STUDIES OF SOME PHYTOCHEMICAL AND CYTOTOXIC PRINCIPLES OF LEAVES AND SEEDS EXTRACTS O F Crotalaria retusa L.

BY

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ZARIA

MAY. 1999

DECLARATION

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

N.F. Amako

Amako, N.F.

27th Aug. 1999.

Date

CERTIFICATION

This thesis titled "**Studies of some Phytochemical and Cytotoxic Principles of Leaves and Seeds Extracts of Crotalaria retusa L.**", meets the regulation governing the award of the Degree of Master of Science of Ahmadu Bello University, Zaria, Nigeria, and is approved in its contribution to knowledge and literary presentation.

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Professor S.A. Ojo Member Supervisory Committee	18/8/99 Date
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DEDICATION

"To my husband, Pat, and my children, Iky, Chuma, Edozie and Eji-e"

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ABSTRACT

The leases and seeds of Nigerian Crotalaria retusa L. were extracted using different solvents 95% Ethanol was found to extract the largest amount of materials Specific methods of isolation of pyrrolizidine alkaloids (PAs) showed that the seeds have higher alkaloidal content than the leaves.

Qualitative chemical tests for various classes of natural products were carried out on ethanolic as well as water extracts of leaves and seeds using standard phylochemical methods These tests revealed the presence of alkaloids, tannins, cardiac glycosides, flavonoids and saponins in ethanolic leaves and seeds extracts Water extracts of seeds and leaves were devoid of alkaloids.

Specific chemical analytical test for PAs was carried out on fresh plant parts (seeds, leaves, flowers, roots) The seeds, leaves and flowers of C. relusa contain PAs and PA-N-oxides. Flowers are particularly rich in these alkaloids The roots are devoid of PAs but have PA-N-oxide, which is apparently reported for the first time

The crude mixture of PAs extracted from leaves was purified by crystallization and cleaning-up methods However the crystallization method produced a purer component than cleaning-up method.

The active extracts were categorised into two broad groups for thin-layer chromatography (TLC), based on the nature of the extracts and suitable solvent systems employed. The TLC of ethanolic leaves extract, $CAPL_1$ and ethanolic seeds extract, $CAPS_1$ when viewed under ultraviolet light (366nm) and visible ligh (254nm) each

produced four spots $CAPL_1$ R_f values (0.32, 0.53, 0.66 and 0.90) while $CAPS_1$ R_F values (0.1 I. 0.25, 0.32 and 0.49). The trichloromethane leaves extract, $CAPL_2$. produced five spots whose R_F. values arc 0.20, 0.71, 0.86, 0.93 and 0.96, when the same solvent system was used.

The TLC of the crude mixture of PAs from leaves CAPL, and seeds CAPS, as well as cleaned-up sample of PAs. $CCAPL_1$, and crystals from crude mixture of PAs. $CCAPL_3$ were carried out using three different solvent systems The R_F values of the components of these samples varied slightly with the solvent systems usee and compared with the R_F values from literature. When chromatograms containing the aforementioned samples were sprayed with visualizing reagents they revealed the preset ce of PAs and PA-N-oxides.

Preparative TLC was used to isolate components of $CAPL_1$ anc $CAPL_2$, three fractions and four fractions were obtained respectively The chromatograms containing these fractions gave positive response for phenolic compounds, steroid glycosides and flavonoids when sprayed with visualizing reagents The fractions obtained from $CAPL_1$ and $CAPL_2$ were available in very small quantities hence they were not used for spectroscopic studies.

The leaves and seeds extracts obtained using different solvents were tested for presence or absence of active principles using Brine Shrimp Lethality Bioassay. The LC_{50} values of the extracts, which are reported in this work for the first time, confirmed the toxicity of C. retusa. It also revealed that the most active principle responsible for the cytotoxicity of C. retusa are PAs.

The antimicrobial screening test of the extracts showed that crude mixtures PAs, CAPL and CAPS as well as cleaned-up sample and crystals from CAPL. have significant antifungal anf antibacterial activities against Candidas albicans and Pseudomonas aeruginosa. However the trichloromethane leaves extract exhibited selective inhibitory properties against some gram negative organisms such as Pseudomonas aeruginosa and Escherichia coli.

The spectroscopic techniques such as Infrared and Ultraviolet-visible were used to identify the functional groups present in the active extracts. These studies revealed the presence of carbonyl and hydroxyl groups in CAPL.CAPS. $CCAPL_3$. and $CAPL_2$ samples. The ultraviolet-visible spectroscopy particularly showed the presence of conjugated systems in $CAPL_2$. The nuclear magnetic resonance spectroscopy confirmed the presence of PAs in CAPL.

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CHAPTER ONE

1.0 INTRODUCTION

The phytochemical investigation of plants for bioactive principles may involve the following - extraction of the plant material, separation and isolation of constituents of interest, characterization of the isolated compounds, investigation of the biosynthetic pathways to a particular compound and quantitative evaluation (Trease and Evans, 1992).

Those active principles elaborated by plants include:- alkaloids, essential oil components, tannins, flavonoids, glycosides, saponins etc. On extraction of plant material its fractionation is monitored by bioassay toward isolating active fractions. In attempt to validate the efficacy of the use of plants, studies have to be carried out on pharmacology and toxicology of the plant extract. Investigations have revealed that plants which are in frequent use in traditional medicine are also found to contain toxic and carcinogenic compounds such as Diterpenes and Pyrrolizidine alkaloids (Sofowora, 1986).

The development of new and improved separation techniques and the availability of modern spectroscopic methods such as ultraviolet, infrared, nuclear magnetic resonance spectroscopy, mass spectroscopy, X-ray diffraction has made characterization of organic compounds from these plants without bioactivity testing a lot easier. The thin-layer chromatography techniques provide a rapid method for separation and monitoring of pyrrolizidne alkaloids (PAs) in biological extracts (Chalmers *et. al.*, 1965). Specific methods of detection of PAs on thin-layer chromatograms have been provided by

Mattocks (1967) and Molyneux *et. al.*, (1980). Spectrophotometric determination to detect quantities of unsaturated PAs down to a few micrograms was given by Mattocks, (1967). Molyneux *et. al.*, (1979) provided a rapid test for quantitative evaluation of PAs and their corresponding N-oxides in samples of senecio species using nuclear magnetic resonance spectroscopy. Culvenor and Woods (1965) tabulated the chemical shifts and coupling constants for the protons of pyrrolizidine nucleus and their derivatives. In addition to monocrotaline which is the major alkaloid of Crotalaria retusa L. (Adams *et. al.*, 1939), three new alkaloids including an incompletely characterized base have been isolated from C. retusa L. (Culvenor *et. al.*, 1957). Their structural evidence was provided by combination of infrared spectroscopy and other chemical analytical procedures.

1.1.0 Plant Products of Pharmacological Interest

Plant products which elicit pharmacological response in animals and man include:- alkaloids, cardiac glycosides, flavonoids, saponins, tannins, etc. A brief discussion of the pharmacological uses of these plant products are outlined below:

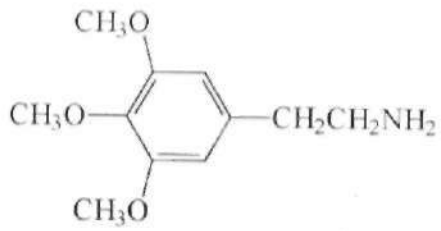
1.1.1 Alkaloids

There is no precise definition of the term alkaloid (alkali-like), or a clear-cut boundary between alkaloids and naturally occuring complex amines. Typical alkaloids derived from plant sources are basic and they contain one or more nitrogen atoms (usually in a heterocyclic ring), and they usually have marked physiological action on man and other animals. It is from these plants containing alkaloids that a large number of drugs are derived.

