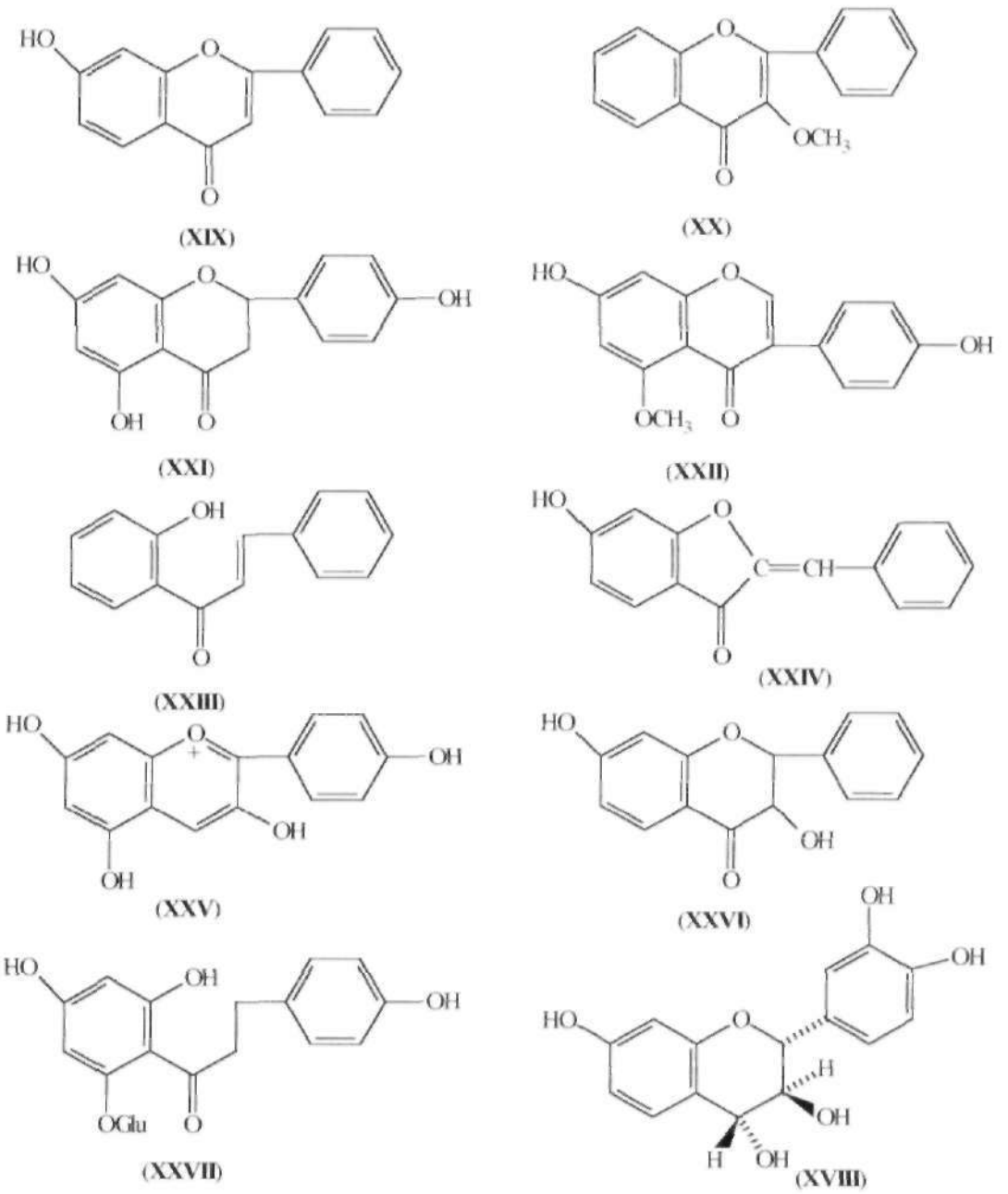


Figure 1.3 Classes of Flavonoids

glycosides include D-glucose, D-galactose and L-rhamnose and less frequent L-arabinose, D-glucuronic acid, and D-xylose (Swain, 1965).

1.1.7 **Carbohydrates**

Sugars are carbohydrates which combine with a wide variety of other compounds to form glycosides. The presence of glycosides in a plant extract is indicated by Fehlings test (Trease and Evans, 1978). Examples of sugars that make up glycosides in nature are pentoses such as D-ribose, D-xylose, D-arabinose, and L-rhamnose, and hexoses such as D-mannose, D-galactose, D-glucose, and L-fructose. The structures of these sugars are depicted in Figure 1.4.

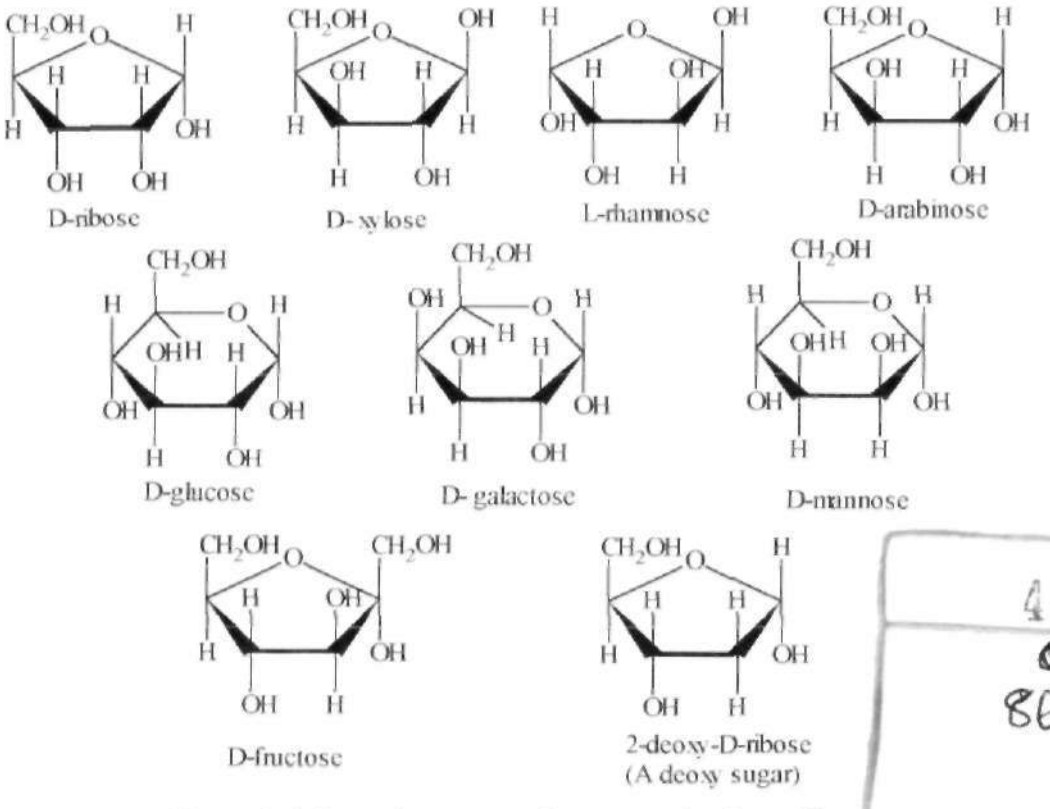


Figure 1.4 Some hexoses and pentoses in glycosides.

46790
QK
866

To understand the nature of the structures of the glycosides present in a plant extract, it is imperative to carry out sugar test so as to determine the type of sugar bonded to the aglycone. Information thus obtained is used as a guide in selecting standards that will be

used to match the sugar in the compounds isolated by TLC. However, sophisticated physical methods such as NMR and mass spectroscopy can be used to determine the pattern of bonding between the sugar and aglycone and also between two or more sugars bonded together.

1.2 **Justification of research.**

Cochlospermum tinctorium is the plant of interest in this work. Chemical and biological investigation was carried out based on the author's personal experience on the efficacy of this plant in remedying typhoid fever. Further impetus was due to the immense therapeutic value and as a source of dyes for traditional dyeing in Northern Nigeria. (see section 2.4.4).

1.3 **Aims/objectives of research**.

To investigate the classes of natural products present in the rhizome of *C. tinctorium* and further to isolate, and characterise and elucidate the structures of these compounds isolated from ethyl acetate extract.

As a result of the known therapeutic value of the plant, cytotoxic effects of extracts obtained by continuous extraction with four solvents of differing polarities, and the antimicrobial activity of some of the crude extracts were investigated. Also, the antimicrobial activity of some isolated pure compounds were investigated so as to establish the compounds responsible for remedying typhoid fever, gastroenteritis and pneumonia.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 GENERAL BOTANICAL FEATURES OF *COCHLOSPERMACEAE*.

The family *Cochlospermaceae* belongs to the dicotyledenous group of plants. It comprises five species namely - *Cochlospermum planchonii*, *Cochlospermum tinctorium*, *Cochlospermum gossypium*, *Cochlospermum gillivraei* and *Cochlospermum angolense*.

C angolense is predominantly distributed in Europe, *C. gillivraei* can be found in Austrialia, C. *gossypium* is widely distributed in tropical Asia while *C. planchonii* and *C. tinctorium* are widely distributed in tropical Africa particularly the savannah region throughout West Africa particularly in Northern Nigeria and Northern Cameroon.

C. gossypium (formerly known as *C. reliosum* Linn.) which is a native of tropical Asia also grows in various parts of West Africa. Likewise, *C. tinctorium* native to Tropical West Africa, grows in temperate regions particularly in parts of America and Europe.

Members of the family Cochlospermaceae are either trees, shrubs or rhizomatous subshrubs; *C. gossypium* is a shrub, *C. gillivraei* exists as a tree and *C. angolense* as rhizomatous shrub. *C. planchonii* and *C. tinctorium* are rhizomatous subshrubs which are similar in outlook but differentiated by the flowering pattern and the size of the leaves. In places where both species exist, hybrids may be found.

All the species of this family are of great medicinal and economic importance. Several studies (Janot and Gonnard, 1938; Rabate', 1939; Hirst and Dunstan, 1953; Aspinal *et al.* 1965; Bate-Smith, 1964; Cook and Knox, 1975; Diallo 1988) have been carried out to determine the chemical constituents of almost all the species. However, *Cochlospermum planchonii* has not been fully exploited.

2.1.1 *Cochlospermum tinctorium* A. Rich: Botanical Character.

The plant is a low shrub. Leaves are variable and palmately lobed. The sepals are 5, unequal. Petals 5, large, imbricate. Stamens numerous, with linear anthers on slender equal filaments. Ovary with 3 - 5 parietal placentas; style slender. Fruit an obovoid often hairy capsule; endocarp rather membranous seeds covered with long cottony hairs which adhere closely to the seeds. The flowers are usually borne near the ground while plant is leafless, and sometimes also near apex of leafy shoots, which seldom exceed 75 cm high; leaves usually deeply lobed with acute lobes, sometimes with lobes narrowly lanceolate, lobes usually serrate; lower surface of mature leaves glabrescent or pubescent (Hutchison *et al.* 1954).

2.2 CHEMICAL COMPOSITION

2.2.1 Polysaccharides.

Janot and Gonnard (1938) studied the methoxyl indices of some gums and observed that the methoxyl index of *Cochlospermum gossypium* 12.6 is smaller than gum tragacarth 18.6-38. The gum exudate of the bark consists of acetylated acidic and neutral polysaccharides. Hirst and Dunstan (1953) identified L-rhamnose, D-galactose and D-galacturonic acid in the hydrolysed product of this gum. Methylation studies established the modes of linkage of the neutral sugar residue. Aspinal *et al.* (1962) studied the mode of linkage of the acidic sugar residue and established the presence of D-glucuronic acid residue as end groups and D-galacturonic acid residue in the inner chains of the molecular structure of the gum.

In order to obtain more information about the location of the acidic sugar unit in the polysaccharide, the following procedure was applied (Aspinall *et al.* 1965):

1. Hydrolysis followed by separation of acidic sugar from neutral sugars by adsorption on an anion-exchange resin and subsequent partition by chromatography of filter sheets.

2. Reduction with sodium borohydride, hydrolysis and paper chromatography.
3. Conversion into the methyl ester methyl glycoside with methanolic hydrogen chloride, reduction with sodium borohydride, hydrolysis and paper chromatography of the hydrolyzate.
4. Reduction with lithium aluminium hydride, methanolysis and gas chromatography of the resulting methylglycoside.

The results are tabulated as shown:

Acidic Oligosaccharide	Method applied	Products
I	1	Galacturonic acid, rhamnose
	2	Galacturonic acid, rhamnitol
	3	Galactose, rhamnose
II	1	Galacturonic acid, galactose
	2	Galacturonic acid, galactitol
	3	Galactose
	4	2,3,4-and 2,3,6-trimethyl glucose
III	1	Glucuronic acid, galacturonic acid, rhamnose
	2	Glucuronic and, galacturonic acid, rhamnitol
	3	Glucose, galactose, rhamnose
	4	Galactose, rhamnose

Oligosaccharide I was found to be 2-*O*-(α-D-galactopyranosyluronic acid)-L-rhamnose an aldobiouronic acid, oligosaccharide II is 4-*O*-(β-D-galactopyranosyluronic acid)-D-galactose, and oligosaccharide III is *O*-(β-D-glucopyranosyluronic acid)-(1→2)-L-rhamnose.

Prior to the work of Aspinal *et al.* (1965), no discrete oligosaccharide containing D-glucuronic acid residue was isolated. Oligosaccharide III has, however, accounted for D-glucuronic acid.

Further, reexamination of the gum showed that the acidic oligosaccharides obtained by partial hydrolysis of the gum comprises these structural unit: GalA →2 Rha...., GalA→ 4Gal....., GA1→ 3Gal A....., GA1 →3 Gal A1→2Rha..... The presence of Gal A1→4 GalA1→2Rha is not certain.

2.2.2 **Pigments**

Two classes of pigments, flavonoids and carotenoids have been isolated from three species of the family Cochlospermaceae. The flavonoids myricetin, quercetin, kaempperol and cyanidin (from leucocyanidin) were isolated from the hydrolysed leaf extract of *C. gossypium* (formerly *C. religiosum*) (Bate-Smith, 1964). Cook and Knox (1975) applied column chromatography and preparative TLC to isolate apigenin (5,7,4′-trihydroxyflavone), narigenin(5,7,4′-trihydroxyflavanone, and (+)-afzelechin(5,7,4′-trihydroxyflavan-2-ol) which were identified by NMR, IR, m.p. and co-TLC and by derivative formation. The glycoside prunin(narigenin 7-*O*-glucoside) and cosmosiin(apigenin - 7-*O*-glucoside) were also identified.

The carotenoids, cochloxanthin and dihydrocochloxanthin in *C. tinctorium* were isolated and characterized using high speed counter current chromatography on a horizontal flow through coil planet centrifuge. HPLC and preparative TLC were not used due to instability of the carotenoids when these analytical methods are utilised (Diallo, 1988).

2.2.3 **Dyes**

A yellowish-red dye whose chemical nature was not determined has been extracted from *Cochlospermum tinctorium* (Mell, 1927A; 1927B).

2.3 **ANTIMICROBIAL AND ANTIVIRAL ACTIVITY.**

Hegenscheid *et al.* (1987) reported the antiviral activity of chloroform and aqueous extracts

of *C. angolense* roots. The chloroform extract had a specific concentration-dependent inhibitory effect on influenza virus A and B type strains cell culture. Neither chloroform or aqueous extract of the roots had any effects on polio virus type 1. And there was only non-specific inhibition of hepatitis A virus.

Alade *et al* (1986) studied the minimum inhibitory concentration (M.I.C) of three known human bacterial pathogens on the crude drug extract of *Cochlospermum tinctorium*. The test organism used were *Neisseria gonorrhoea*, *Salmonella typhi* and *Shigella dysenteriae*.

The antibacterial activity of the crude drug extract was tested on 1:10, 1:100 and 1:1000 serial dilutions of the test organisms. From the result obtained it was observed that at 1:10 dilution of *Salmonella typhi* (typhoid fever causing bacteria) the drug had tremendous positive effect but had no effect on lower population of the test organism that is in 1:100 and 1:1000 dilution. Surprisingly the crude drug extract had antibacterial activity on 1:10 dilution of *Neisseria gonorrhoea* (gonorrhoea causing bacteria), the drug, was only active against very low concentration of *Shigella dysenteriae* (dysentry causing bacteria) specifically at 1:1000 dilution. It is important to point out that the drug is not used locally in Northern Nigeria to cure gonorrhoea. However, the result of this research (by Alade and co-workers) shows that the drug can effectively cure gonorrhoea.

2.4 APPLICATIONS OF THE FAMILY *COCHLOSPERMACEAE*.

2.4.1 *Cochlospermum angolense*.

This plant is used as an antiviral agent (Hegenscheid *et al*. 1987).