Two broad divisions are recognised:

(a) Non-heterocyclic alkaloids (biological amines). It consists of six groups, which include Mescaline and Colchicine.

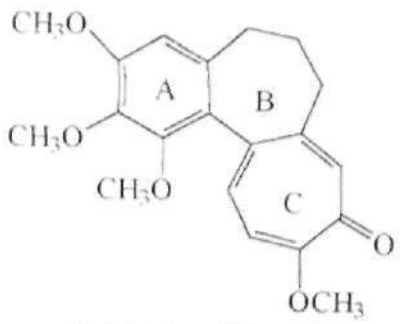
(b) Heterocyclic or typical alkaloids consist of twelve groups. According to their ring structure these include: - Tropane, Quinoline, Indole, Imidazole, Purine and Pyrrolizidine. The basic nuclear structure of each group is shown below



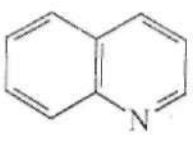
Mescaline (**I**)



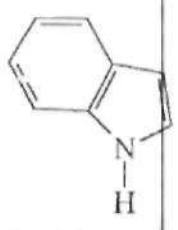
Tropane(**II**)



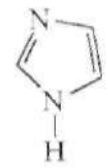
Colchicine (**III**)



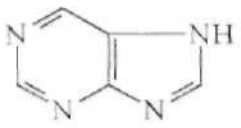
Quinoline (**IV**)



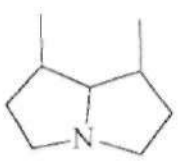
Indole (**V**)



Imidazole (**VI**)



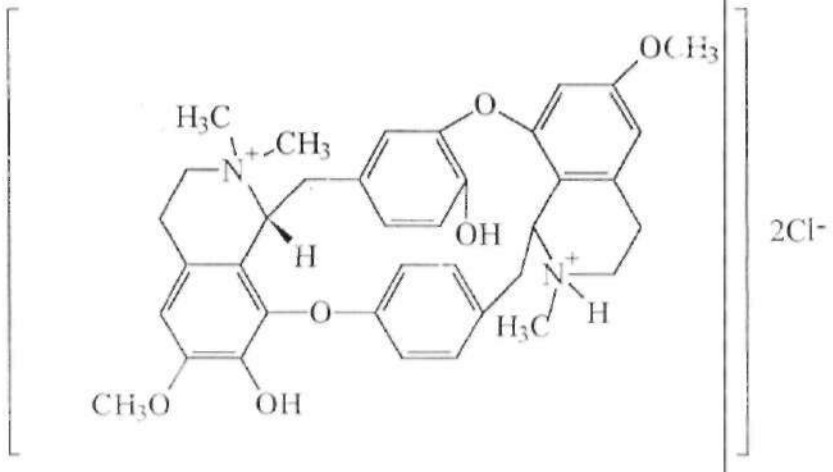
Purine (**VII**)



Pyrrolizidine (**VIII**)

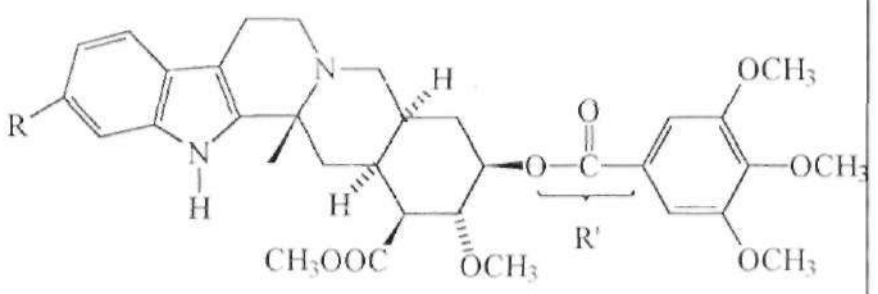
Alkaloids show great variation in their chemical structure and pharmacological actions.

Curare genus (South American arrow poisons) family Menispermaceae, Logamaceae are plants which have been observed to paralyze animals but does not kill them. Investigation into their constituents showed the presence of alkaloids, the most important being D-tubocurarine which has been reported to have muscle-relaxant properties (Trease and Evans 1992; Sofowora, 1993).

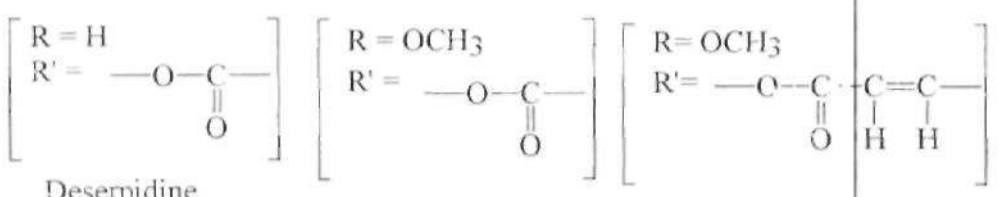


D-Tubocurarine chloride (**IX**)

Rauwolfia vomitoria (African Rauwolfia) has been used to sedate mentally disturbed patients by traditional healers in Nigeria and also reportedly used in many African countries (Dalziel, 1955; Watt and Breyer-Brandwijk 1962). Over a hundred alkaloids have been isolated from roots of Rauwolfia species, these alkaloids include Reserpine, Desperdine, and Rescinamine (Sofowora, 1993).

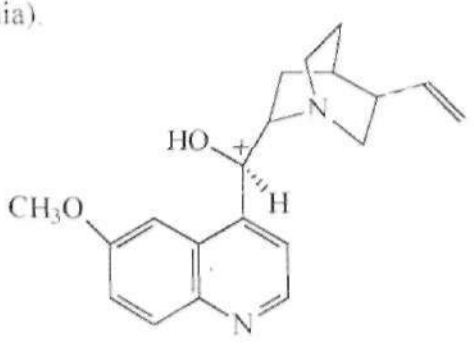


(X)



Deserpidine Reserpine Rescinamine

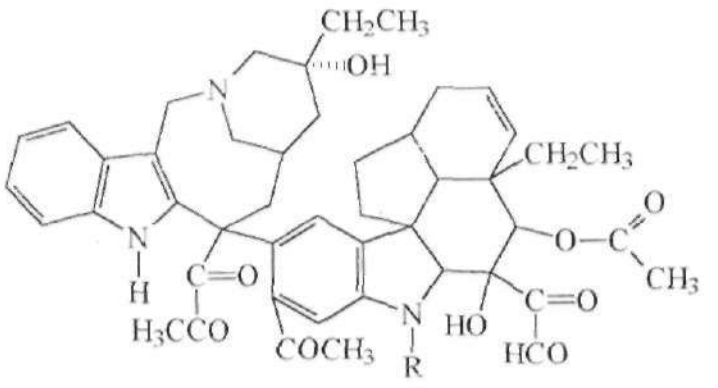
Reserpine together with some of other Rauwolfia has been shown to be responsible for sedative properties of their roots. This alkaloid also exhibits antihypertensive properties and is used in orthodox medicine to treat cases of mild hypertension and anxiety. It is used in Japan for treatment of Cardiac arrhythmias. It is cheaper to produce this alkaloid from the root of Rauwolfia species than do so synthetically (Sofowora, 1993, Trease and Evans, 1992). They also reported the active constituents of Cinchona bark which are alkaloids-quinine (antimalaria) and quinidine (useful for cardiac arrhythmia).



Quinine (**XI**)

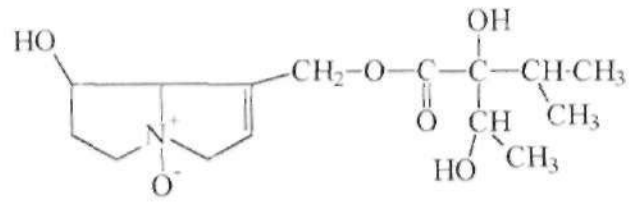
Belladona leaf (Atropa belladona L.- deadly night shade) has been shown to contain alkaloid hyoscyamine. This drug is used for internal preparation such as sedatives and to depress nerve endings to the secretory glands.

The Madagascan periwinkle, Catharanthus roseus G. Don is famous for its antileukaemic and antidiabetic properties which has been attributed to the presence of dimeric alkaloids the most important being vincristine and vinblastine; (Sofowora, 1993).



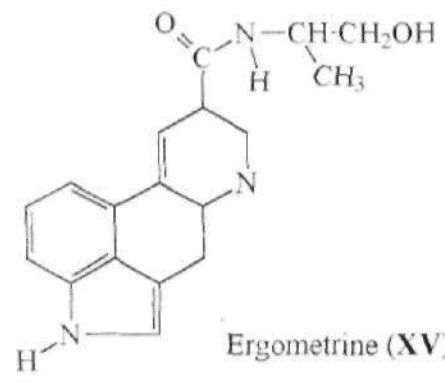
(XII) Vincristine R=CHO
Vinblastine R=CH_3

The genus Senecio (Compositae) well known for their toxicity are known to contain pyrrolizidine alkaloids, several of which have shown antitumuor activity. One such compound is Indicine N-oxide from Heliotropuim indicum. It showed no evidence of hepatoxicity and possessed significant antitumuor activity (Trease and Evans, 1992).



Indicine N-oxide (**XIV**)

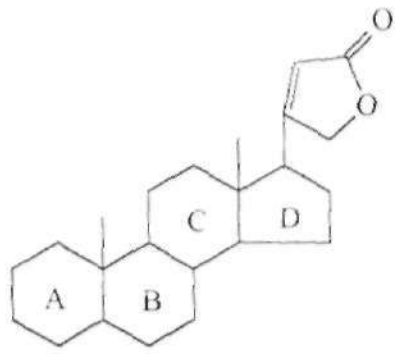
Ergot (Ergot of Rye) is the dried sclerotum of a fungus, Claviceps purpurea tulashe (Fam. Clavicipitaceae) contains a mixture of alkaloids the major alkaloid of therapeutic importance are ergometrine which is used in labour to promote and strengthen uterine contractions during child birth (Trease and Evans 1992, Sofowora, 1993).



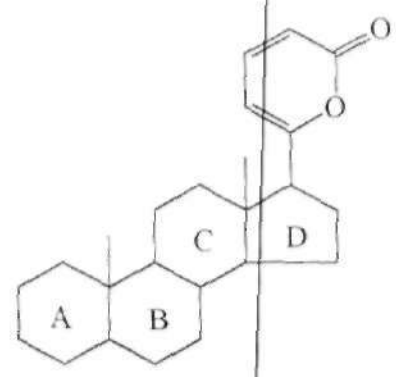
Ergometrine (**XV**)

1.1.2.0 Glycosides

The glycosides are a large and varied group of naturally occuring plant products. They include cardiac glycosides, saponins, flavones and their glycosides etc. They are characterized by the fact that on hydrolysis by either acid or enzyme they yield one or more sugars together with a non-sugar moiety, the aglycone or genin. Two types of genins are recognised according to whether there is a five or six-membered lactone ring. These are known as cardenolides (e.g digitoxigenin) and bufanolides e.g scillarenin).



Cardenolide (**XVI**)

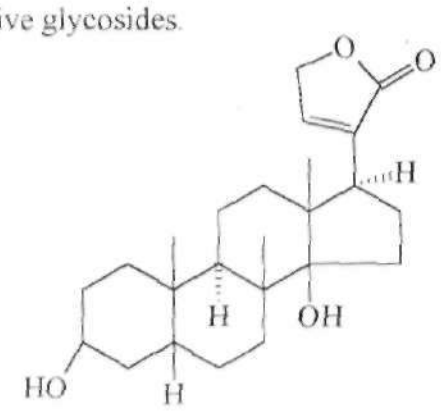


Bufanolide (**XVII**)

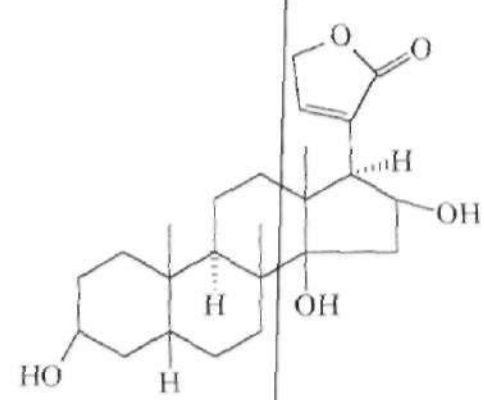
1.1.2.1 Cardio-active Glycosides

These are the cardenolide group and they are the most important medicinally. All the medicinal preparations of the drugs are derived from natural sources. The cardiac glycosides act directly on the heart muscles. They are used for the treatment of failing heart and show beneficial effect in some cardiac arrhythmias. They exert on failing heart a slowing and strengthening effect. The heart-arresting properties of these glycosides render them most effective as arrow poisons (to secure muscular relaxation in surgical operations and in certain neurological conditions).

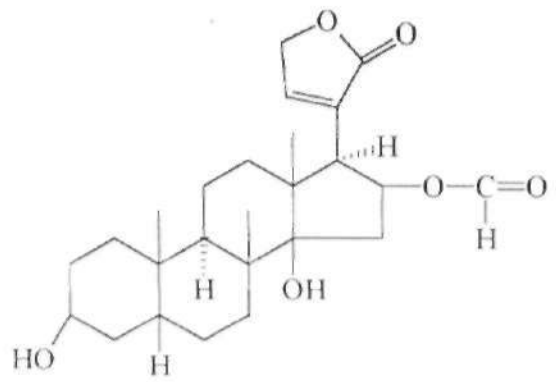
In Western medicine the glycosides of various Digitalis species are extensively employed. The following formulae indicate the major aglycones of digitalis purpurea cardio-active glycosides.



Digitoxigenin (**XVIII**)

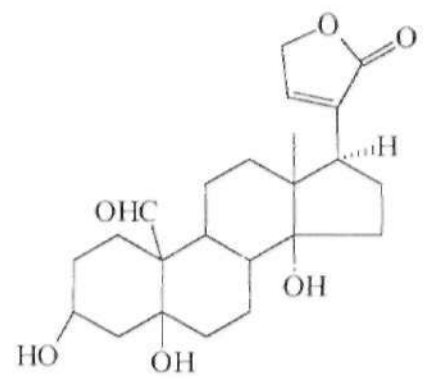


Gitoxigenin (**XIX**)



Gitaloxigenin (**XX**)

More than twelve genera of the family Apocynaceae are known to contain cardio-active glycosides. Seeds of East African Strophanthus Kombe consists of a mixture of glycosides or cardiac aglycones the major ones are K-Strophanthoside, K-strophanthin-β and cymarin all based on the genin strophanthidin.