2.4.2 *Cochlospermum gossypium*

This species formerly known as *Cochlospermum reliosum* is native to tropical Asia and

now recently cultivated in West Africa, can be used as a substitute for gum tragacanth. *C. gossypium* gum as we have seen earlier (Section 2.2.1) consists of mainly D-galacturonic acid residues. Likewise gum tragacanth consists of D-galacturonic acid residue (McIlory, 1948). Because of this similarity in chemical composition *C. gossypium* gum is recommended as a substitute for gum tragacanth.

C. gossypium gum contains the same chemical components with most gum derived from *Streculia* species. Therefore, it can be used in place of gums derived from *Streculia* species (also known as Khaya gum) (Aspinall, 1965). Interestingly *C. gossypium* has found application as a stabilizer in the preparation of viscous emulsions (Mukerjee and Shulka, 1970).

2.4.3 *Cochlospermum planchonii*.

The roots of this plant are used in cooking soup in Southern Nigeria when oil is not available. In this case, it imparts colour and flavour. The roots are also used medicinally in Northern Nigeria and Sierra Leone for treatment of fevers generally, and other medicinal uses applicable to *Cochlospermum tinctorium*. The roots yield a yellow dye which is used to dye textile materials and shoes in Hausa speaking areas of Northern Nigeria and Nupe (Irvine, 1961).

2.4.4 *Cochlospermum tinctorium*

The roots (rhizome) of this plant yield a yellow or brownish - yellow dye, called in Hausa rawaya which is often included with other yellow dyes. The dye is used for cloth, thread, and mat fibres. It is also used to dye leather particularly in Guinea and Northern region of Ghana. The floss is used for stuffing pillows. The root is also used to flavour and colour cooking oil. The bark affords a useful fibre. Beautiful when in flower, the plant is worth cultivating for ornamental purpose. It is considered as a possible fodder plant in Northern Ghana (Dalziel, 1955).

C. tinctorium is used in local cosmetics. The yellow root is used in many local remedies. Mixed with shea butter and other oils it is applied for burns. A root decoction or infusion is taken with other herbs for stomach troubles and urethral discharges. The Mossi tribe of Upper Ivory Coast (Cote d'Ivoire) use the roots for treating indigestion and stomach pains. It is used in parturition and as an emmenagogue, and by the Fulani in Guinea to arrest diarrhoea in calves. The root decoction is drunk for orchitis, worms, and fevers in general. It is also used as a liniment for epilepsy, pneumonia, intercostal pains, bronchial affections and swellings, and it is said to make a good sitz-bath cure for piles, and to be used for drops for conjunctivitis. The powdered root, in water or millet-beer, is supposed to cure jaundice and local application to cure bites of poisonous snakes. It is included with other constituents in local remedies for leprosy. It has been reported that chewing of the roots is good as a tonic and the seed oil is used to treat leprosy (Irvine, 1961). In Northern Cameroon the drug when mixed with other herbs is used to make arrow poison (Castagnou *et al.* 1965).

CHAPTER THREE

3.0 EXPERIMENTAL

3.1 Sample Collection

The rhizome of *C. tinctorium* were collected at the valley behind Ahmadu Bello University Press, Zaria within Ahmadu Bello University ground in Samaru-Zaria, on 19th and 20th April, 1996. At this period of the year the plant is fully mature, and it is characterized by withering of leaves and appearance of seeds

The bark of the rhizome was removed and the fleshy part was chopped into pieces and dried immediately (since it contains mucilage which is susceptible to bacterial and fungal attack) in an oven at 60°C for 7 days. The dried rhizomes were then pounded in a mortar and sieved using a laboratory test sieve of 1.40mm mesh size.

3.2 Materials Used.

3.2.1 Materials for Phytochemical tests

(i) 1% hydrochloric acid

(ii) 4% ferric chloride solution

(iii) 10% ammonia solution

(iv) 2% sodium nitroprusside solution

(v) 5.7% sodium hydroxide solution

(vi) 20% sodium hydroxide solution

(vii) 2% solution of 3,5-dinitrobenzoic acid in methanol

(viii) acetic anhydride

(ix) resorcinol

(x) phloroglucinol

(xi) 10% aqueous sulphuric acid

(xii) sulphuric acid

(xiii) *Molisch's reagent* (10% solution of α-naphthol in alcohol)

(xiv) *Gelatin-salt reagent* (1% solution of gelatin containing 10% sodium chloride)

(xv) *Mayer's Reagent*: (1) 1.36 g of mercuric chloride was added into 60 ml of distilled water (2) 5 g of potassium iodide was added into 20 ml of water. Solutions (1) and (2) were mixed and sufficient water added to make up 100 ml.

(xvi) *Wagner's Reagent*: 2 g of iodine and 3 g of potassium iodide were dissolved in water and sufficient amount of water was added to make up 100 ml.

(xvii) *Dragendorff's Reagent*: 0.85 g of bismuth nitrate in 10mls of acetic acid and 40mls of distilled water were mixed together to give stock solution (A). 8.0 g of potassium iodide was dissolved in 20 ml of distilled water to give stock solution (B). 5 ml each of the stock solution (A) and (B) were mixed with 20 ml of 33% acetic acid followed by 100 ml of distilled water to produce Dragendorff's reagent.

(xviii) *Fehling's solution*

Solution (A): about 7 g of copper sulphate (or cupric sulphate) and 0.1 ml of sulphuric acid were mixed with sufficient water to produce 100 ml

Solution (B): 35.2 g of sodium potassium tartrate and 15.4 g of sodium hydroxide were dissolved in sufficient water to make up 100ml.

Equal volumes of solutions A and B were mixed immediately before use.

(xix) *Ammoniacal silver nitrate*: 3 g of silver nitrate was dissolved in 50 ml of water and ammonia solution was added until the initial precipitate of silver oxide was redissolved. The resulting solution was diluted with water to 100 ml.

3.2.1 **Solvents for extraction**

(i) Petroleum spirit 40-60 °C analytical reagent.

(ii) Peroxide-free diethyl ether

(iii) Acetone

(iv) Methanol

(v) Ethyl acetate

(vi) n-Butanol

(vii) Chloroform.

3.2.1.1. **Purification of solvents for extraction**:

1. **Ether:** peroxide was removed by shaking 1000 ml of ether with 10 ml of a solution comprising 6.0 g ferrous sulphate and 6ml conc. H_2SO_4 in 110 ml of water. The ether was then washed with water, dried for 24 hr with $CaCl_2$, filtered and dried further by adding sodium metal chips until it remains bright. The ether is stored in a dark cool place, distilled from sodium just before use.
2. **Methanol:** technical grade methanol was purified by adopting the method of Lund and Bjermun (1931) 5 g of magnesium turnings and 0.5 g of iodine were warmed with 75ml of methanol in a flask until the iodine disappeared and all the magnesium turnings was converted to the methoxide. Up to 1litre of methanol was added and after refluxing for 3 hours, it was distilled excluding moisture from the system. Redistillation from sulphanilic acid removed basic impurities and traces of magnesium methoxide.
3. **Chloroform**: ethanol added to chloroform as a stabilizer was removed by shaking with several small portions of conc. H_2SO_4, washed thoroughly with water, and dried with fused $CaCl_2$ overnight, filtered and distilled. The distilled chloroform was kept in the dark to avoid photochemical formation of phosgene which is usually formed when chloroform is exposed to light.
4. Other organic solvents were distilled before use.

3.2.2. **Materials for TLC**.

(i) Kieselgel 60HR reinst (silica) from Merck was used as stationary phase.

(ii) Twenty (20 x 20cm) plates

(iii) Microscope slides

(iv) Gallenkamp spreader

(v) Shandon Chromatographic tank for TLC

(vi) Ultravoilet lamp (254 and 366nm)

(vii) Capillary tubes for sample application.

3.2.2.1 **Plate preparation**

(i) **Preparation of Thin layer plates (microscopic slides)**

Four 20 x 20 cm glass plates were placed on a Gallenkamp spreader. 30 microscope slides are arranged in the plates side by side, a drop of water was placed underneath each slide to facilitate proper adhesion so that when coating, the slide will not fall off. The slides were cleaned with petrol (40-60 °C) to remove grease and dirty particles. 30 g of kieselgel (silica) was mixed with 70 ml of distilled water in a 500 ml round-bottom flask. The mixture was stirred and allowed to stand for 15 minutes. The flask was agitated moderately for 5 minutes to render the slurry into a uniform paste. The slurry was then used to coat the slide 0.25 mm thick. The plates were air - dried for 15 hours and activated for 30 minutes at 120 °C, stored in a dessicator until when required for experiment.

(ii) **Preparation of plates for preparative TLC.**

1mm thick plates were prepared as follows: 120 g of kieselgel (silica) was mixed with 280 ml of distilled water in a 1L round-bottomed flask. The mixture was stirred and allowed to stand for 15 minutes. After 15 minutes the flask was agitated moderately for 5 minutes to render the slurry into a uniform paste.

The slurry obtained was used to coat five 20 x 20 cm plates. Prior to coating, the plates were cleaned with petrol (40-60 °C) to remove grease and oil. The plates were air-dried for 15 hours. Thereafter they were activated for 3 hours at 120°C and used immediately.

3.2.2.2 **Preparation of spray reagent for TLC**.

(i) 1% ethanolic $AlCl_3$

(ii) 0.5% methanolic magnesium acetate

(iii) concentrated ammonia solution

(iv) Benedict's reagent: 1.73 g of copper sulphate was dissolved in 10 ml of water. 17.3 g of trisodium citrate and 10 g of anhydrous sodium carbonate were dissolved in 80 ml of water, with the aid of heat; this solution was poured into the copper sulphate solution and the mixture diluted to 100 ml.

3.2.3 **Reagents for UV Spectroscopy**

Preparation of reagent stock solution and solids.

Reagent	Method of Preparation
Sodium methoxide (NaOMe)	Freshly cut metallic sodium (2.5 g) was added cautiously in small portions to dry spectroscopic methanol (100 ml)
Aluminum Chloride ($AlCl_3$)	Five grammes of fresh anhydrous reagent grade $AlCl_3$ were added cautiously to spectroscopic methanol (100 ml)
Hydrochloric acid (HCl)	Concentrated reagent grade HCl (50 ml) was mixed with distilled water (100 ml)
Sodium acetate (NaOAc)	Anhydrous coarsely powdered reagent grade NaOAc was used
Boric acid (H_3BO_3)	For procedure I: Anhydrous powdered reagent grade H_3BO_3 was used. For procedure II: Spectroscopic methanol (100 ml) was saturated with anhydrous reagent grade H_3BO_3.

3.2.4 **Materials for Brine Shrimp Lethality Assay**

(i) Sea water

(ii) Shallow rectangular dish (150 mm x 5 mm) with plastic divider

(iii) Dimethylsulphoxide (DMSO)

(iv) Yeast solution (prepared by dissolving 3 mg of yeast in 5 ml of water)

(v) Shrimp eggs

3.2.5 **Materials for Antimicrobial test**.

(i) Mueller Hinton Agar (Oxoid)

(ii) Test microorganisms: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Escherichia coli*.

(iii) Petri - dishes (150mm x 15mm)

3.3 **PHYTOCHEMICAL SCREENING**

3.3.1 **Extraction**

Two extraction processes were used in this study namely - continuous and batch extraction. A soxhlet extractor was used for both processes and four solvent systems namely - methanol, 80% aq. acetone, peroxide-free ether and petroleum spirit (40 - 60 °C). 40 g of the powdered rhizome was extracted batch-wise exhaustively with 300 ml of petroleum spirit (40 - 60 °C), ether, 80% aq. acetone and methanol for 18, 28, 11 and 8 hours respectively. Likewise 40 g of the powdered rhizome was extracted continuously with 300 ml of light petroleum spirit, ether, 80% aq. acetone and methanol for 18, 40, 36 and 24 hours respectively. The extracts obtained from both extraction processes were concentrated *in vacuo* using a rotavapor.

3.3.2 **Phytochemical test**

After extraction is completed, phytochemical test was carried out on each extract to determine the presence of alkaloids, tannins, saponins, anthraquinones and carbohydrates.

3.3.2.1 **Test for alkaloids**

About 0.05 g of each extract was stirred with 4 ml of 1% HCl on a steam bath; 1ml of the filtrate was treated with a few drops of Mayer's, Wagner's and Dragendorff's reagents. A creamy white precipitate with Mayer's reagent and a reddish-brown precipitate with Wagner's and Dragendorff's reagents are taken as preliminary evidence for the presence of alkaloids.

Confirmatory test for alkaloids were performed. Non-alkaloidal compounds capable of giving false - test results with either reagents were carried out. The procedure involves using 1ml of the acidic aqueous extract, prepared as described above and treating it with 28% ammonia solution until the solution was distinctly alkaline to litmus paper and then extracted several times with chloroform (ethanol free). The chloroform extracts were combined and concentrated *in vacuo* to about 2 ml, and then extracted with an equal volume of 1% HCl. The 1% HCl extract was divided into three equal parts and each was treated with a few drops of Mayer's, Wagner's and Dragendorff's reagents respectively. A creamy white or reddish-brown precipitate was taken as confirmatory evidence for the presence of alkaloids.

3.3.2.2. **Test for tannins**:

About 0.05 g of each plant extract was dissolved in 10 ml of distilled water, boiled to solubilise sample, if insoluble in cold water and filtered. Ferric chloride solution was added to one-half of the filtrate and gelatin-salt reagent was added to the other half. To the solution to which ferric chloride solution was added a blue or blue-black precipitate

indicates the presence of condensed tannins. A white precipitate when gelatin-salt reagent is added indicates the presence of tannins generally.

3.3.2.3 **Test for anthraquinones.**

(i) *Borntragers test*: the test is used to identify the presence of free anthraquinones. 0.05 g of each plant extract was shaken with 10ml benzene, filtered and 5 ml of 10% ammonia solution added to the filtrate. The mixture was shaken and the presence of pink, red or violet colour in the ammoniacal (lower) phase indicate the presence of free anthraquinones.

(ii) *Combined anthraquinone test:* Combined anthraquinones are simply referred to as anthraquinone glycosides, they are identified as follows - 0.05 g of each plant extract was boiled with 5 ml aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 3 ml benzene, the benzene layer separated and half its own volume of 10% ammonia solution added. A pink, red or violet colouration in the ammonia phase (lower layer) indicates the presence of anthraquinone derivatives in the extract.

3.3.2.4 **Test for saponins**:

0.05 g of each extract was shaken with 1 ml distilled water in a test-tube for 1 min. It was kept aside and observed for persistent frothing for 15 mins. If the frothing persists this indicates the presence of saponins (Wall *et al.* 1952).

3.3.2.5 **Test for cardiac glycosides**:

3.3.2.5.1 *Legal test*: a small portion of the extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside together with a few drops of 20% sodium hydroxide solution were added. A deep red colour which faded to brownish-yellow indicates the presence of cardenolides.

3.3.2.5.2 *Kedde test*: 1ml of 8% solution of the extract in methanol was mixed with 1 ml of a 2% methanolic 3,5-dinitrobenzoic acid and 1 ml of a 5.7% aqueous sodium hydroxide. A reddish-brown to brownish-yellow colour indicate the presence of cardenolides and the presence of a whitish crystalline solid in the solution indicate the presence of a lactone ring in the cardenolides.

3.3.2.5.3 *Keller - Kiliani Test*: 0.05 g of each extract was boiled with 5 ml of 70% ethanol for two to three minutes and filtered. The filtrate was diluted with an equal volume of water. This solution was shaken with 1-2 drops of strong lead acetate solution to precipitate other pigments and filtered. The filtrate was shaken with 5 ml of chloroform in a 50 ml separatory funnel and chloroform layer collected into a porcelain dish and gently evaporated to dryness on a water-bath. The cooled residue was dissolved in 3 ml of glacial acetic acid containing 2 drops of 5% ferric chloride solution. This solution was transferred into a test-tube containing 2ml of concentrated sulphuric acid a reddish-brown ring at the interface between the two liquid indicates the presence of deoxy sugar, a pale green or blue colour at the upper layer indicates the presence of a steriodal ring as the aglycone portion of the cardiac glycosides.

3.3.2.5.4 *Liebermann - Burchardt's test*: 0.05 g of each plant extract was dissolved in 1 ml of acetic anhydride and cooled in ice. Sulphuric acid was then carefully added. A colour change from blue to green indicated the presence of a steroidal nucleus as the aglycone portion of the cardiac glycoside. A pink colour indicates the presence of terpenes.

3.3.2.5.5 *Salkowski test*: 0.05 g of the extract was dissolved in 1 ml of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring as the aglycone portion of the cardiac glycoside.

3.3.2.6 **Test for flavonoids**

Flavonoids are classified as condensed tannins. Specific tests can be carried out on extracts

to determine the nature of flavonoids present.