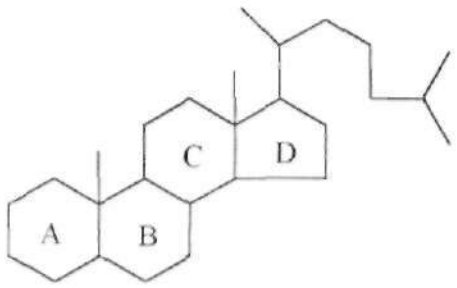


Strophanthidin (**XXI**)

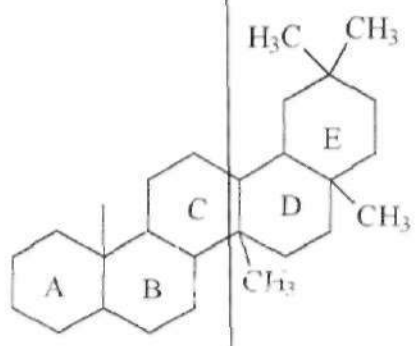
Bufanolides have found little use as drugs (Trease and Evans 1992).

1.1.2.2 Saponins and Steroidal Sapogenins

Saponins are glycosides, which produce stable foams when their aqueous solutions are shaken. They have haemolytic properties when injected into the blood stream and are highly toxic. When taken by mouth saponins are comparatively harmless e.g. Sarsaparilla is rich in saponins but is widely used in the preparation of non-alcoholic beverages (Trease and Evans 1992). As glycosides they are hydrolysed by acids to give an aglycone (sapogenin) and various sugars. According to the structure of the aglycone two kinds of saponins are recognized : (i) Steroidal (tetracyclic triterpenoids) and (ii) pentacyclic triterpenoid.



Steroid skeleton (**XXII**)

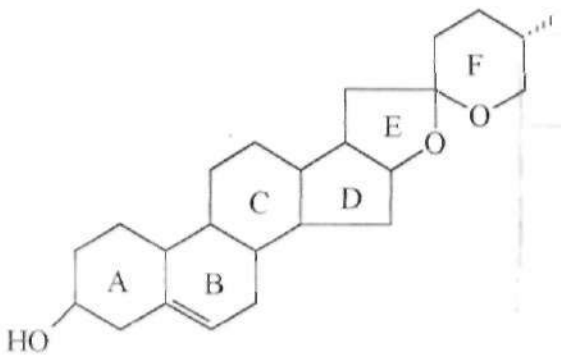


Pentacyclic triterpenoid (**XXIII**)

Steroidal saponins are of great importance and interest due to their relationship with compounds such as the sex hormones, cortisone, diuretic steroids, vitamin D and cardiac glycosides. Some are used as starting material for the synthesis of these compounds. Steroidal saponins are less widely distributed in nature.

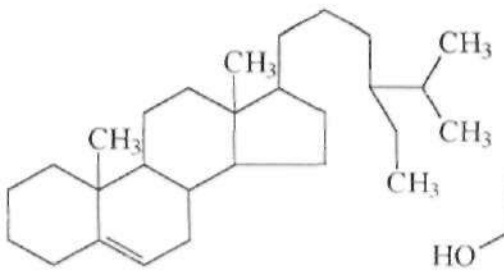
Phytochemical surveys have shown their presence in families like Dioscoreaceae (Dioscorea species), Amaryllidaceae (Agava species), Leguminosae (Fenugreek), Solanaceae (Solanum) etc. Diosgenin is the principal sapogenin which is isolated from tuber of many Dioscorea species (yams). Until 1970 it is the sole source for steroidal contraceptive manufacture.

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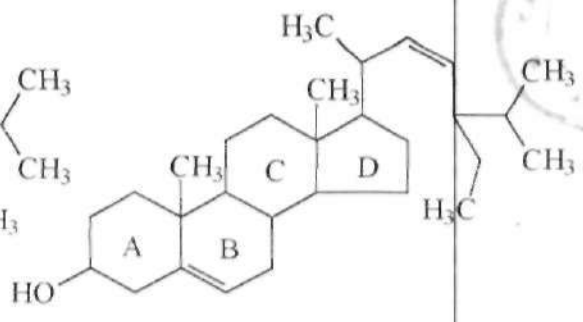


Diosgenin (**XXIV**)
(Δ^5 25 -Spirosten-3β-ol)

Memordica charantia L. (Curcurbitaceae) African cucumber whose fruits are used locally as purgative agents and Vermifuges; have been reported to contain steroidal glycosides in the fruits which consist of a mixture of (β-D-glucose of β-sistosterol and of $\Delta^{5,22}$ - stigmastadiene-3-β-ol (Sofowora, 1993).



β-Sistosterol (**XXV**)

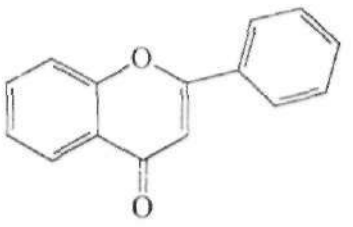


$\Delta^{5,22}$ -Stigmastadiene-3-ol (**XXVI**)

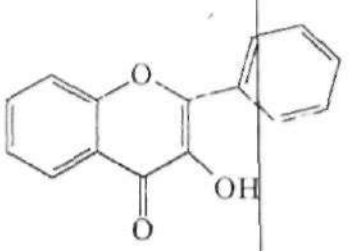
Fatope *et al*, (1990) reported a new cucurbitane triterpenoid from M. charantia L. Triterpenoid saponins are abundant in many dicotyledonous families.

1.1.2.3 Flavone and Related Flavonoid Glycosides

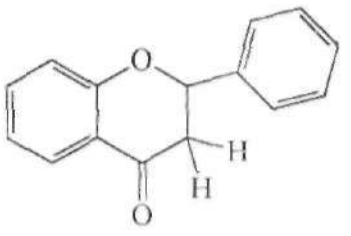
This group of compounds derived its name from the Latin word "*Flavus*" which means yellow. It occurs both in free state and combined as glycosides. It is widely distributed in plant kingdom. They form the largest group of naturally occuring phenols common in higher plants and in young tissues where they occur in cell sap. They are abundant in families Leguminosae, Rutaceae, Polygonaceae and compositae. Most flavones are O-glycosides. Isoflavones are particularly abundant in family Leguminosae (e.g. in dyers broom, Genista tinctoria).



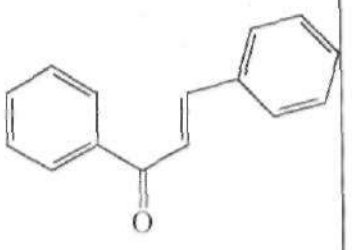
Flavone (**XXVII**)
(2-phenyl-8-chromone)



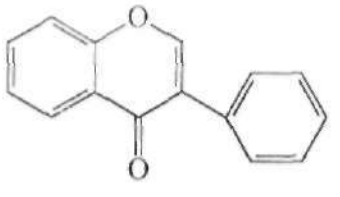
Flavonol (**XXVIII**)
(3-hydroxyflavone)



Flavanone (**XXIX**)



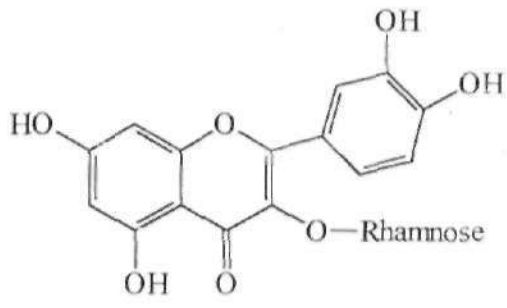
Chalcone (**XXX**)



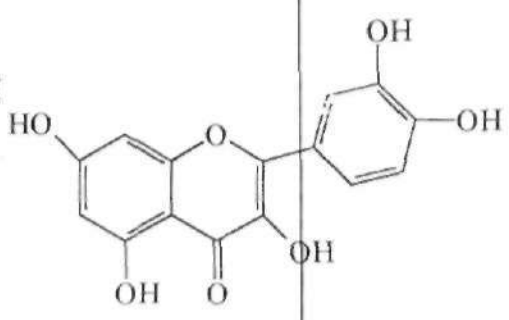
Isoflavone (**XXXI**)
(3-phenyl-8-chromone)

The therapeutic usefulness of flavonoids is yet to be fully realized. This group is known for its anti-inflammatory, anti-allergic effects, anti-thrombitic, and vasoprotective properties, inhibition of tumuor promotion and protective for gastric mucosa. These effects have been attributed to the influence of flavonoids on arachiolonic acid metabolism (Trease and Evans 1992). Some flavonoids have anti-bacterial and anti-fungal properties e.g fustic from the wood of Morus tinctoria.

Citrus and other fruits in addition to ascorbic acid and other compounds provide flavonoids which decrease capillary fragility and therefore used in cases of hypertension and radiation injuries. They contain a mixture of rhamnoglucosides of a tetrahydroxyflavone and hesperetin. A similar glucoside, rutin, the rhamnoglucoside of quercetin is found in many plants. Commercial quantities are found in tobacco residues.

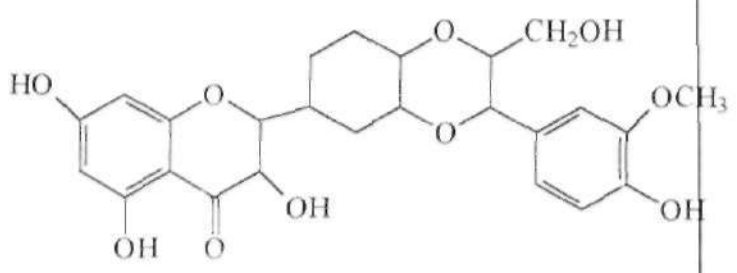


Quercetin-3-rhamnoside (**XXXII**)



Quercetin (**XXXIII**)

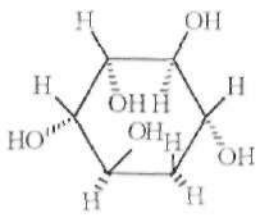
A number of flavonolignans e.g silybin have anti-hepatoxic properties. Extracts of plants of Silybum marianum are widely used in Germany for treatment of liver ailments.



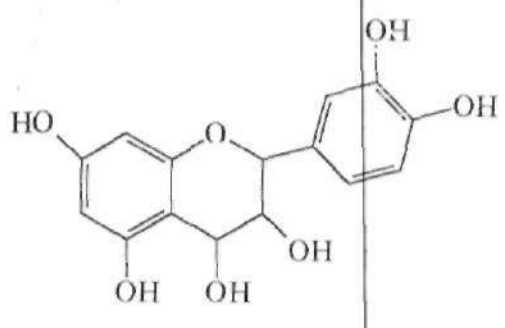
Silybin (**XXXIV**)

Euphorbia hirta L. (commonly known as asthma herb) is used for the treatment of asthma. In East and West Africa extracts of the decoction of the plant collected in the flowering and fruiting stages are used in asthma and respiratory tract inflammations. In Malagasy and Australia it is prescribed for its pectorial anti-spasmodic principles and is used for coughs, chronic bronchitis and other pulmonary disorders.

A preliminary screening of the stem and leaf of the plant has shown that it is rich in flavonoids. Power and Browning (1913) isolated quercetin. This flavonoid is assumed to be present as 3-rhamnoside. Other compounds isolated include Leucocyanidol, and quercitol.

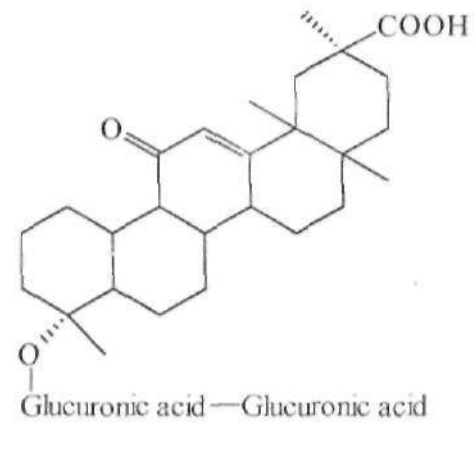


Quercitol (**XXXV**)

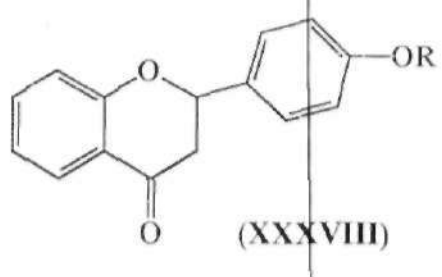


Leucocynidinol (**XXXVI**)

Liquorice root (Licorice root – Glycyrrhiza) family Leguminosae consists of roots and stolons of Glycyrrhiza glabra L. of different varieties. The yellow colour of this root is due to the presence of flavonoids which has received considerable study since the anti-gastric effect of flavonoid rich fraction was recognized. These flavonoids include liqueritin, isoliqueritin (a chalcone) which occurs as a glycoside and during drying it is converted into liquiritigenin and isoliquerifigenin (chalcone form) (Trease and Evans 1992).



Glycyrrhizin (**XXXVII**)
- mixture of Ca^{2+} and Na^{+} salts of glycyrrhizinic acid



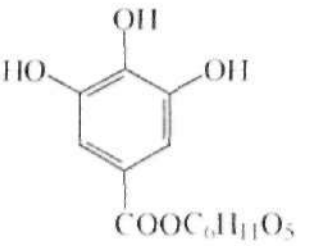
(XXXVIII)

Liquitrin R=glycosyl
Liquiritigenin R=H

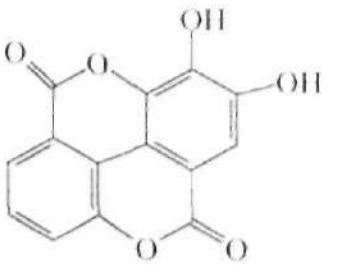
Liquorice roots have long been employed as flavouring agents, demulcent, mild expectorant, treatment of rheumatoid arthritis various inflammatory conditions. The flavonoid component possesses anti-microbial properties as well as anti-ulcerogenic activity, it also gives symptomatic relief from peptic ulcer pain (Trease and Evans 1992).

1.1.2.4 Tannins

An old definition of tannins denotes them as compounds present in plant extracts which are able to combine with protein of animal hides, prevent their putrefaction and convert them to leather. This definition excludes simpler phenolic substances often present with tannins such as gallic acid, Catechins and Chlorogenic acid. Many tannins are glycosides e.g. glucogallin.



Glucogallin (XXXIX)
(glycoside of gallic acid)



Ellagic acid (**XL**)

Two main groups of tannins are recognized

(i) Hydrolysable tannins, hydrolysed by acids or enzymes. Their solutions turn blue with iron salts. Hydrolysable tannins consists of two main types:- gallitannins and ellagitannins.

(ii) Condensed tannins (proanthrocyanidins) includes all true tannins, they are more resistant to breakage. They are related to flavonoid pigments (gives characteristic red colour to many drugs such as cinchona bark, tealeaves, etc). Their solution is turned green with iron (III) chloride.