(i) *Ammoniacal silver nitrate test*: 0.02 g of the sample was dissolved in 1ml of water and a few drops of ammoniacal silver nitrate was added. The colour was observed before and after heating for 5 minutes. A brown, blue or black colour before heating with the blue turning black or brownish-black and black still remains black is indicative of presence of flavonol glycosides. No colour before and after heating indicates flavanone glycosides. The only exception in this class of flavonoid is hesperidin which turns black after heating. A reddish-brown colour before heating which still remains the same colour after heating indicates flavone aglycones. Chalcones do not form colour before heating except 2',3,4-trihydroxychalcones, it forms a reddish-brown or black colour after heating.

(ii) *Test on untreated crude extract:* 0.02 g of the extract was dissolved in 1ml of 50% ethylacetate solution. The colour in the upper layer is observed. A pale yellow colour in visible light indicates the presence of flavonol glycosides. Flavanone glycosides do not give colour change except hesperidin which is pale yellow in visible light (daylight), flavone aglycones do not give colour in visible and ultraviolet light. Chalcones do not give colours except 2',3,4-trihydroxychalcone which is yellow. Flavone aglycones do not give colours in visible light except Isocogonin and Norivogonin which are pale yellow.

3.3.2.7 **Test for carbohydrate**

3.3.2.7.1 *Molisch's test:* 0.02 g of each extract was dissolved in 1ml of water. 3 drops of α - naphthol solution was added and sulphuric acid poured gently along the side of the test-tube. A pink coloured ring at the interface indicates the presence of carbohydrate. When the amount of carbohydrate was high in an extract the pink colour spreads over the upper layer.

3.3.2.7.2 *Fehling's solution test*: 0.02g of the extracts were heated on a water bath with 1 ml of dilute sulphuric acid for 2-3 minutes. Sodium hydroxide solution was added dropwise

until the solution becomes distinctly alkaline to litmus. The solution was then treated with Fehling's solution. A reddish-brown precipitate indicates the presence of reducing sugars.

3.3.2.7.3 *Selivanoff's test*: this test is used to identify ketoses. 0.02 g of each extract was dissolved in 1ml of water. A few crystal of resorcinol was added and warmed on a water-bath with an equal volume of concentrated hydrochloric acid. A reddish-brown precipitate indicate the presence of ketoses.

3.3.2.7.4 *Test for pentoses*: 0.02 g of each extract was dissolved in 1 ml of water in a test-tube with an equal volume of hydrochloric acid containing a little phloroglucinol. Formation of a red or reddish-brown precipitate indicates the presence of pentoses.

3.4 **Bulk extraction.**

250 g of the powdered rhizome was exhaustively extracted continuously with petroleum spirit (40 - 60 °C), ether, 80% aqueous acetone and methanol in a soxhlet extractor for 18, 40, 36 and 24 hours respectively. The extracts were concentrated using a rotavapor to yield 5.01 g, 3.43 g, 9.51 g and 8.65 g respectively.

3.4.1 **Flavonoid extraction**.

7 g of 80% aq. acetone extract was taken up in 300 ml of water and extracted at room temperature continuously with EtOAc and n-BuOH (4 x 300 ml each). Subsequently, the solutions were concentrated *in vacuo* using a rotavapor to obtain EtOAc, n-BuOH and wter extracts.

3.4.2 **Isolation of flavonoids**

3.4.2.1 Thin Layer Chromatography

Thin Layer chromatography (TLC) was used to separate the flavonoid mixture into its components. Since it resolved the components better, compared to column chromatography and it takes less time to develop. The only shortcoming is that too many plates are handled to effect proper separation of desired amount of flavonoid mixture.

3.4.2.1.1 **Choice of mobile phase**.

TLC on microscopic slides were used to determine which mobile phase would render good resolution of the desired compounds in the mixture. Various ratios of Petrol-EtOAc were tested, these are 1:1, 1:2, 1:3, 1:4, 1:8, 1:9, 3:7, 2:3, 3:2, 7:3. The process involves spotting the sample (EtOAc) extract on the thin layer plate and developing it in a small tank suitable for the slide containing the solvent system and the resulting chromatogram, after air drying, viewed under short wavelength (254 nm) and long wavelength (366 nm) ultraviolet light. The separated spots are then marked and recorded. The resulting chromatogram are compared, culminating in selection of the best solvent system for large scale separation. The result for this experiment are discussed in section 4.1.3.

3.4.2.1.2 **Separation of flavonoid mixture**.

2.0 g of EtOAc extract was separated by preparative TLC as follows. 0.5 g of the extract was dissolved in 5 ml of methanol and 1 ml each was streaked on a 1mm thick (20 x 20 cm) plate. 20 plates were used to separate 2.0 g. The plates were developed in a rectangular TLC tank using petrol-EtOAc (3:2) as mobile phase for 45 minutes at 30 °C. It is pertinent to emphasize here that 1 ml of the extract corresponding to 0.1 g (100 mg) was streaked to avoid over loading of plates.

Seven bands were observed with R_f values 0.91, 0.67, 0.62, 0.54, 0.49, 0.40, and 0.00 respectively. The bands were then scraped into 500 ml beakers labeled 1 - 7 and each extracted exhaustively with methanol and subsequently concentrated *in vacuo* to give fractions, 1 (37.3 mg), 2 (13.6 mg), 3 (9.7 mg), 4 (26.2 mg), 5 (19.9 mg) and 6 (49.2 mg). Each fraction was tested for homogeniety by re-spotting on analytical TLC plates (0.25 mm thick) and developed with three solvent system - n-BuOH-AcOH-H_2O (4:1:5), 60% AcOH and Benzene-Dioxane-AcOH (90:25:4) Fractions 1 - 6 were found to be homogenous except fraction 7 which was a mixture. The result for this experiment are discussed in section 4.2.1.1.

3.5 CHARACTERISATION OF ISOLATED FLAVONOIDS

Three methods were used to characterize and elucidate the structure of the four major fractions (1, 4, 5 and 6) and two minor fractions 2 and 3 ultraviolet spectroscopy, reaction with various spray reagents and R_f values determination by analytical TLC.

3.5.1 Analytical thin layer chromatography

Analytical TLC (0.25 mm thick) on kiesegel 60HR (silica) was performed with three solvent systems: 60% acetic acid, Butanol-Acetic acid-Water (4:1:5) (upper phase) and Benzene - Dioxane-Acetic acid (90:25:4) Spots were identified under ultraviolet light at 254 and 366 nm. The R_f value for each component/fraction determined. The results are depicted in Table 4.10.

3.5.2 Reaction with Spray reagent.

Three spray reagents were used to determine the type of flavonoid, namely - 1% ethanolic aluminum chloride, fuming ammonia solution and Benedict reagent. In addition 0.5% methanolic magnesium acetate was used to determine if any of the fractions is an anthraquinone.

The following procedure was employed: the first six TLC fractions obtained from the mixture were spotted on a 20 x 20 cm plate of 0.25 mm thickness and developed with one or the other of the solvent systems in section 3.5.1 above. After development the plates were viewed before spraying with reagent in visible light (that is daylight) and ultraviolet light (366 nm) Afterwards the plates were then sprayed with one or the other of the aforementioned reagents. Each spray reagent requires a freshly developed plate containing the components. The plates were then viewed at visible (daylight) and under ultraviolet light as mentioned earlier . In cases where ammonia was used as the spray reagent the plate was exposed to fumes of concentrated ammonia and viewed immediately at visible and ultraviolet light. The results for this experiment are in Table 4.11.2 - 4.11.5.

3.5.3 **Ultraviolet Spectroscopy**

Steps in the UV spectral analysis:

The test reagents prepared in section 3.1.4 were used for the following.

(a) A stock solution of the flavonoid was prepared by dissolving a small amount of the compound in about 10 ml of spectroscopic grade methanol. The concentration was adjusted so that the absorptivity (optical density) of the major absorption peak between 250 and 400 nm lies in the region of 0.6 to 0.8 in a 1 cm cell.

(b) The 'methanol spectrum' was measured using 2-3 ml of stock solution of the flavonoid in methanol.

(c) The 'NaOMe spectrum' was measured immediately after the addition of three drops of the NaOMe test reagent to the solution used for step (b). After 5 min, the spectrum was rerun to check for flavonoid decomposition.

(d) The '$AlCl_3$ spectrum' was measured immediately after the addition of six drops of the $AlCl_3$ test reagent to 2 - 3 ml of fresh stock solution of the flavonoid in methanol.

(e) The '$AlCl_3$/HCl spectrum' was recorded immediately after the addition of three drops of the stock HCl solution to the cuvette containing the flavonoid solution and $AlCl_3$ solution (from step (d)

(f) The 'NaOAc spectrum' of the flavonoid was determined by adding coarsely powdered anhydrous reagents grade NaOAc with shaking, to 2 - 3ml of fresh stock methanol solution of flavonoid in a cuvette until an excess of NaOAc was present. In order to be certain that the solution was saturated the cuvette was stoppered and set aside for about 2 - 5 min before measuring the spectrum . A second spectrum was run after 5 - 10 min to check for decomposition.

(g) The 'NaOAc/H_3BO_3 spectrum' was determined by two methods. The methods depend on whether or not decomposition of the flavonoid was observed during the recording of 'NaOMe spectrum'. If decomposition was observed then the 'NaOMe spectrum was rerun after five minutes, then procedure I was employed. When decomposition of the flavonoid in the presence of NaOMe did occur, procedure II was used for the NaOAc/H_3BO_3 spectrum.

Procedure I: Sufficient powdered anhydrous reagent grade H_3BO_3 to give a saturated solution was added with shaking to the cuvette (from step f - which contained the NaOAc). The spectrum is then recorded.

Procedure II: Five drops of the H_3BO_3 stock solution were added to 2 - 3 ml of fresh stock methanol solution of the flavonoid. The solution was then quickly saturated with coarsely powdered reagent grade NaOAc and the 'NaOAc/H_3BO_3 spectrum' recorded.

3.6 BIOLOGICAL ACTIVITY

In this work two methods of measuring biological activity were employed namely, brine shrimp lethality assay to determine the toxic levels of the crude drug extracts and anti-microbial sensitivity test, to determine the extent to which each extract can inhibit the growth of certain bacteria.

3.6.1 **Brine Shrimp Lethality Assay**

3.6.1.1 **Sample preparation**

0.5 ml of sea water was poured into all vials. A 'control' is prepared for each extract by adding 0.5 ml of sea water and 0.5 ml of dimethyl sulphoxide (DMSO).

Samples were prepared by dissolving 30 mg of each crude extract in 3ml of DMSO to give stock solutions. Five different concentrations namely 1000 mg, 500 mg, 250 mg, 125 mg and 62.5 mg were prepared in triplicate by (adding 0.5 ml of stock solution of crude extract to vials labelled 1000 mg, another 0.5 ml was also transferred to vials labelled 500 mg. 0.5 ml of extract solution is then transferred from the vial labelled 500 mg into 250 mg, similarly, 0.5 ml of extract solution was transferred from 250 mg vials into 125 mg and finally 0.5 ml was transferred from 125 mg into vials labelled 62.5 mg) 3 ml of sea water is added into vials at all concentration levels containing sample and 'control'

3.6.1.2 **Hatching the shrimp.**

Brine shrimp eggs were hatched in a shallow rectangular dish (150 mm x 5 mm) filled with sea water. A plastic divider was clamped in the dish almost touching the base to make two unequal compartments. The eggs (*ca* 50 mg) were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated. After 48 hours the phototropic nauplii were collected using dropping pipettes from the lighted side having been separated by the divider from their shells.

3.6.1.3 **Bioassay**

Ten shrimps were transferred to each of the prepared vial of varied concentrations using a dropping pipette with a long tip and the total volume in the vial adjusted to 5ml with sea water. The nauplii were counted under the microscope in the stem of the pipette against a

lighted background. The vials were maintained under illumination. Survivors were counted after 24 hours, the total death and the percent deaths at each dose level and 'control' were determined. For each extract or crude sample the LC_{50} was calculated at 95% confidence interval by linear regression analysis. The result thereof are in Table 4.13 - 4.13.4

3.6.2 **Antimicrobial Sensitivity Test**.

3.6.2.1 **Sample Preparation**

500 mg of 80% acetone, methanol, n-BuOH and EtOAc extract and 1 mg of isolated fraction 1,4,5 and 6 were respectively dissolved in 1ml of DMSO. Three drops were taken from each crude drug solution and poured on filter paper disks 1 mm diameter. The disks were allowed to dry.

3.6.2.2. **Sensitivity test**.

Two experiments were performed. The first involves determining the extent to which 80% acetone and methanol extracts inhibited the growth of selected bacteria sp. The other involves determining the extent to which EtOAc, n-BuOH and MeOH extracts, and the effect of fractions 1,4,5 and 6 on the growth of selected bacteria species.

Method: Each of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli* were preincubated in 5 ml of tryptic soy broth for 2 hours, mixing the culture with 25 ml of melted Mueller-Hinton agar cooled at 45 °C and making a 30 ml pour plate in a large (150 x 15 mm) petri dish, and allowed to solidify and used immediately.

3.6.2.2.1 **Sensitivity test for 80% acetone and methanol extract**.

The 80% acetone and methanol extracts were dispensed immediately on the agar surface (as

prepared in 3.6.2.2 above) containing all the bacteria except *Escherichia coli*. After 24 hours the zones of inhibition around each disk were measured by measuring the diameter (mm).

3.6.2.2.2 **Senstivity test for EtOAc, n-BuOH, and MeOH crude extracts including fractions 1,4,5 and 6.**

Antibiotic disk containing isolated fraction 1,4,5 and 6 and n-BuOH, EtOAc, and MeOH crude extracts were immediately dispensed on the agar surface of the petri dishes containing all bacteria (as prepared in 3.6.2.2). After 24 hours the plates were examined for inhibition of bacterial growth. This is done by recording positive for inhibition of growth and negative for none inhibition.

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

4.1 PHYTOCHEMICAL SCREENING

4.1.1 Extraction

The yields of extracts obtained by continuous and batch extraction methods are shown in Tables 4.1 and 4.2 respectively. The total amount of extract obtained by using continuous extraction, which was 14.06%, greatly exceeded the amount extracted by batch process. This is due to the fact that continuous extraction method is superior to batch extraction method in that each solvent selectively extracts some specific class of compounds thereby rendering the complex mixture of compounds in a given plant easy to handle for analysis.

In continuous extraction process the solvents are used in increasing polarity. Four solvent systems were used namely petroleum spirit 40 - 60°C to extract fats and lipophilic colouring material (flavonoids and carotenoids). Ether was used to extract compounds containing no free -OH groups and carboxylic acids containing no free phenolic group. Ether also extracts aglycones and some glycosides (rhamnosides). 80% aqueous acetone, and finally methanol extract mainly glycosides which could be saponin, flavonoids, anthraquinone and cardiac glycosides (Stahl, 1965).

In both extraction methods, 80% aqueous acetone was used in place of pure acetone because generally, aqueous solvents are superior to non-aqueous solvents for extracting glycosides. Water has been found to improve the efficiency of less polar solvents particularly pure acetone (Keinanen, 1993). Besides, 80% aqueous acetone extracted more component compared to pure acetone. The result in Table 4.2 clearly shows that 80% acetone extracted more material by weight compared to methanol despite the greater polarity of methanol. This trend is in consonance with the earlier observation by Keinanen

(1993). However, the aqueous acetone extracts less material by weight compared to methanol when used in continuous extraction (Table 4.1). This is an indication that acetone extractable components (based on batch extraction) has been extracted by the preceding two solvents namely - petroleum spirit and ether.

Table 4.1. Amount of extract from 40g powdered sample by continuous extraction.

Solvent	Weight of extract (g)	Percent extract
Petrol 40-60°C	0.70	1.75
Ether	2.30	2.30
80% aq. acetone	1.95	4.88
Methanol	2.05	5.13

Table 4.2 Amount of extracts from 40 g powdered sample by batch extraction.

Solvent	Weight of extract (g)	Percent extracted
Petrol 40 - 60°C	0.70	1.75
Ether	1.28	3.20
80% aq. acetone	3.40	8.50
Methanol	2.57	6.43

4.1.2 Phytochemical Test

4.1.2.1 Alkaloid

Alkaloid tests were performed on extracts of *C. tinctorium* to determine which solvent extract of the plant contain alkaloid. Table 4.3.1 and 4.3.2 clearly shows the presence of alkaloids in the rhizome of *C. tinctorium*. Alkaloids were detected in the methanolic extract obtained by both extraction methods. Dragendorff's and Wagner's reagents gave positive

tests in contrast to Mayer's reagent which was negative. The presence of alkaloids in this plant as established in this study, confirms previous report (Thoms, 1909). Persions and Quimby (1967) failed to detect alkaloids probably because of their restrictive use of Mayer's reagent only.

Tanble 4.3.1 Influence of alkaloidal reagent on extract from continuous extraction.

Sample	Observation			Inference
	Dragendorff	Wagner	Mayer	
Petrol 40 - 60°C	-	-	-	Alkaloid absent
Ether	-	-	-	Alkaloid absent
80% acetone	-	-	-	alkaloid absent
Methanol	+	+	-	Alkaloid present.

Table 4.3.2 Influence of alkaloidal reagent on extracts from batch extraction.

Sample	Observation			Inference
	Dragendorff	Wagner	Mayer	
Petrol 40 - 60°C	-	-	-	Alkaloid absent
Ether	-	-	-	Alkaloid absent
80% acetone	-	-	-	Alkaloid absent
Methanol	+	+	-	Alkaloid present

Key: + = reddish -brown precipitate

\- = *no precipitate.*

4.1.2.2 **Anthraquinone**

The presence of anthraquinones were investigated due to their therapeutic effect as purgative drugs (as previously discussed in 1.1.2). The profile in Table 4.4.1 and 4.4.2 shows that the plant contains only combined anthraquinone since it reacted positively with

the modified Borntrager reagent only but failed the simple Borntrager's test. It is important to point out that free anthraquinones respond to Bontragers test but very stable anthraquinone glycosides or reduced anthraquinones of the anthranol type do not respond to simple Borntrager's test.

However, the 80% acetone and methanol extracts from batch extraction respond positively to the modified Borntrager's test indicating the presence of combined anthraquinone. This is not suprising as anthraquinones are polar in nature, and hence extractable by polar solvents, like methanol and acetone. In the continuous extraction procedure, only the aqueous acetone extract showed positive test for anthraquinone glycoside. It is not surprising that the methanol extract does not contain anthraquinone glycosides, since the polar acetone which is the preceding solvent is capable of extracting all the anthraquinone glycoside.

It is note worthy to point out that this is the first time that the presence of anthraquinone glycosides are reported from this plant. There is no previous report on detection and isolation of anthraquinone in this plant.

Table 4.4.1 Detection of anthraquinones in extracts from continuous extraction.

Sample	Observation		Inference
	Borntrager test	Combined anthraquinone test	
Petrol 40 - 60°C	-	-	Anthraquinone absent
Ether	-	-	Anthraquinone absent
80% acetone	-	+	Anthraquinone glycopside present
Methanol	-	-	Anthraquinone absent

Key: + = pink colour in ammoniacal phase

\- = No colour in ammoniacal phase.

Table 4.4.2. Detection of anthraquinones in extracts from batch extraction.

Sample	Observation		Inference
	Borntrager test	Combined anthraquinnone test	
Petrol 40 - 60°C	-	-	Anthraquinone absent
Ether	-	-	Anthraquinone absent
80% acetone	-	+	Anthraquinone glycoside present
Methanol	-	+	Anthraquinone glycoside present.

Key: + = pink colour in ammoniacal phase

\- = No colour in ammoniacal phase.

4.1.2.3 **Cardiac glycosides.**

The presence of cardiac glycosides in this plant was investigated. Tables 4.5.1 and 4.5.2 shows the reponses of various reagents on the plant. All extracts from both extraction methods except methanol extract from continuous extraction respond positively to Legal, Kedde, Keller-Kiliani (K-K) and Liebermann-Burchardt (L-B) test. However, all the extracts from both extraction methods respond to Salkowski test.

A positive Legal test is indicative of a cardenolide. Kedde test indicates that they are *Digitalis* - like in nature possessing a deoxy sugar reflected by the reddish - brown ring formed at the interface (Trease and Evans, 1978). A positive response to Liebermann - Burchardt test with a blue to green colour shows that the cardenolide possesses steroidal nucleus as the aglycone. The Salkowski test responds to the same compound as Liebermann-Burchardt test. An appraisal of Table 4.5.1 shows that all test employed to detect cardenolides, in the methanol extract with the exception of Salkowski test failed. Consequently, this trend implies that Salkowski test is the most sensitive among the reagents

used. Thus, we can clearly say that methanol extract from successive extraction contains cadenolides but in small quantities.

Based on the result of these tests, the likely cardenolides in *C. tinctorium* are of the type exemplified by compound (**X**) (see page 5) in which the hydrogen of the hydroxyl groups are replaced by sugars. The presence of the cardenolides in all the extraction solvents can be explained from the structural nature of the compounds which allow them to be soluble in both polar and non-polar solvents.

Table 4.5.1 *Detection of cardiac glycosides in extract obtained from continuous extraction*.

Sample	Observation					Inference
	Legal	Kedde	K-K	L-B	Salkowski	
Petrol 40-60°C	Brownish-yellow colour	Yellowish-brown colour	A reddish-brown ring at interface	Colour change from blue to green	A reddish-brown ring at interface	Cardiac glycoside present
Ether	Colour change from deep red to brownish-yellow	Reddish-brown colour	Reddish-brown ppt at interface	Colour change from violet to blue to green	A reddish-brown ring at interface	Cardiac glycoside present.
80% acetone	Colour faded from red to reddish-brown	Brown ppt	A reddish-brown ppt at interface	Violet to blue	Reddish-brown ring at interface	Cardiac glycoside present
Methanol	No response	No response	No ring at interface	No response	Reddish-brown ring at interface	Cardiac glycoside present.

Table 4.5.2 Detection of cardiac glycosides in extract from batch extraction.

Sample	Observation					Inference
	Legal	Kedde	K-K	L-B	Salkowski	
Petrol 40-60°C	Brownish - yellow	Yellowish-brown	Reddish-brown ring at interface	Colour change from violet to blue to green	A reddish-brown ring at interface	Cardiac glycoside present.
Ether	Brownish-yellow	Brownish-yellow	Reddish-brown ring at interface	Colour change from violet to blue to green	Reddish-brown ring at interface	Cardiac glycoside present
80% acetone	Brownish-yellow	Brownish-yellow	Reddish-brown ring at interface	Colour change from violet to blue to green	Reddish-brown ring at interface	Cardiac glycoside present
Methanol	Brownish-yellow	Yellowish - brown	Reddish-brown ring at interface	Colour change from violet to blue to green	Reddish-brown ring at interface	cardiac glycoside present.

4.1.2.4 **Saponins**

The phytochemical test as indicated by Tables 4.6.1 and 4.6.2 shows the presence of saponins in all extract obtained by batch and continuous extractions in varying degrees. The saponin was determined by the time taken for the foam to disappear. For the batch

extraction method the extent of foaming decreased in this order, 80% acetone > methanol > ether petroleum spirit. However, the result from continuous extraction reveals that, ether does not respond to frothing test, this may likely be due to the fact that the preceding solvent which is petroleum spirit extract the same saponin(s) which the ether is expected to extract. The saponin content in each extract obtained by continuous extraction decrease in this order 80% acetone > methanol > petroleum spirit.

4.1.2.5 Tannins

Most of the extracts tested responded to ferric chloride test and gelatin-salt test as depicted in Tables 4.7.1 and 4.7.2, indicative of the presence of tannins. Petrol 40 - 60 °C extract contains condensed tannin due to the green colour imparted with ferric chloride. Ether extracts a mixture of hydrolysable and condensed tannins as evident by the dark blue-green colour imparted with ferric chloride. 80% acetone extract contains condensed tannins due to the green colour imparted with ferric chloride. However, methanol extract responds blue - green to ferric chloride indicative of the presence of condensed and hydrolysable tannins.

Table 4.6.1 Detection of Saponins in extracts from continuous extraction.

Sample	Observation for frothing test	Inference*
Petrol 40-60 °C	Foam formed persisted for 3 mins	Saponin present
Ether	No foam	Saponin absent
80% acetone	Foam formed persisted for 15mins	Saponin present
Methanol	Foam formed pesisted for 5 mins	Saponin present

* The longer the time for foam to last, the greater the amount of saponin in the plant extract.

Table 4.6.2 Detection of Saponins in extracts from batch extraction

Sample	Observation for forthing test	Inference*
Petrol 40-60 °C	Foam persisted for 3 mins	Saponin present
Ether	Foam persisted for 7 mins	Saponin present
80% acetone	Foam persisted for 18 mins	Saponin present
Methanol	Foam persisted for 10 mins	Saponin present

* The longer the time for foam to last, the greater the amount of saponin in the plant extract

Table 4.7.1 Detection of tannins in extracts from continuous extraction.

Sample	Observation		Inference
	Gelatin-salt	$FeCl_3$	
Petrol 40-60 °C	White precipitate	Green	Presence of condensed tannins
Ether	White precipitate	Black-green	Presence of hydrolysable and condensed tannins.
80% acetone	White precipitate	Green	Presence of condensed tannins
Methanol	White precipitate	Blackish-green	Presence of hydrolysable and condensed tannins

Table 4.7.2 Detection of tannins in extract from batch extraction.

Sample	Observation		Inference
	Gelatin-salt	$FeCl_3$	
Petrol 40-60 °C	White precipitate	Green	Presence of condensed tannins
Ether	White precipitate	Black- green	Presence of hydrolysable and condensed tannins
80% acetone	White precipitate	green	Presence of condensed tannins
Methanol	White precipitate	Dark blue-green	Presence of hydrolysable and condensed tannins.

4.1.2.6 **Flavonoids**

A preliminary test to investigate the types of flavonoid present in each extract was conducted by noting the colour of the flavonoid in water saturated ethyl acetate (untreated) and in ammoniacal silver nitrate. Tables 4.8.1 and 4.8.2 shows the response of flavonoids in extract obtained from both extraction methods. The results showed that when the extracts are not treated with ammoniacal silver nitrate they all give yellow, greenish-yellow and brownish-yellow colouration which is indicative of the probable presence of flavonol glycoside, chalcones or flavanone glycosides. A somewhat different pattern emanates when the extracts are reacted with ammoniacal silver nitrate. The result obtained by using this reagent is more definitive. From Table 4.8.1 for successive extraction, petroleum spirit extract respond by giving a greenish-yellow colour before and brown colour after treatment with ammoniacal silver nitrate, indicative of the presence of a broad spectrum/any class of flavonoid compounds. Ether extract respond with a greenish-brown colour before it turns reddish-brown after heating, indicative of the presence of flavonol glycosides. 80% acetone extract responds with a brown colour before it turns green which indicates the probable presence of flavonol, flavanone, flavane and chalcone glycosides. Methanol extract

responds with a brown colour before it turns black after heating, indicative of flavonol glycosides and most probably chalcones and flavanone glycosides in addition. Interestingly, the pattern observed for batch extraction method differs to some extent from that of continuous extraction as shown in Table 4.8.2. Petroleum spirit extract gave similar response with that obtained from continuous extraction. Ether extract gave a yellow colour before and brown after heating indicating the presence of flavonoids. However, 80% acetone extract contains probably chalcone, flavanone or flavonol glycosides since it gave a brown colour before it turned green after heating. Conversely, methanol extracts contain a mixture of classes of flavonoid since it gave a green-yellow colour before it turned brown after heating. It is noteworthy to say that the results obtained by continuous extraction method shows that more classes of flavonoid are extracted compared to batch extraction. For example, the ether and methanol extracts from continuous extraction contain predominantly flavonol glycosides which belong to a class of flavonoids while from batch extraction, they contain a mixture of flavonoids. To further ascertain the class of flavonoids present in the plant extracts, attempt were made to isolate the component flavonoids. However, only the 80% acetone extract gave significant results.

Table 4.8.1 Detection of flavonoids in extracts from continuous extraction.

Sample	Observation			Inference
	Untreated	Ammoniacal silver nitrate		
		Before heating	After heating	
Petrol 40-60 °C	Yellow	Greenish-yellow	Brown	Flavonoid present
Ether	Yellowish-green	Greenish-brown	Reddish-brown	Flavonoid present
80% acetone	Yellow	Brown	Green	Flavonoid present
Methanol	Yellow	Brown	Black	Flavonoid present

Table 4.8.2 Detection of flavonoids in extracts from batch extraction.

Sample	Observation			Inference
	Untreated	Ammoniacal silver nitrate		
		Before heating	After heating	
Petrol 40-60 °C	Yellow	Greenish-yellow	Brown	Flavonoids present
Ether	Yellow	Yellow	Brown	Flavonoids present
80% acetone	Yellow	Brown	Green	Flavonoids present
Methanol	Yellowish-brown	Greenish-yellow	Brown	Flavonoids present.

4.1.2.7 **Carbohydrate**

Tables 4.9.1 and 4.9.2 are profiles of the nature and the types of sugar present in each extract obtained by both extraction methods. All extracts from both extraction methods responded to Molisch's test, indicative of the presence of a carbohydrate. Extracts from both extraction method possess reducing sugars except petroleum spirit 40-60 °C extract since they tested positive to Fehling's solution. The result of this test also indicated that the extracts contained glycosides. All the extracts tested positive to Selivanoff's test implying that they contained compound(s) bonded to ketoses. The test for pentose sugars was also found to be positive in all extracts obtained from batch extraction except petroleum spirit, while petroleum spirit 40-60 °C and ether extracts from continuous extraction methods were both negative for pentose sugars.

Table 4.9.1 Detection of sugar in extracts obtained by continuous extraction process

Sample	Observation				Inference
	Molisch's test	Fehling's test	Selivanoff's test	Pentose test	
Petrol 40-60 °C	Purple colour at interface	No response	Faint red	No response	Sugar present
Ether	Purple colour at interface	Brick-red precipitate	Red colour	No response	Sugar present
80% acetone	Purple colour at interface	Brick-red precipitate	Reddish-brown precipitate	Red colour turns brown on prolong heating	Sugar present
Methanol	Purple colour at interface	Brick-red precipitate	Highly intense reddish-brown precipitate	Red colour turns brown on prolong heating	Sugar present.

4.2 SEPARATION AND CHARACTERIZATION OF FLAVONOIDS.

4.2.1 SEPARATION

4.2.1.1 **Choice of mobile phase.**

In the selection of appropriate mobile phase microscope slide TLC was employed as suggested by Touchstone (1992). The diagrams in Appendix I depict the pattern of separation of EtOAc extract mixture into its components. Since the extract under study was

obtained by EtOAc extraction, it implies that the sample possess the same polarity as EtOAc. Therefore EtOAc as solvent is unsuitable for effecting separation (see Appendix I slide XI). To achieve separation a non-polar solvent was therefore added to EtOAc to reduce its polarity and improve its selectivity by retarding the movement of some components.

Table 4.9.2 Detection of sugar in extracts obtained by batch extraction process.

Sample	Observation				Inference
	Molisch test	Fehling's test	Selivanoff's test	Pentose test	
Petrol 40-60 °C	Purple colour at interface	No response	Faint red colour	No response	Sugar present
Ether	Purple colour at interface	Brick - red precipitate	Reddish-brown colour	Red colour turns brown on prolong heating	Sugar present
80% acetone	Purple colour at Interface	Brick red precipitate	Reddish-brown precipitate	Red colour turns brown on prolong heating	Sugar present
Methanol	Deep purple solution instead of ring	Brick red precipitate	Reddish-brown colour	Red colour turns brown on prolong heating	Sugar present.

Petrol - EtOAc mixture, was used as mobile phase starting from (1:1) and gradually increasing the EtOAc in petrol. The effect of large amounts of EtOAc was investigated by using (1:8) to (1:9) From the diagrams, we can observe that the higher the amount of

EtOAc the poorer the separation becomes. A comparison of (1:1) and (2:3) at first glance looks similar but when reexamined closely (1:1) is superior because the distance between spots 1 and 2 is more, similarly the distance between 2 and 3 is more. In contrast, 2 and 3 are too close in (2:3). However, the effect of a three-fold increase in petrol and two fold in EtOAc on (1:1) to give (3:2) reveals a pattern which is somewhat better. In (3:2) the spots are well separated and without tailing. (7:3) gave fairly good separation compared to ratio (1:1). Considering, the aforesaid explanation petrol - EtOAc ratio of (3:2) was adopted for use in bulk separation of EtOAc extract. On careful development, spot 2 split into four components/bands when the plates were rerun for the second time. Further observation and explanation on this spot is discussed in section 4.2.2.1.

4.2.2. CHARACTERIZATION

4.2.2.1 Analytical TLC

4.2.2.1.1 R_f Value determination

Table 4.10 shows R_f values of each component obtained by developing TLC plates with three different solvent systems. The three solvent systems used gave high R_f values. However Benzene - Dioxane - AcOH (90:25:4) is adjudged to be the best, since the R_f values are fairly far apart in contrast to the other two solvent systems whose R_f values are very close.

Table 4.10 R_f values of isolated flavonoid fractions in three solvent systems.