Galls contain 50 – 70% of tannin known as tannic acid, also present are ellagic and gallic acids. Japanese and Chinese galls are of commercial importance and are produced by insects – aphis and are valued as astringents and styptics. An injectible form of this drug is used for treatment of haemorrhoids (Trease and Evans 1992)

1.2.0 Justification of Research

In many instances where PAs and toxicity of Crotalaria species have been identified the seeds have been the organ that are usually investigated. Reports have shown that leaves of C. retusa are eaten as vegetable in India and East Africa.

Inspite of the lush growth of C. retusa which should attract grazing animals, observation and personal communications have revealed that animals do not browse on C. retunsa. In Nigeria, C. retusa is abundant in grazing fields and this may be a source of contamination in hay making. No reports have been found on the Brine shrimp lethality bioassay of C. retusa.

1.3.0 Aims/Objectives

The main objective of this work is to isolate the relevant chemical constituents of C. retusa based on their cytotoxic effects, anti-microbial activity, and characterize these using spectroscopic methods. Accordingly efforts are made to supply information in these areas.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General Botanical Characteristics of Crotalaria Linn. Species (Rattle box)

The genus Crotalaria (Family: Leguminosae) is known by general name 'Rattle box', and different vernacular names in Nigeria: Hausa – '*birana*', Yoruba – '*koropo*' or '*pekepeke*' and Ibo – '*akidimuo*'. Crotalaria species is the largest genus in tropical Africa. It comprises of about 600 species and over 400 are found in Africa (Baker, 1914, Milne-Redhead 1961, and Polhill, 1971. Out of 63 species found in West Africa (Polhill, 1982), 37 species are found in Nigeria (Hutchinson *et. al*, 1958).

Crotalaria Linn are herbaceous plants common in grassland and widespread in the tropics. Leaves are simple, l-foliolate or digitately 3(-7)-foliolate, usually petiolate. Stipules are filiform or may be lacking. Flowers are usually yellow variously marked, less often white or blue, usually terminal, leaf-opposed. Bract and paired bracteoles are usually present. Calyx usually with 5-subequal lobes, sometimes the lower 3 on a short lip, sometimes 2-lipped with long lobes. Standard almost always with 2 appendages at the base inside, hairy outside, beaked keel. Stamens all joined in a sheath at least at the base, anthers alternately long, basifixed and smaller dorsifixed.

Style is curved with leaves on the upper stigma usually small, rarely bilobed. Inflated legume which are subsessile to long – stipulate, almost always dehiscent and l-many seeded. Seeds mostly oblique-cordiform to oblong – reniform with definite hilar sinus and sometimes conspicous aril (Polhill, 1982). Some species common in Nigeria and other African countries include :- Crotalaria arenaria benth, C. burkeana benth,

C.barkae Schweinf; C. domiana Bak; C. atrorubens Hochst; C. glauca willd; C. goreensis Robyns; C. lachnosema Stapf; C. microcarpa Hochst; C. juricea; C. naragutensis Hutch; C. retusa Linn.

2.1.1. Crotalaria retusa Linn

An erect annual or short-lived perenial 0.5 – 1.5m tall; usually well branched. Leaves are simple, blade oblanceolate to oblong – obovate usually rounded at the apex. Racemes 10 – 30cm long, many flowered. Calyx glabrous to appressed puberulous, longer than the tube. Standard obovate – circular, pale yellow, purplish-veined inside and reddish purple diffused outside. Wings oblong – obovate, longer than the keel, bright yellow; keel rounded with a slightly incurved twisted beak. Legume shortly stipulate, oblong-clavate ±12 – 20 seeded. Seed finely papillose yellowish brown (Hepper, 1958).

C. retusa is well distributed as a fibre plant and as a green manure, readily naturalised. Most common around the coasts of tropical Africa and in areas with reasonably equable rainfall (Polhill, 1982)

2.2.0 Chemical Constituents of Crotalaria Species

Various species of Crotalaria have been investigated for presence of bioactive principles. Those bioactive agents reported includes amino acids, pyrrolizidine alkaloids and phenolic glycosides such as steroids, triterpenoids, hydroxyanthracene, flavonoids and saponins.

2.2.1 Amino Acids

When accumulated may have varied roles as nitrogen reserves, alkaloid precursors, and toxins. Pilbeam and Bell (1979) have studied and identified free amino acids in seeds of 163 crotalaria species. The distribution of the amino acids in the genus was found to be in line with the classification proposed by Polhill (1971) and Bisbay and Polhill (1973).

Bell (1968) noted the presence of the neurotoxin-α-amino-β-oxalyamino propionic acid (ODAP) in seeds of C. incana L. and C. pallida Ait. The presence of this amino acid was later reported in all other Crotalaria species (Quereshi *et. al.*, 1977). δ–Hydroxynorleucine has been isolated from seeds of C. juncea L. (Pant *et. al.*, 1974) and identified in seeds of C. tetragonia Roxb; and nine other crotalaria species (Pilbeam *et. al.*, 1979). The amino acid isowillardine has been isolated from seeds of C. ochroleuca G. don (Pilbeam *et. al.*, 1979) and found to be toxic to Callosobruchus maculatus larvae (Janzen *et. al.*, 1977). ODAP has been found to be toxic to chicks, young rats, guinea pigs, dogs, monkeys and yeast (Mehta *et. al.*, 1972, Pilbeam *et. al.*, 1979) -α-amino-℘-oxlylaminobutyric and (ODAB) has been shown to be toxic to chicks (Rao *et. al.*, 1979) while α-℘-diaminobutyric acid (DAB) was reported by Ressler (1961) to be toxic to rats and larvae of the Southern Cowpea weevil – Callosobruchus maculatus. (Janzen *et. al.*, 1977). δ-hydroxynorleucine is toxic to fungi Alternaria alternata and Phomalingcan. Yadava *et. al.*, (1994) reported that C. laburnifolia is a rich source of essential amino acids: - alanine, aspartic, arginine, leucine, glutanic, tyrosine, histidine, norvaline and β-phenylalanine.

The difference in amino acid patterns varies between species as shown in the Table 1

Table 1: Amino Acid Patterns in Different Crotalaria Species (Pilbeam and Bell 1979)

Species	*ODAP*	*ODAB*	*ADAP*	*DAB*	*Glutyr*	*Isowill*
C.barkae Schweinf	+	+	-	-	-	-
C.brevidens Benth	-	-	-	-	+	+
C.bukeana Benth	+	+	-	+	-	-
C.caudata	-	-	-	+	-	-
C.doniana Baker	+	+	-	-	-	-
C.glauca Willd	-	-	-	+	-	-
C.gorensis	-	+	-	+	-	-
C.laburnifolia L	+	-	+	-	+	-
C.lanceolata	-	-	-	-	+	+
C.lachnosema Staph	-	-	-	+	+	+
C.lachnophora A.Rich	-	-	-	-	+	+
C.naragutensis	-	-	-	-	+	+
C.ochroleuca	-	-	-	+	+	+
C.pallida	+	-	-	-	+	+

ODAP - α-amino-β-oxalylaminopropionic acid

ODAB - α-amino-ω-oxalyldiaminobutyric acid

ADAP - acetyldiamino propanoic acid

DAB - α-ω-diaminobutyric acid

Glutyr - ω-glutaanyltyrosine

Isowill - *isowillardine*

The presence of one or more of ODAP, ODAB, ADAP, and DAB in seeds and seed-bearing plant of these species are potentially toxic to livestock and poultry and hence the reported toxicity of C. barkae and C. pallida which contain them.