Component	n-BuOH-AcOH-H_2O (4:1:5)	60%AcOH	Benzene-Dioxane-AcOH (90:25:4)
1	0.87	0.85	0.80
2	0.78	0.86	0.78
3	0.81	0.85	0.66
4	0.80	0.87	0.68
5.	0.84	0.80	0.58
6.	0.90	0.89	0.59

4.2.2.22 **Reaction of with spray reagent.**

Reaction of component/fraction on TLC plates sprayed with selected reagents have proved to be a valuable tool in the charcterisation of flavonoids. This method gives an insight on the nature of the type of flavonoid being investigated. Flavonoid with a carbonyl group at position 4 on the heterocyclic ring respond to spray reagents, by their ability to fluoresce by chelation when sprayed with metallic salt reagents, for example, aluminium chloride or by formation of the corresponding phenolate anion achieved by fuming with ammonia vapour (Haslam, 1966). However, fuming ammonia vapour is the most suitable for routine flavonoid identification while chelation with salts of metals like aluminium chloride have been found to enhance the limits of detection, particularly for flavones, isoflavones and leucoanthocyanin (Seikel, 1962)

In this study the effect of spray reagent on each flavonoid fraction isolated was determined and the response of each was examined when the TLC plates were viewed under visible light (that is daylight) and ultraviolet light (366 nm). Table 4.11.1 gives the profile of the response for each fraction untreated with spray reagent under visible and ultraviolet light while Table 4.11.2, 4.11.3, and 4.11.5 shows the responses when treated with fuming ammonia vapour, 1% ethanolic aluminium chloride, Benedict's reagent and 0.5% methanolic

magnesium acetate respectively. Appraising Tables 4.11.1-4.11.3 we can observe that compounds 1 and 2 respond as flavanones, compounds 3 and 4 as flavonols and fractions **5** and **6** as chalcones. Benedict reagent has not been fully applied to the identification of flavonoids. Gage *et al* ., (1951) were the first to apply it over a relatively wide range of flavonoids, but regrettably their work was inconclusive. The colours obtained after spraying were matched with those obtained by Gage and co-workers (see table 4.11.4). Fraction 1 respond as chalcone, this observation is not in agreement with those observed with fuming ammonia and aluminium chloride, fraction **2** as flavone or flavonol glycosides, **3** and **4** as flavonol glycoside which is in agreement with the trend observed with ammonia and aluminum chloride. The identification of fractions **5** and **6** as flavonol glycosides is not in agreement with result obtained when ammoniacal and aluminum chloride were used .

EtOAc extract was obtained from 80% acetone fraction. From the phytochemical test 80% acetone extract tested positive for combined anthraquinone. To confirm whether any of the isolated compounds from the EtOAc extract could be anthraquinone glycosides, the components were further characterized by using the method of Shibata *et al.*, (1950). This method involves spraying with 0.5% magnesium acetate. However, all the compounds tested failed to give positive response, implying none is an anthraquinone glycoside (See Table 4.11.5)

Table 4.11.1 Flavonoid response, untreated with spray reagent.

Spot	Reagent	None	None	Inference
	Light source	Visible	UV	
1		Colourless	Colourless	Flavanone
2		Colourless	Colourless	Flavanone
3		Yellow	Green	Flavonol
4		Yellow	Green	Flavonol
5		Yellow	Orange	Chalcone
6		Orange yellow	Orange	Chalcone

Table 4.11.2 *Influence ammonia on isolated flavonoids*

Spot	Reagent	NH_3	NH_3	Inference
	Light Source	**Visible**	**UV**	
1		Colourless	Colourless	Flavanone
2		Colourless	Colourless	Flavanone
3		Pale yellow	Brown	Flavonol
4		Yellow	Brown	Flavonol
5		Yellow	*Orange*	*Chalcone*
6		*Orange-yellow*	orange-brown	Chalcone

Table 4.11.3 Influence of 1% ethanolic aluminium chloride on flavonoids

Spot	Reagent	$AlCl_3$	$AlCl_3$	Inference
	Light Source	***Visible***	**UV**	
1		Colourless	Colourless	Flavanone
2		Colourless	Colorless	Flavanone
3		Yellow	Yellow	Flavonol
4		Yellow	Yellow	Flavonol
5		Yellow	Orange	Chalcone
6		Yellow	Orange	Chalcone

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Table 4.11.4 Influence of Benedict Solution on isolated flavonoids.

Spot	Reagent	Benedict Solution		Inference
	Light source	Visible	UV	
1		Colourless	Colourless	Flavanone/Chalcone
2		Yellow	Brown	Flavanone/Flavonol glycoside
3		Yellow	Orange-yellow	Flavonol glycoside
4		Yellow	Orange-yellow	Flavonol glycoside
5		Orange-yellow	Orange-yellow	Flavanol aglycone / glycoside
6		Orange-yellow	Orange-yellow	Flavonol aglycone / glycoside

Table 4.11.5 Influence of 0.5% methanolic magnesium acetate on isolated extracts

Compound	Before heating	After heating	Inference
1	Colourless	Colourless	-
2	Colourless	Pale yellow	-
3	Yellow	Yellow	-
4	Yellow	Yellow	-
5	Yellow	Yellow	-
6	Yellow	Yellow	-

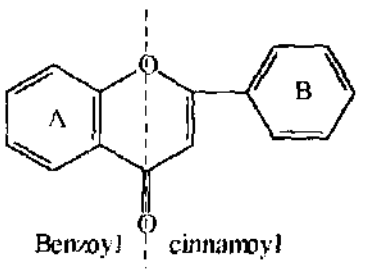
Key: - implies negative test that is no anthraquinone glycoside present.

4.2.2.23 **Ultraviolet Spectroscopy.**

Ultraviolet spectroscopy has become a major technique for the characterization of flavonoid compounds. The value of spectral data in the identification and structural analysis of these plant pigments has been increased considerably by the use of reagents much as aluminium chloride ($AlCl_3$), aluminium chloride/hydrochloric acid $AlCl_3$/HCl, sodium methoxide (NaOMe), sodium acetate (NaOAc) and boric acid-sodium acetate (NaOAc/H_3BO_3) to produce shifts in the absorption maxima in accordance with the location of the various functional groups in the flavonoid molecule. On the basis of the UV spectra alone the structure of most flavonoid compounds can be determined (Jurd, 1962; Mabry *et al.*, 1970; Markham and Mabry, 1975).

The UV spectra of most flavonoids consist of two major absorption maxima one of which occurs in the range 240-285 nm (band II) and the other in the range 300 - 400 nm (band I). In general terms the band II absorption may be considered as having originated from the A - ring benzoyl system and band I from the B-ring cinnamoyl system (see structure below) (Markham and Mabry, 1975).

Table 4.12 contains the λ_{max} of each flavonoid in MeOH and five shift reagents which are NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc, and NaOAc/H_3BO_3. The shift reagent responses depend on the orientation of certain groups and chromophores in the flavonoid structure. The λ_{max} of each flavonoid was extrapolated from the corresponding UV spectra depicted in Appendix IIA - G.

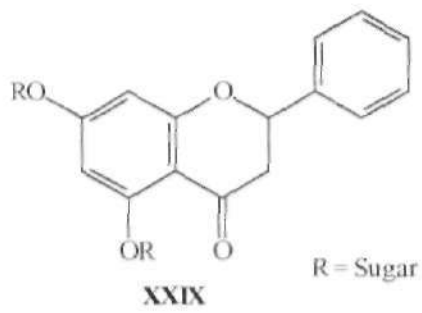


XVIII

Extract 1.

The MeOH spectrum of this compound exhibit a band II λ_{max} at 282 nm characteristic of a flavanone and dihydroflavonol. The band does not shift in the NaOMe induced shift spectrum, indicative of the absence of A ring hydroxylation. Implying that the hydroxyl groups at position 5 and 7 are possibly substituted. However since the NaOAc spectrum is stable the compound is not a dihydroflavonol but a flavanone. The shift induced by $AlCl_3$ and $AlCl_3$/HCl is insignificant indicative of the absence of *o*-dihydroxyl groupings.

Based on the foregoing information the structure of extract 1 is likely to be similar to the structure **(XXIX)**.



R = Sugar moiety or CH_3

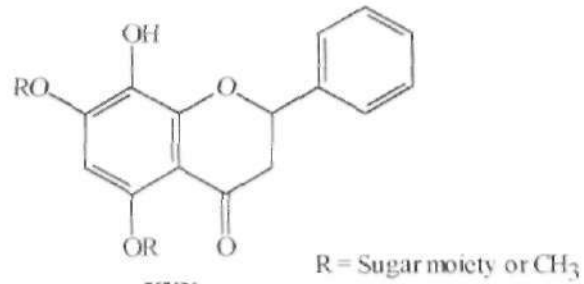
XXIX

Extract 2

The MeOH spectrum of this compound shows a dominant band II, λ_{max} 286 nm characteristic of flavanone and dihydroflavonol. band I λ_{max} 302 nm appears as a shoulder. NaOMe spectrum shows a Band II bathochromic shift of 56 nm, indicative of the absence of 5-hydroxyl group. In addition a shift of this magnitude indicates the presence of A-ring hydroxylation. The NaOAc spectrum shows a bathochromic shift of 56 nm indicative of the presence of a 7-hydroxyl group in a 5-deoxyflavanone or dihydroxyflavonol. The NaOAc spectrum does not decompose with time implying the compound is not a dihydroflavonol but a flavanone. NaOAc/H_3BO_3 shows a band II bathochromic shift of 58 nm which is as high as the expected shift for 6, 7 and 7, 8 dihydroxyl grouping. 5,6-dihydroxyl do not show such a shift.

$AlCl_3$/HCl spectrum exhibit a Band II bathochromic shift of 22 nm indicative of the presence of 5 - hydroxyl group. However, Band II shows a bathochromic shift of 26 nm in $AlCl_3$ over that observed with $AlCl_3$ /HCl this indicates the presence of *o*-dihydroxyl grouping at position 6,7 or 7,8. Since the NaOMe and NaOAc are stable this rules out the presence of alkali sensitive groupings like 5,6,7-, and 5,7,8 trihydroxyl groups.

Considering the information obtained from NaOAc, $AlCl_3$, $AlCl_3$/HCl we can say that the extract possesses alkali sensitive grouping but if we are to consider the NaOMe spectrum the hydroxyl group at position 5 is absent. Conversely, the $AlCl_3$/HCl spectrum shows a bathochromic shift indicative of the presence of 5-hydroxyl group. To reconcile this anomaly it is assumed that the 5-hydroxyl group is substituted, until proved otherwise by mass spectra measurement and nmr. Thus, the proposed structure of extract 2 is likely to be similar to the structure **(XXX)**.



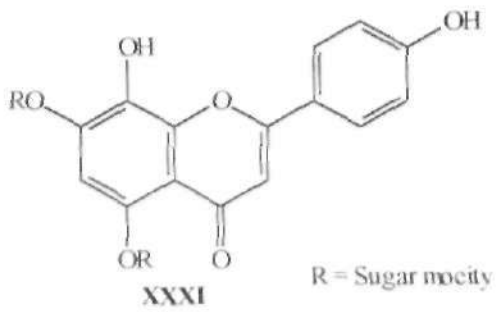
XXX

Extracts 3 and 4

The MeOH spectra of Extracts 3 and 4 show a Band I λ_{max} of 362 and 364 nm respectively which are characteristic of a flavonol. The NaOMe spectrum of both extracts lack a band between 320-330 nm indicative of the probable presence of glycosylation at the 7-hydroxyl group. NaOMe and NaOAc induced shift spectrum for both extracts are similar, indicative of presence of a substituted 7-hydroxyl group in a 4′-hydroxyl flavonol.

The shift induced by $AlCl_3$, $AlCl_3$/HCl and NaOAc/H_3BO_3 are insignificant, indicative of the absence of *o*-dihydroxyl groups at 6,7;7,8 and 5,6,. However, the NaOMe and NaOAc

spectrum, for both extracts do not decompose with time, indicative of the absence of alkali sensitive groupings which as 5,6,7-, 5,7,8-, and 3,3′; 4′-trihydroxyl system. Thus, from the foregoing information, it seems that extracts 3 and 4 might be similar or the same. They are probably isomeric compounds. Thus we propose that structure for extracts 3 and 4 are likely to be similar to the structure (**XXXI**)

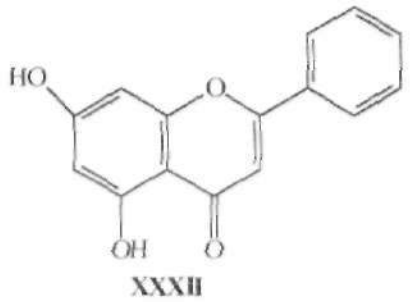


XXXI

Extract 5

This extract exhibits a band II λ_{max} 286 nm in MeOH and band I λ_{max} 362 nm was a shoulder characteristic of a flavanone and dihydroflavonol. band II shows a bathochromic shift of 38 nm in NaOMe indicative of a flavanone and dihydroflavonol. This also indicates the presence of hydroxylation of A-ring and the presence of a 5,7-dihydroxyl system. NaOMe spectrum does not degenerate with time indicative of the probable absence of 3′4′-dihydroxyl system in a flavanone.

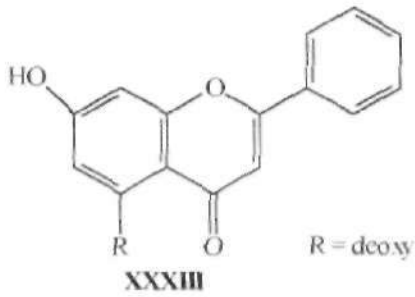
NaOAc spectrum is stable with time indicative that extract **5** is not a dihydroflavonol. The band II bathochromic shift in $AlCl_3$ which is 24 nm is 8nm over and above that observed with $AlCl_3$/HCl indicative of the absence of *o*-dihydroxyl grouping at 6,7 or 7,8. The NaOAc/H_3BO_3 spectrum exhibit band I bathochromic shift of 10 nm indicative of the absence of 6,7 and 7,8 *o*-dihydroxyl grouping. Considering the above information, the structure of extract **5** is likely to be similar to the structure (**XXXII**).



XXXII

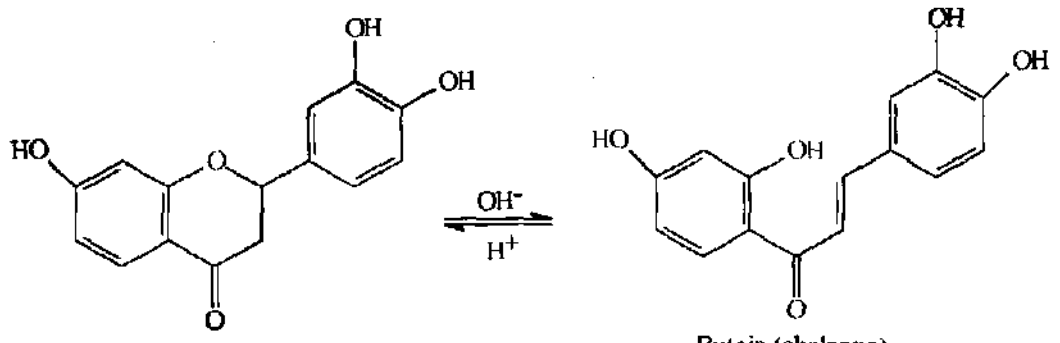
Extract 6

This extract shows a band II λ_{max} 282 nm and band I λ_{max} 366 nm as a shoulder. By implication, the extract is likely to be a flavanone or dihydroflavonol . In NaOMe spectrum there is a band II bathochromic shift of 84 nm indicative of hydroxylation in A - ring. The NaOMe spectrum does not degenerate with time indicative of the absence of 5,6,7- and 5,7,8-trihydroxyl system. Band I shoulder at 366 nm is indicative of the lack of a free 5-hydroxyl group which usually isomerize to chalcones. NaOAc spectrum exhibits a band II bathochromic shift of 56 nm indicative of a 7-hydroxyl group in a 5-deoxyflavanone or dihydroflavonol. Since the NaOAc spectrum does not decompose with time, implying there are no alkali sensitive grouping in the A-ring and extract 6 is not a dihydroflavonol. Since the difference between $AlCl_3$ and $AlCl_3$/HCl bathochromic shift is 2 nm, the extract lacks *o*-dihydroxyl grouping in the A-ring (at 6,7 and 7,8). However, NaOAc/H_3BO_3 shows a band II bathochromic shift of about 18nm indicative of the absence of *o*-dihydroxyl groupings in the A-ring. Based on the aforesaid information the structure of extract 6 is likely to be similar to the structure (**XXXIII**).



XXXIII

The information obtained from the UV spectra for extracts 1-4 are the same as those observed when the extracts are sprayed with reagents. Conversely, there is no agreement for extracts **5** and **6**. UV spectral analysis shows that extracts **5** and **6** are flavanones whilst they respond as chalcone when sprayed with reagent (See Tables 4.11.2 - 4.11.4). The spray reagent used are NH_3 and Benedict's reagent which are alkaline and $AlCl_3$ which is alkaline in solution. To account for this seeming ambiguity, it is important to point out that chalcones and flavanones are isomeric and undergo interconversion readily. This phenomenon is typified by butin isomerising into butein as shown in the figure below.



Butin (flavanone)
XXXIV

Butein (chalcone)
XXXV

Acid or alkali acts as catalyst for this reversible process. Usually in acidic medium the formation of the flavanone is more favoured and in alkaline medium the chalcone. The presence of a hydroxyl in the 6′- position of chalcone or 5 - position of the flavanone plays a significant part in the isomerisation. When present the flavanone becomes predominantly stable and the corresponding chalcones cannot be isolated. If this hydroxyl group is absent, usually an equilibrium mixture is produced (Seshadri, 1962; Dean, 1963). On the basis of the foregoing explanation extract **5** which is suspected to be 5-hydroxy flavanone is expected to be stable, however its isomerisation is catalysed to the corresponding chalcone by the spray reagent used. extract **6** lacks a 5-hydroxyl group so it easily isomerises when treated with spray reagent. However extracts 1 and 2 are flavanones which when treated with spray reagent do not give the corresponding chalcones. This development indicates that they are stable flavanones.

4.3 BIOLOGICAL ACTIVITY.

4.3.1 Brine Shrimp Lethality Assay

Brine shrimp (*Artemia salina* LEACH) is used as a simple bioassay tool for cytotoxicity test for plant extracts. The procedure determines LC_{50} values (that is lethal concentration to cause 50 percent death) in μg/ml of active compounds and extracts in the brine medium. The activities of known active compounds and extracts are manifested as toxicity to the shrimps. The advantages of this method are being rapid results, reliability, inexpensive and convenient assay (Meyer *et al.*, 1982). Table 4.13 shows the LC_{50} value (median lethal concentration) for four extracts determined by calculation at 95% confidence interval using the values of probits and log dose for each extract as depicted in Tables 4.13.1 - 4.13. 4. The LC_{50} value for EtOAc extract was not determined since 100% death of the shrimps was observed even at low concentration. EtOAc extract is the most potent, followed by 80% acetone extract and lastly the n-BuOH and MeOH extracts respectively. The equations used to calculate LC_{50} values are shown in appendix III A- C and probit values were extrapolated from the table shown in appendix IV. *t* values were obtained from the table shown in appendix V.

4.3.2 Antimicrobial Sensitivity Test

The pour plate procedure was used for this experiment because of its simplicity and rapidity (Sellers, 1970). 80% acetone and methanol extract were tested against four bacteria species based on the type of ailment cured by the plant (see section 3.3.2). The bacteria used are three enteric gram negative bacteria namely *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonella typhi*. A cocci, *Staphylococcus aureus* was also used. *K. pneumoniae* causes about 2% bacterial pneumonia and urinary tract infection or enteritis in children. *Ps aeruginosa* causes meningitis, infection of wounds, enteritis and necrotizing pneumonia, *S. typi* causes enteric fever, commonly referred to as typhoid fever. While *Staph. aureus* causes skin infections, such as boils and furuncle. *Staph. aureus* is also

responsible for such serious infections as osteomyelitis, a form of pneumonia and septicemia (Cohen, 1970). Interestingly two crude extracts tested were effective in inhibiting the growth of these four bacteria. Table 4.14.1 gives a profile of the zones of inhibition in mm (obtained by measuring the diameter of the clear zones) of 80% acetone extract and MeOH extracts on four bacteria. The 80% acetone extract inhibits *S. typhi* and *K. pneumoniae* more than methanol extract. This means that the former extract is more effective in arresting typhoid fever and bacterial pneumonia than the latter. However, MeOH extract inhibits *Ps aerugionsa* and *Staph. aureus* more than 80% acetone extract, implying that it is more effective in arresting enteritis, wound infections, necrotizing pneumonia and skin infections.

Four isolated extracts **1**, **4**, **5** and **6** (Table 4.14.2) were tested against the four aforementioned bacteria with an additional enteric gram negative bacteria *Escherichia coli* which causes inflammation of lungs, biliary tract, meninges and peritoneum. It also causes urinary tract infection (Cohen, 1970). Extract **5** inhibits *K. pneumoniae* and *E. coli*. Hence it can be used to cure bacterial pneumonia and inflammation of the lungs, meninges and peritoneum. Extracts **1**, **4** and **6** do not inhibit the test bacteria.

Crude extracts of n-BuOH, EtOAc and MeOH inhibit the growth of all bacteria tested. It is noteworthy to point out that EtOAc extract inhibits the growth of *S. typhi* more than any of the bacteria tested. This implies that it contained compound(s) that are active against *S. typhi*. However, extracts **1**, **4**, **5** and **6** were isolated from EtOAc extract. Unfortunately, they could not inhibit the growth of *S. typhi*. This phenomenon can be attributed to any of these three factors. Firstly the concentration of the extract administered might be in sufficient. Secondly, the active extract(s) could be any of the untested extracts **2** and **3**. Thirdly, the solvent system used for TLC to effect separation might not have extracted the active principle that inhibit *S. typhi* if any..

Table 4.12 Ultraviolet absorption spectra of isolated flavonoid extracts

Flavonoid	MeOH λ_{max} (nm)	NaOMe λ_{max} (nm)	$AlCl_3$ λ_{max} (nm)	$AlCl_3$/HCl λ_{max} (nm)	NaOAc λ_{max} (nm)	NaOAc/H_3BO_3 λmax (nm)
1	324sh	324sh	312sh	280	310	None
	282	282	282			
2	302sh	342	336sh	314	342	350sh
	286	286	334	308	286	344
		262sh	286	282		282
3	362sh	362	312	310	376	356sh
	312	312	292	288	362sh	312
	292	292			312	292
					292	
4	364sh	362	364sh	314	354sh	358sh
	310	312	314	292	312	314
	294	298	302		298	310
						298
5	362sh	354	310	308sh	378sh	372
	286	286	282	302sh	340sh	362
				282	284	278
6	366	366sh	380	304sh	352sh	374sh
	358sh	354	306sh	282	338sh	366
	350	294	282		282	300
	334sh					280
	304					
	282					

Table 4.13 LC_{50} Value obtained at 95% confidence limits

Extracts	LC_{50} µg/ml
EtOAc	lethal at all concentration
n-BuOH	437 ± 8
80% acetone	240 ± 3
MeOH	871 + 5

Table 4.13.1 Toxicity test for 80% acetone extract.

Dose µg/ml	log dose	Number of dead shrimp out of 30	Percentage Response	Probit
1000	3.000	27	90	6.28
500	2.699	21	70	5.52
250	2.398	15	50	5.00
125	2.097	7	23	4.26
62.5	1.796	6	20	4.16

Table 4.13.2 Toxicity test for n-Butanol extract.

Dose µg/ml	log dose	Number of dead shrimp out of 30	Percentage Response	Probit
1000	3.000	27	90	6.28
500	2.699	12	40	4.75
250	2.398	10	33	4.56
125	2.097	5	17	4.05
62.5	1.796	0	-	-

Table 4.13.3 Toxicity test for ethylacetate extract

Dose µg/ml	log dose	Number of dead shrimp out of 30	Percentage Response	Probit
1000	3.000	30	100	-
500	2.699	30	100	-
250	2.398	30	100	-
125	2.097	30	100	-
62.5	1.796	30	100	-

Table 4.13.4 Toxicity test for methanol extract

Dose µg/ml	log dose	Number of dead shrimp out of 30	Percentage Response	Probit
1000	3.000	20	67	5.44
500	2.699	15	50	5.00
250	2.398	9	30	4.48
125	2.097	7	23	4.26
62.5	1.796	6	20	4.16

Table 4.14.1 Zones of inhibition of methanol and 80% acetone extract on bacteria

Extract	*Staph. aureus*	*Ps aeruginosa*	*K. pneumoniae*	*S. typhi*
80% acetone	14 mm	12 mm	15 mm	20 mm
Methanol	15 mm	15 mm	10 mm	15 mm

Table 4.14.2 Antibacterial activity test of four isolated flavonoid and some crude extracts.

Extract	Organisms				
	S. typhi	*Staph. aureus*	*K. pneumoniae*	*E. coli*	*Ps aeruginosa*
1	-	-	-	-	-
4	-	-	-	-	-
5	-	-	+	+	-
6	-	-	-	-	-
n-BuOH	+	+	+	+	+
EtOAc	+++	+	+	+	+
MeOH	+	+	+	+	+

Key: - = negative test, + = positive test, +++ = extremely positive.

CHAPTER FIVE

5.0 CONCLUSION

Cochlospermum tinctorium A. Rich is a plant of great economic importance. The phytochemical screening of the plant reveals some class of natural product hitherto not detected particularly anthraquinone glycoside. However, the separation of EtOAc extract by preparative TLC results in the isolation of six flavonoid compounds in pure state. The biological activity of the crude extracts were investigated using brine shrimp lethality assay and antimicrobial sensitivity test. The LC_{50} value obtained for crude extracts reveals a strikingly interesting pattern. EtOAc extract was shown to be lethal at all concentrations. The extract therefore, contains active principles of immense antimicrobial activity. From the LC_{50} value for EtOAc extract we can predict with high degree of accuracy that it can inhibit selected bacterial growth tremendously. The antimicrobial sensitivity test also shows that the 80% acetone, methanol, EtOAc and n-BuOH extract can effectively suppress the growth of *Salmonella typhi*, that is they contain active principles that can be used to remedy typhoid fever. The extracts also possess active principle that can be used to remedy gastroenteritis and pneumonia since they are active against *Staphylococcus aureus*, *Esherichia coli* and *Klebsiella pneumoniae*. Isolated pure flavonoid component **5,** a flavanone, is qualitatively active against *Klebsiella pneumoniae* and *Escherichia coli* implying that it can be used to cure bacterial pneumonia and gastoenteritis.

Preliminary structural investigation shows that isolated extracts **1** - **6** are flavonoids. **1, 2, 5** and **6** are flavanones while **3** and **4** are flavonols.

5.1 RECOMMENDATION.

It is evident from the contents of this research work that more work needs to be done. A case in point is the aspect of structural elucidation. Instrumental methods like mass spectroscopy is required to confirm the aglycone portion of the flavonoid glycosides and the

type of sugar bonded to the aglycone. Also, the position of glycosylation needs to be further established. Nuclear magnetic resonance (NMR) spectroscopy is required in determining the number of sugar units attached to the aglycone. NMR is also needed to establish the absolute configuration of the atoms that make up the compounds. Techniques like long and short range COSY (^{1}H-^{1}H and ^{1}H - ^{13}C), NOESY, HMBC, HOMO and DEPT must be done in order to establish the actual structure of the isolated compounds.

Another area that also needs further work is antimicrobial sensitivity test of the isolated compounds, MIC and MBC must be carried out so as to determine the minimum concentration required to suppress bacterial growth. Alade *et al.* (1986) showed MeOH extract is active against *Neisseria gonorrhea*. It is recommended that extracts from continuous extraction and pure compounds be tested on these bacteria in order to know which compound inhibits their growth and subsequently determine the MIC and MBC. Furthermore, more bacteria and possible viruses should be tested so as to discover new uses for the drugs aside from its local use or those mentioned in the literature.

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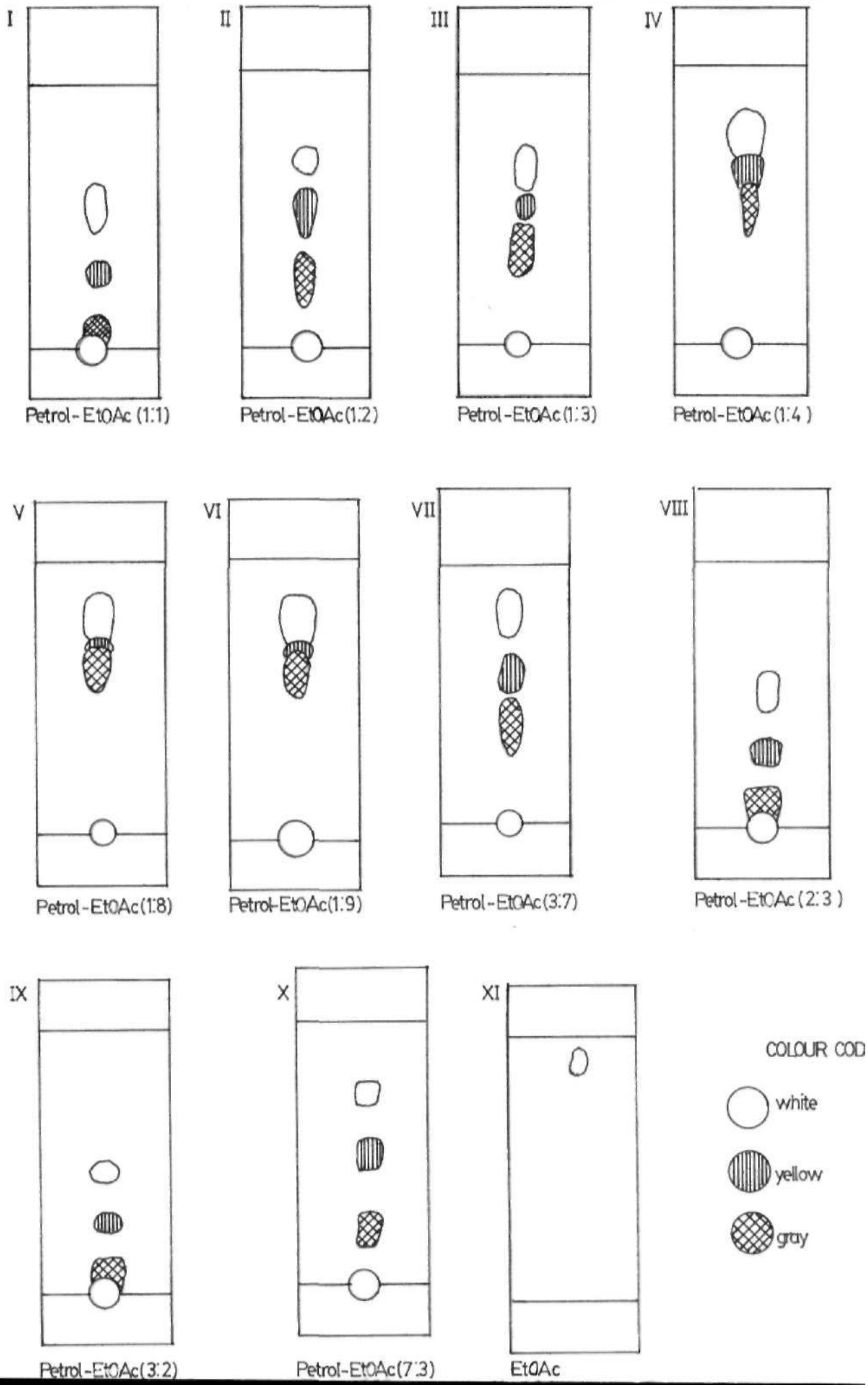
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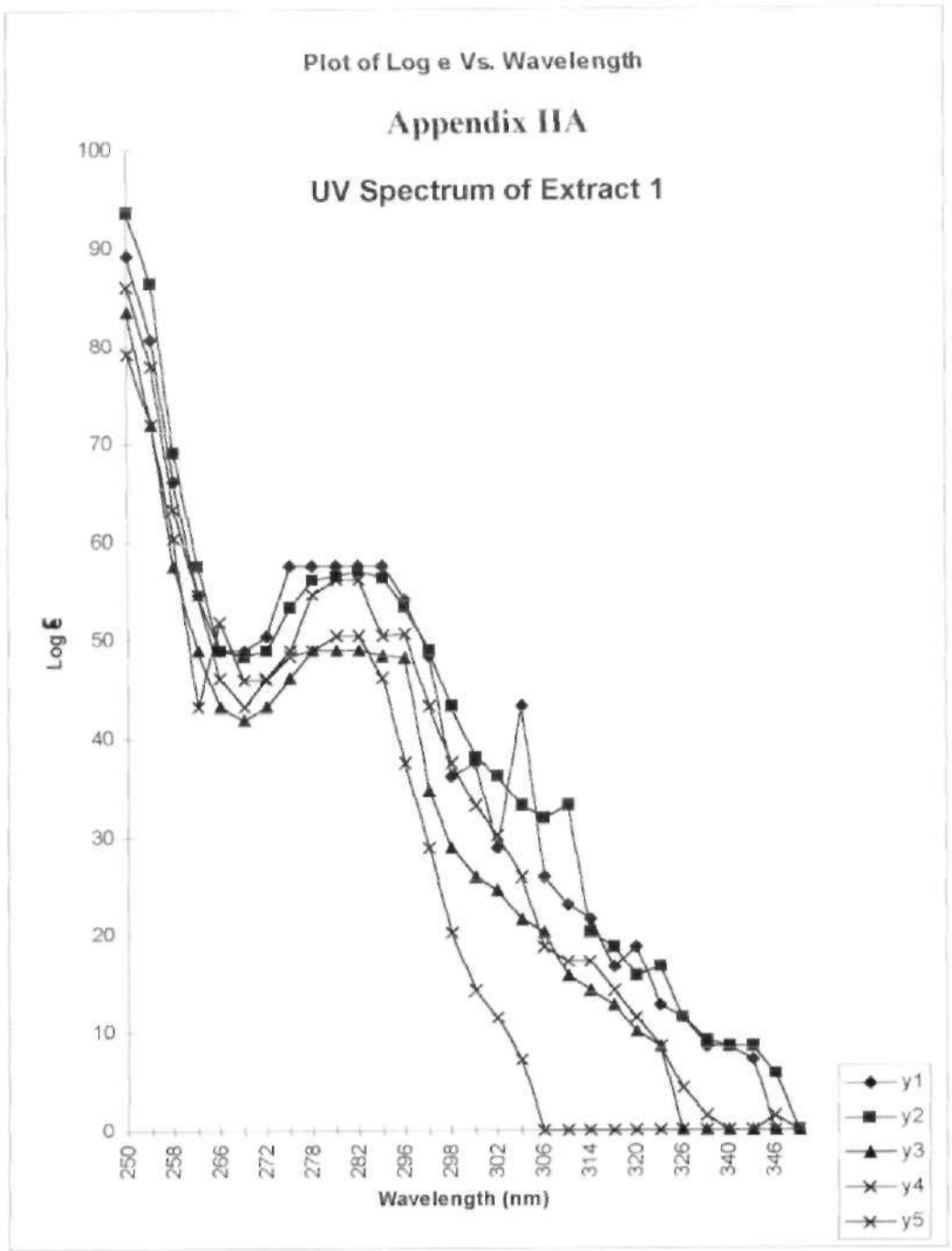
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APPENDIX I