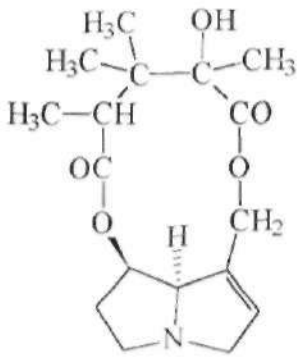
The amino acid pattern of C. juncea is different from that of any other crotalaria species due to high concentrations of δ-hydroxynorleucine (Pilbeam *et. al.*, 1979). This reflect the fact that C. juncea has been, the most extensively cultivated crotalaria species.

2.2.2 Alkaloids

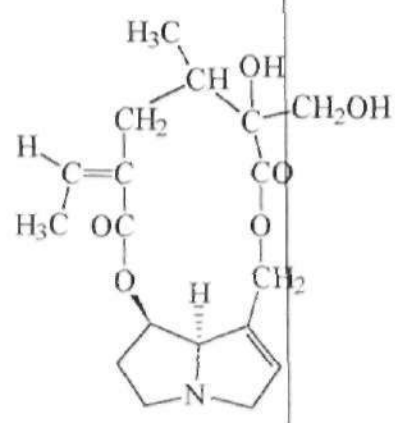
Investigations over the last 40 years have confirmed that the families Senecio and Boraginaceae and genus Crotalaria are the main sources of hepatotoxic Pyrrolizidine alkaloids (PAs) (Pilbeam *et. al.*, (1979); Smith and Culvenor, 1981; Polhill, 1982) noted the alkaloid levels in 85 African species. The presence of PAs in crotalaria species provides a notable chemical marker for the genus as they have not been detected in any generic subdivision of other legumes. The alkaloid monocrotaline tends to be characteristic of the generic subsection Crotalaria which include C. retusa, and C. spectabilis while 1-methylene pyrrolizidine and 7β-hydroxy-1-methylene-8β-pyrrolizidine have been found to be characteristic of subsection stipulosae which includes C. damarensis, C. goreensis, C. lachnophora (Polhill 1982).

PAs are esters of aminoalcohols known as necines and carboxylic acids called necic acids. PAs derived from saturated aminoalcohols are non-hepatotoxic while those derived from unsaturated amino alcohols are known to be hepatotoxic. A common feature present in all the hepatotoxic PAs is the unsaturation in the basic moiety that is a 3-pyrroline ring. Usually the most hepatotoxic are the cyclic diesters (Mattocks, 1968).

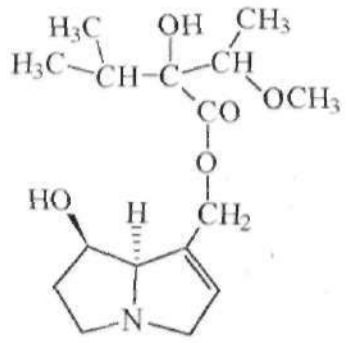
The PAs usually exhibit their toxicity due to their conversion *in vivo* during metabolic activities to pyrrole derivatives (secondary metabolites). PAs may be present in large proportion as N-oxides which are neither basic nor bitter and are not rejected in feeds by animals. Culvenor and Smith (1957) in addition to monocrotaline isolated, three new alkaloids - retusine, retusamine and retronecine N-oxide and an incompletely characterized base with m.p. 130-132°C from C. retusa. Roeder *et al.*, (1993) isolated six PAs from C. aegyptiaca using spectroscopic methods. Two of them were new PAs – Croaegyptine and Crotalarine lactone.



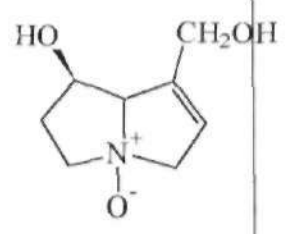
Monocrotaline (**LII**)



Retrorsine (**LIII**)

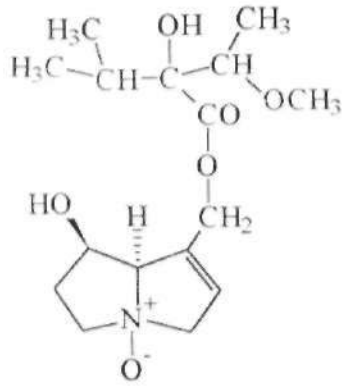


Heliotrine (**LIV**)

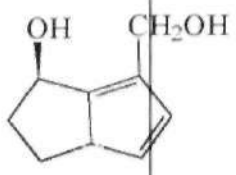


Helitridine N-oxide (**LV**)

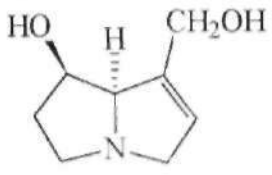
Figure 1-Structures of some hepatotoxic pyrrolizidine alkaloids



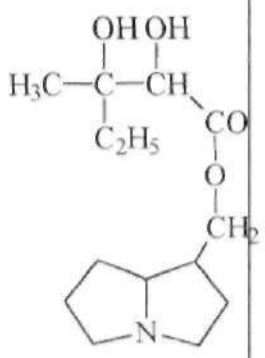
Heliotrine N-oxide (**LVI**)



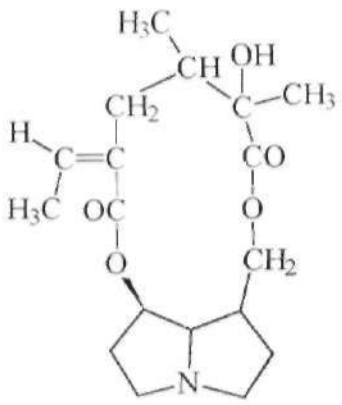
Dihydrohelitridine (**LVII**)



Retronecine (**LVIII**)



Strigosine (**LIX**)



Rosmarinine (**LX**)

Figure 2 – Structures of some non-hepatotoxic pyrrolizidine alkaloids

Table 2: Some Crotalaria Species Containing Hepatotoxic PAs

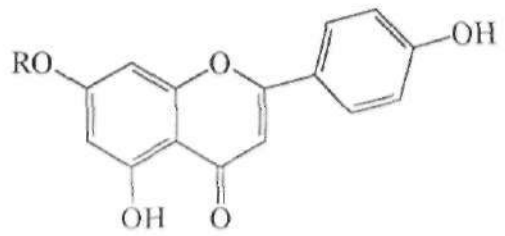
Plant	Constituent alkaloid	Part Investigated	Reference
C.brevidens Benth	Integerrine	Seed	Smith and Culvenor 1981
C. dura Wood and Evans	Dicrotaline	Aerial	Smith and Culvenor 1981
C. grahamiana Wight and Arn.	Monocrotaline, grahamine,	Aeriel and Seed	Smith and Culvenor 1981
C. intermedia kotschy	Usaramine	Seed	Smith and Culvenor 1981
C. juncea L.	Senecionine, seneciphylline, riddelline, trichodes-mine, junceine	Seed	Watt and Breyer-Brandwijk 1962
C. lachnosema	Dicrotaline, acetyl dicrotaline	Aeriel	Mattocks and Nwude 1988
C. mucronata	Usaramine, nilgrine	Seed	Smith and Culvenor 1981
C. nana Burn	Crotananine, cronaburnine	Seed	Smith and Culvenor 1981
C. naragutensis	Nilgrine, intergerri-mine, acetyl intege-rrimine, usaramine	Seed	Mattocks and Nwude 1988
C. retusa L	Monocrotaline, retusine, retusamine, retronecine	Seed	Culvenor *et. al.*, 1957