Influence of various ratios of Petrol-EtOAc mixture on EtOAc extract





y1 = MeOH

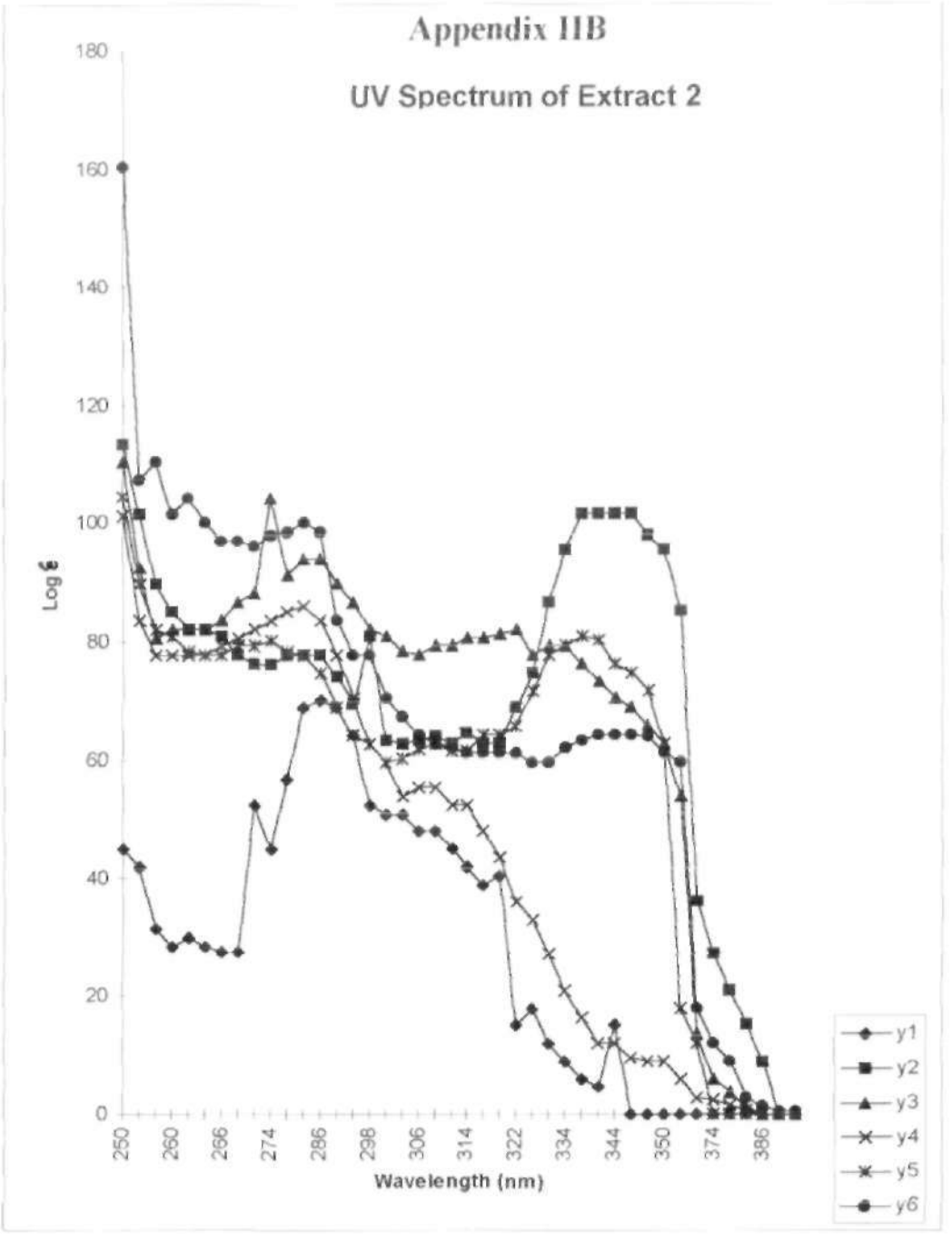
y2 = NaOMe

y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

Appendix IIB



y1 = MeOH

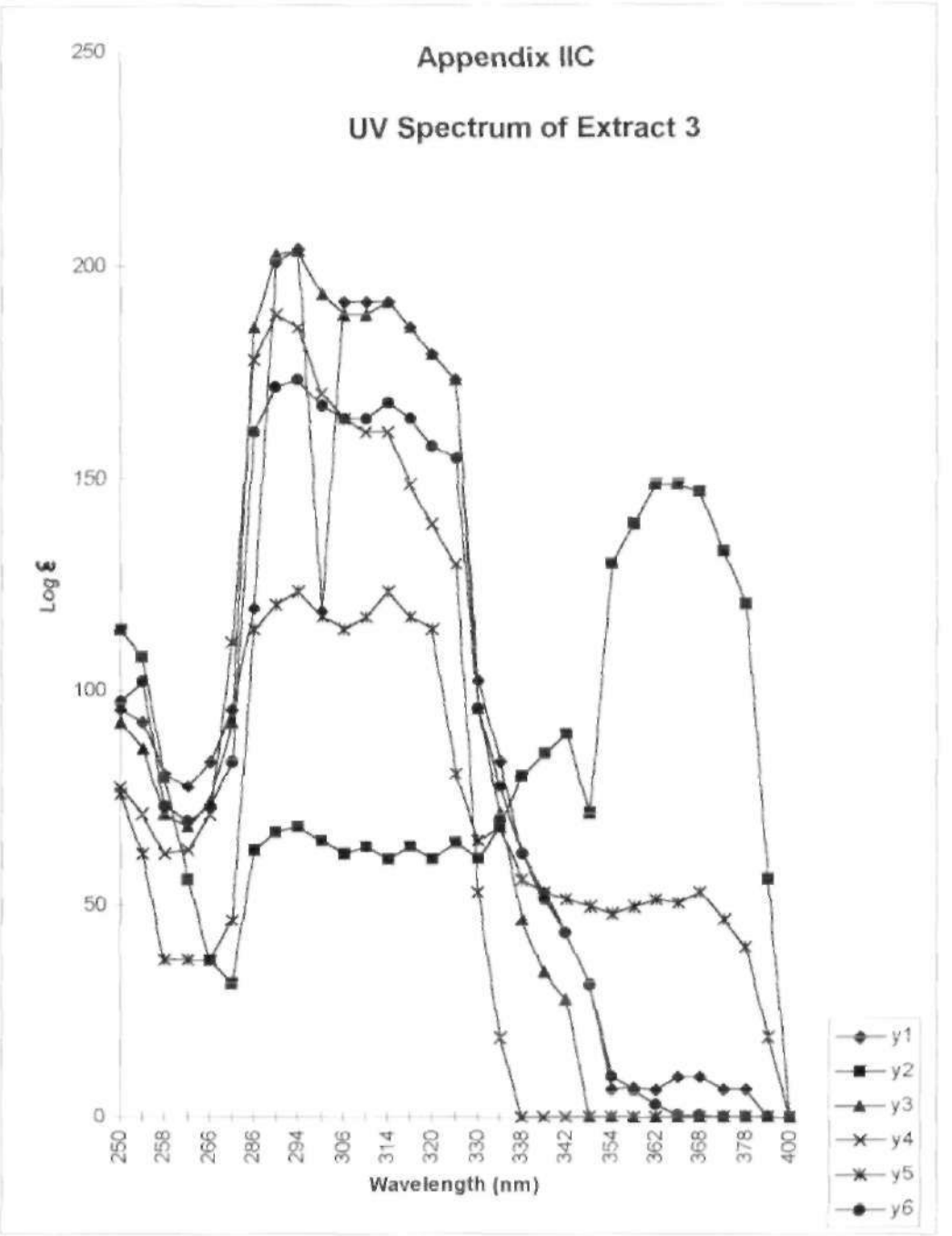
y2 = NaOMe

y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

y6 = NaOAc/H_3BO_3



y1 = MeOH

y2 = NaOMe

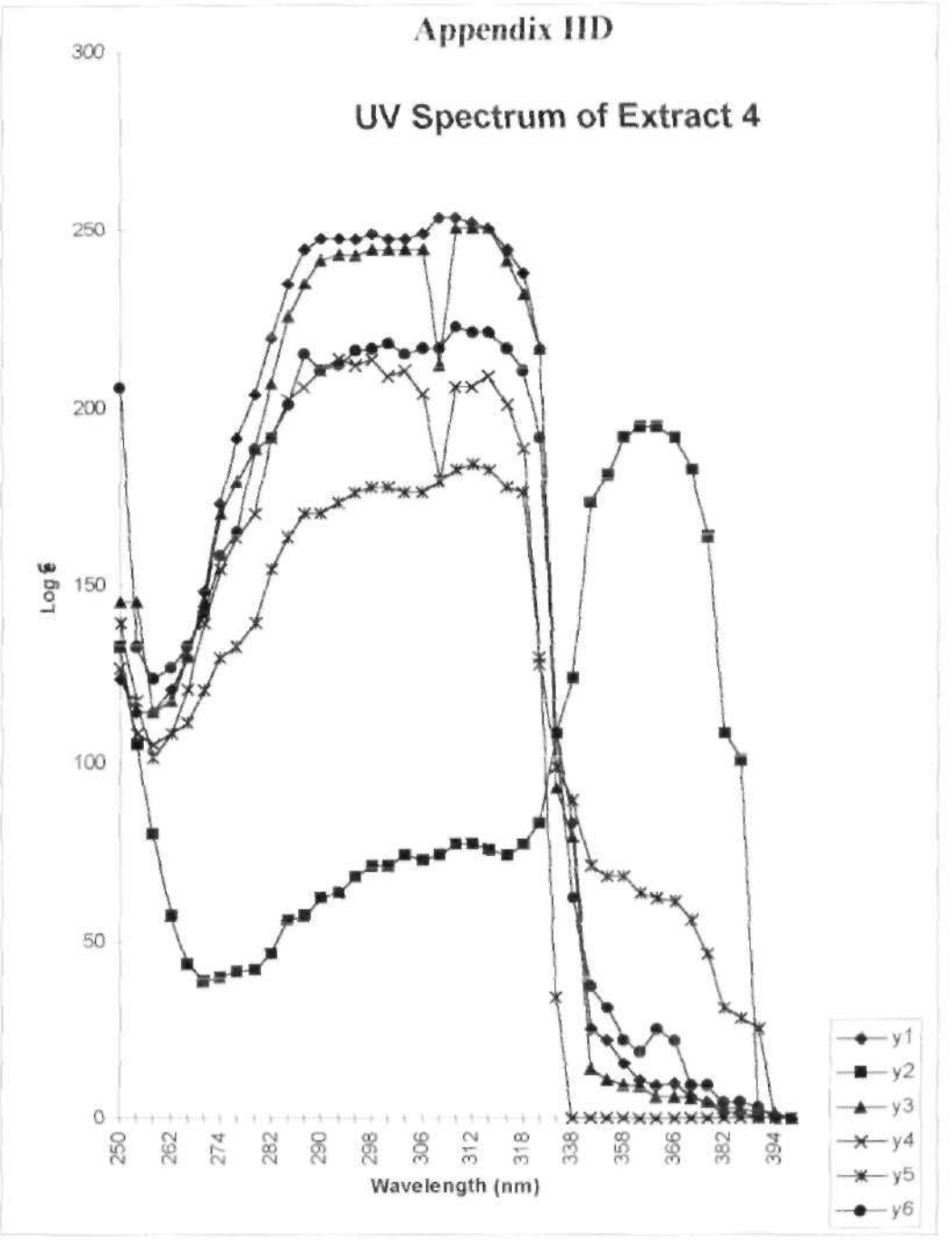
y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

y6 = NaOAc/H_3BO_3

Appendix IID



y1 = MeOH

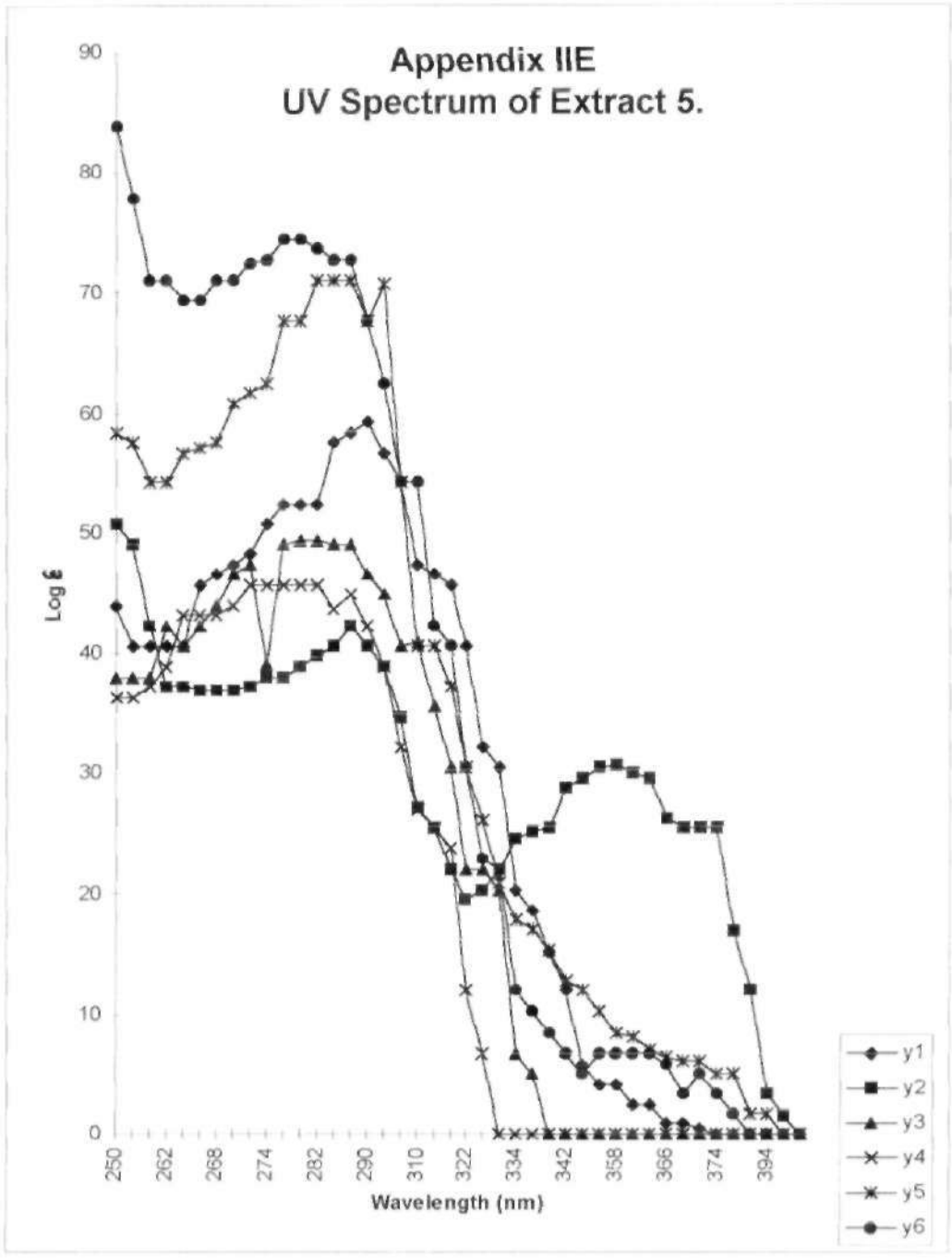
y2 = NaOMe

y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

y6 = NaOAc/H_3BO_3



y1 = MeOH

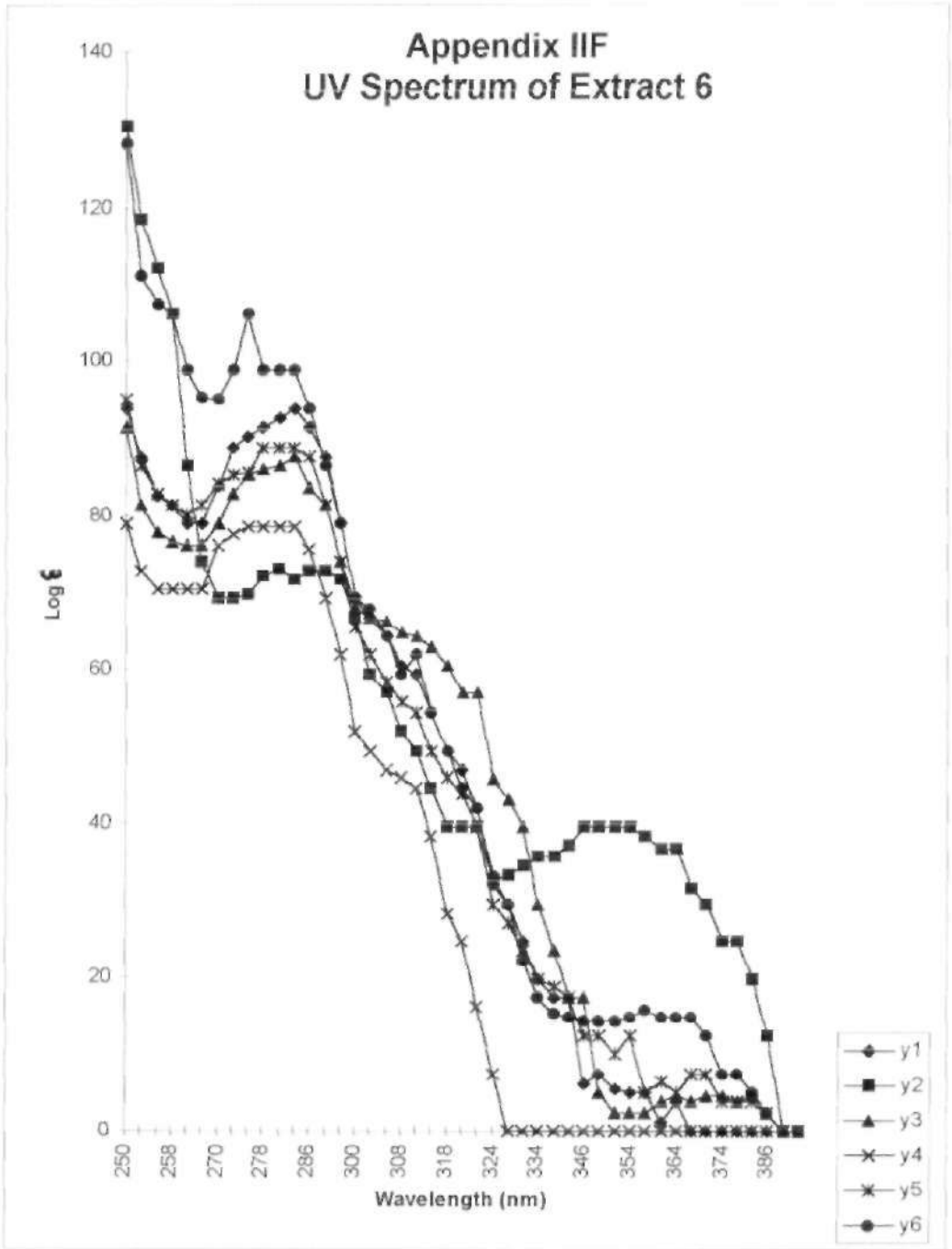
y2 = NaOMe

y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

y6 = $NaOAc/H_3BO_3$



y1 = MeOH

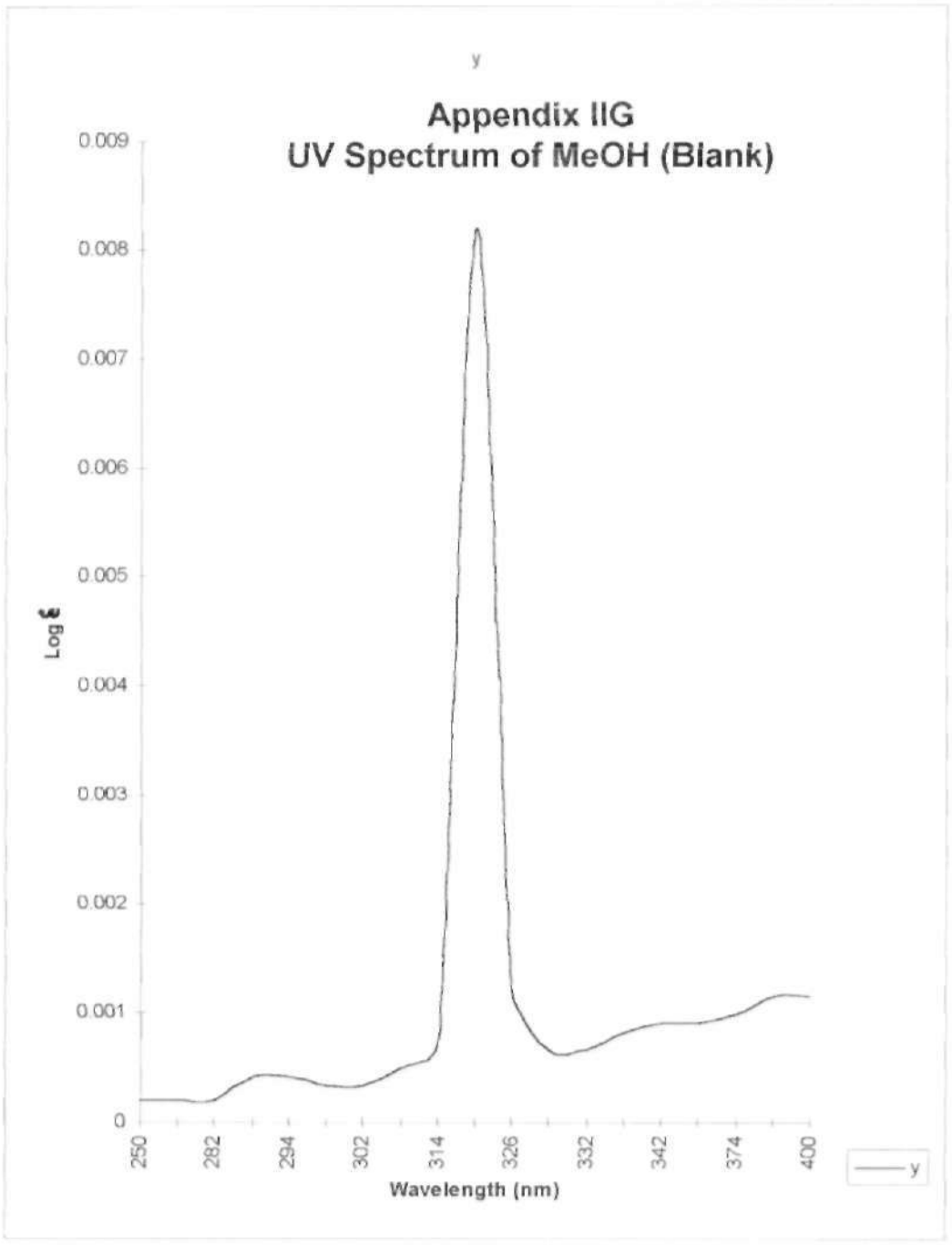
y2 = NaOMe

y3 = $AlCl_3$

y4 = $AlCl_3$/HCl

y5 = NaOAc

y6 = NaOAc/H_3BO_3


y
Appendix IIG
UV Spectrum of MeOH (Blank)
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Log ε
250
282
294
302
314
326
332
342
374
400
Wavelength (nm)
y

APPENDIX IIIA

Let Y = Probit and X = log dose

LC_{50} value calculation for 80% acetone extract

Y	X	Y^2	X^2	XY
6.28	3.000	39.44	9.00	18.84
5.52	2.699	3.47	7.29	14.90
5.00	2.398	25.00	5.75	11.99
4.26	2.097	18.15	4.40	8.93
4.16	1.796	17.31	3.23	7.47
$\Sigma Y = 25.22$	$\Sigma X = 11.99$	$\Sigma Y^2 = 130.37$	$\Sigma X^2 = 29.67$	$\Sigma XY = 62.13$

$$\overline{X} = \frac{\sum X}{N} = \frac{11.99}{5} = 2.40$$

$$\overline{Y} = \frac{\sum Y}{N} = \frac{25.22}{5} = 5.04$$

The correlation coefficient r, is given by:

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

$$\therefore r = \frac{62.13 - 11.99 \times {}^{25.22}/_{5}}{\sqrt{\left(29.67 - {}^{11.99^2}/_{5}\right)\left(130.37 - {}^{25.22^2}/_{5}\right)}} = \frac{1.65}{1.71} = 0.96$$

This value for r is greater than the theoretical value r for $P' = 0.05$ and $\phi = N - 2 = 3$, i.e. 0.88 (see APPENDIX VI). There is therefore a significant correction between X and Y, and so it is worth proceeding with the regression analysis.

The regression coefficient is given by

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

$$= \frac{62.13 - 11.99 \times 25.22 / 5}{29.67 - 11.99^2 / 5} = 1.80$$

The regression line equation is:

$$y - \bar{Y} = b(x - \bar{x})$$

Hence

$$y - 5.04 = 1.80 (x - 2.40)$$

$$\therefore y = 1.80x + 0.72$$

$$\sum d_y^2 = \sum (Y - \bar{Y})^2 = \sum Y^2 - \frac{\sum^2 Y}{N}$$

$$= 130.37 - 25.22^2 / 5$$

$$= 3.16$$

$$\sum d_x^2 = \sum (X - \bar{X})^2 = \sum (X^2) - \frac{\sum^2 (X)}{N}$$

$$= 29.67 - 11.99^2 / 5$$

$$= 0.92.$$

The variance V_y of Y about the regression line is given by:

$$V_y = \left(\sum d_y^2 - b^2 \sum d_x^2\right) / \phi$$

$$= (3.16 - 1.80^2 \times 0.92) / 3 = 0.06.$$

The variance of V_x of a value Xpredicted from the regression line, when Y = 5.00 that is probit 5 implying 50% death in given by:

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

$$= \frac{0.07}{1.79^2}\left[1 + \frac{1}{5} + \frac{3.16}{1.80^2} \bullet \frac{1}{0.92}\right]$$

$$= 0.022$$

Hence $\sqrt{Vx} = \sqrt{0.022} = 0.15$

Limits of errors are calculated using the value of t for $\phi = 3$ and $P = 0.95$ that is 95% confidence internal obtained from a table of t values (see Appendix) are $\pm tV_x = \pm 3.18 \times 0.15 = \pm 0.48$

The value of X calculated from the regression line equation for $Y = 5$ is given by

$$y = 1.80x + 0.72$$

$$5 = 1.80x + 0.72$$

$$1.80x = 4.26$$

$$x = 2.38$$

$$\therefore x = 2.38 \pm 0.48.$$

Since this value are logarithmic their antilog is obtained to get the actual value.

$$\therefore x = 239.88 + 3.01$$

$$= 240 \pm 3\ \mu g/ml$$

$$\Rightarrow LC_{50} = 240 \pm 3\mu g/ml.$$

APPENDIX IIIB

LC_{50} value calculation for n-BuOH extract.

Let Y = Probit and X = log dose

The Y and X values for this calculation are taken from Table 4.13.2.

Y	X	Y^2	X^2	XY
6.28	3.000	39.44	9.00	18.84
4.75	2.699	22.56	7.29	12.82
4.56	2.398	20.79	5.75	10.94
4.05	2.097	16.40	4.40	8.49
-	1.796	-	3.22	-
$\Sigma Y = 19.64$	$\Sigma X = 11.99$	$\Sigma Y^2 = 29.19$	$\Sigma X^2 = 29.66$	$\Sigma XY = 51.09$

Degrees of freedom, ϕ = N - 2 = 3

$$\overline{X} = \frac{\sum X}{N} = 2.40, \qquad \overline{Y} = \frac{\sum Y}{N} = 3.93$$

Correlation coefficient, r is given by:

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

$$= 0.89$$

This r calculated is greater than the theoretical value for r for $P' = 0.05$ and $\phi = 3$, i.e. 0.88, so we proceed with the regression analysis

Regression coefficient is given by

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

$= 4.40$

Regression line equation is

$$y - \overline{Y} = b\left(x - \overline{x}\right)$$

$y - 3.93 = 4.40\ (x - 2.40)$

$\Rightarrow y = 4.40x - 6.63.$

$$\sum d_y^2 = \sum \left(Y - \overline{Y}\right)^2 = \sum Y^2 - \frac{\sum^2 Y}{N}$$

$$= 99.19 - 19.69^2 / 5$$

$$= 21.65.$$

$$\sum d_x^2 = \sum \left(X - \overline{X}\right)^2 = \sum (X^2) - \frac{\sum^2 (X)}{N}$$

$$= 0.91$$

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

$$V_y = \left(\sum d_y^2 - b^2 \sum d_x^2\right) \Big/ \phi$$

$= 1.34$

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

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

$= 0.089$

Hence, $\sqrt{V_x} = 0.29$

Limits of errors are $\pm t\sqrt{V_x}$ at P = 0.05 for $\phi = 3$ which is $\pm 3.18 \times 0.29 = \pm 0.92$.

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

$y = 4.40x - 6.63.$

$5 = 4.40x - 6.63$

Thus $x = 2.64$

$\therefore x = 2.64 \pm 0.92.$

To get actual value we take antilog of x

$\therefore X = 436.52 \pm 8.32$

$= 437 \pm 8$

$LC_{50} = 437 \pm 8$ µg/ml.

APPENDIX IIIC

LC_{50} value calculation for MeOH extract

Let Y = Probit and X = log dose

The Y and X value for this calculation are taken from Table 4.13.4

Y	X	Y^2	X^2	XY
5.44	3.000	29.59	9.00	16.32
5.00	2.699	25.00	7.29	13.50
4.48	2.398	20.07	5.75	10.74
4.26	2.097	18.15	4.40	8.93
4.16	1.796	17.31	3.23	7.47
$\Sigma Y = 23.34$	$\Sigma X = 11.99$	$\Sigma Y^2 = 110.21$	$\Sigma X^2 = 29.67$	$\Sigma XY = 56.96$

$$\overline{X} = \frac{\sum X}{N} = 2.40, \qquad \overline{Y} = \frac{\sum Y}{N} = 4.70$$

Degrees of freedom ϕ = (N - 2) = 5 - 2 = 3

Correlation coefficient, r is given by

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

= 0.92

This r calculated is greater than the theoretical value for r for P′ = 0.05 and ϕ = 3, i.e. 0.88.

So we proceed with the regression analysis

Regression Coefficient is given by

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

$$= 1.10$$

The regression line equation is

$$y - \overline{Y} = b\left(x - \overline{x}\right)$$

$$y - 4.70 = 1.10\ (x - 2.40)$$

$$\Rightarrow y = 1.10x + 2.06$$

$$\sum d_y^2 = \sum \left(Y - \overline{Y}\right)^2 = \sum Y^2 - \frac{\sum^2 Y}{N} = 1.26$$

$$\sum d_x^2 = \sum \left(X - \overline{X}\right)^2 = \sum (X^2) - \frac{\sum^2 (X)}{N} = 0.92$$

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

$$V_y = \left(\sum d_y^2 - b^2 \sum d_x^2\right) \Big/ \phi = 0.05$$

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

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

$$= 0.053$$

Hence $\sqrt{V_x} = 0.22$.

limits of errors are $\pm t\ V_x$ at P = 0.95 for $\phi = 3$

which is $\pm\ 3.18 \times 0.22 = \pm 0.70$

Value of X calculated from the regression line equation for Y = 5 is

$$Y = 1.10x + 2.06$$

$$5 = 1.10x + 2.06$$

Thus $x = 2.94$

$$\therefore X = 2.94 \pm 0.70$$

Antilog $X = 871 \pm 5$ µg/ml

$LC_{50} = 871 \pm 5$ µg/ml.

APPENDIX IV

PROBITS CORRESPONDING TO PERCENTAGES

Probits										
percentages	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	4.45	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 V

	Value of *t*		Degrees of freedom ϕ		value of t
Degrees of freedom	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	00	1.96	2.58

APPENDIX VI

Theoretical values of the correlation coefficient (P′ = 0.05)

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