Table 3 : Crotalaria species containing non-hepatotoxic PAs

Plant	Constituent Alkaloid	Part investigated	Reference
C.aridicola Domin	1-Methoxymethyl-1,2-dehydropyrrolizidine, 7β-hydroxy-1-methoxymethyl-1,2- dehydropyrrolizidine, 7β-acetoxy-1-methoxymethyl-1,2- dehydropyrrolizidine	Aeriel and Whole	Smith and Culvenor 1981
C.damarensis Engl.	1-Methylenepyrrolizidine	Whole	Smith and Culvenor 1981
C.goreensis Gull et Perr.	7β-hydroxy-1-methylene-8β-pyrrolizidine, 7β-hydroxy-1-methylene-8α-pyrrolizidine,	Aeriel and Seed	Smith and Culvenor 1981

2.2.3 Phenolic Glycosides

C. laburnifola on phytochemical investigation yielded β-sitosterol as well as flavonoid glycosides –orientin, iso-orientin and quercetin-O-galactose (Subramanian *et. al.*, 1968). On screening C. retusa and C. striata Subramanian *et. al.*, (1969) reported the presence of β-sitosterol, free quercitin and flavonol-3-O-glycoside; no flavonoid C-glycoside was identified from C. retunsa while C. striata yielded luteolin, vitexin, and its O-xyloside. Bulhoes *et. al.*, (1977) screened for presence or absence of alkaloids, steroids, flavonoids and saponins in aerial parts of C. retusa. Suri *et. al.*, (1978) reported the presence of triterpenoid in Crotalaria species. C. anagyroids yielded a substantial amount of two compounds, which were identified as β-amyrin and β-sitosterol. A novel flavonone glycoside – Aromadendrin-7-0-α-L- rhamnopyranosyl (1→ 4)–O–β– D galactopyranoside and another new flavone glycoside were isolated from seed of C. laburnifolia (Yadava, 1994).



(LXI)

R= 7-o-B-D-glucopyranosyl
-(1→4)-o-B-D-xylopyranoside

Khalillulah *et.al.*, (1993) reported the presence of a new prenylated chalcone with uncommon type of hydroxylation in β-ring (2, 4, 5–positions) in C. ramossisima. Yadava *et.al.*, (1995) isolated a novel flavonol glycoside –3'–4'–Di-o-methylquercetin–3–O–α–1–rhamnopyranosyl–(1→2)–O–β–D–glucopyranoside from the stem of C. verrucosa. Yadava *et. al.*, (1995) isolated a new cardenolide–5,6–dehydrosarmentogenin–3–O–β–D–glucopyranosyl–(1→6)–O–β–D–galactopyranoside from the seeds of C. retusa.

2.3.0 Toxicity of Crotalaria Species

The genus Crotalaria has been incriminated as a main toxic species involved in a number of diseases of both human and livestock. The toxicity of Crotalaria species has been attributed to the presence of hepatotoxic PAs (Bull. *et. al.*, 1968, and Molyneux, *et. al.*, 1979).

2.3.1 Toxicity in Humans

The effect of ingestion of PAs from contaminated grains, herbal teas, or medicines are cumulative and may become serious months or even years after ingestion. The insidious nature of their effects prevents the population at risk from recognizing the toxicity of the plants.

Crotalaria fulva Roxb may be a component of bush teas which cause veno-occlusive disease of children in Jamaica (Schoental, 1963). Also bread poisoning in South Africa (Selzer *et. al.*, 1951) and toxic hepatitis with ascites in central Asian Republics, of USSR have been attributed to poisonous PAs which are present in most Crotalaria species. Major outbreaks were reported in 1931 and 1945 and was found to be due to the contamination of wheat, barley and millet with seed of Heliotropium lasiocarpum (Milenkov and Kizhailan 1952). A common disease of children in Jamaica described by Hartwell *et. al.*, (1951) as veno-occlusive disease was later reported as due to consumption of bush teas made from Crotalaria species (McLean, 1970). The effect in all cases was acute to subacute and mortality was high. Occurrences of Crotalaria species poisoning is summarised below:

Table 4 Occurrences of Crotalaria Species poisoning

Plant	Country	Reference
Crotalaria species	Central India	Tandon *et.al.*, 1976 Krishnamachari *et.al.*, 1977
C. fulva	Jamaica	McLean 1970
C. juncea	Ecuador	Lyford, *et al.*, 1976

So far there has been no recognized report of tumours in humans due to intake of PAs. In human cases the clinical reports of the disease speak of recovery after supportive treatment (Culvenor, 1980).

2.3.2 Toxicity in Livestock

Twenty African species have been implicated in livestock poisoning (Watt and Breyer – Brandwyk, 1962, Polhill, 1982). Forty species in Zimbabwe were regarded toxic depending on the state of maturity and on soil and climate condition. None of the

species in Zimbabwe is eaten in the green state by livestock (Watt and Breyer – Brandwijk, 1962). The crotalaria species put on new growth and flower before the rain starts and attract livestock at this stage. Also the plant may contaminate silage, food pellets and chaff feed as diet to the animals.

The effect upon animals upon consumption of toxic Crotalaria species is generally chronic characterized by Cirrhosis - like condition of the liver (Watt and Breyer – Brandwijk, 1962). Instances like lamitic crotalism in cattle, styfsiekte in South Africa was attributed to C. barbae, C. burkeana, C. incana, C. lachnocarpoides and C. polyspermae. While C. spectabilis, C. retusa and C. fulva were implicated in a more lethal form of Crotalism which affects the nervous system, lungs and liver (Kingsburry, 1964 and Polhill, 1982). This toxic hazard occurs at a lesser extent in sheep than horse, cattle, poultry and pig. Natural outbreaks of Crotalaria species poisoning are summarized below:

Table 5 Crotalaria Species Known or Suspected of Causing Poisoning in Livestock

Livestock	Species Implicated	References
Horse	C. aridicola Dominin, C. dura Wood and Evans, C. globifera, C. juncea, C. sagittalis Roth, C. spectabilis, C. crispata C. retusa	Culvenor *et.al.*, 1962, Watt and Breyer-Brandwijk, 1962 Peterson and Culvenor, 1983 Rose *et.al.*, 1957
Cattle	C. barkae Schweinf C. burkeana Benth C. pallida Ait	Watt and Breyer-Brandwijk, 1962 Miller, 1967 Kingsburry, 1964
Sheep	C. burkeana Benth, C. dura Woods and Evans, C. juncea C. pallida, C. rhodesiae, C. mucronata	Watt and Bryer-Brandwijk, 1962 Laws, 1968
Pig	C. retusa, C. spectablis Roth	Hooper and Scalan, 1977 Emmel *et.al.*, 1935
Poultry	C. goreensis, C. mucronata C. retusa, C. spectabilis	Norton *et.al.*, 1979; Bierer *el. al*; 1960; Hooper and Scalan 1977; Thomas, 1934

2.3.3 Toxicity in Experimental Animals

The PAs are known to produce toxicity and death in laboratory animals at very low dose rates e.g. in pigs with 8ppm monocrotaline in diet (Hooper and Scalan, 1977). For some alkaloids dosing at lower dose rates induced malignant tumours in the liver (Culvenor, 1980). Also continuos dosing of lasiocarpin at 7ppm in the diet in rats was effective, retrorsine produced tumours after a single high but lethal dose. Bulhoes, *et.al.*, (1977) reported that C. retusa contain antibiotics and Kalaichelvan, *et.al.*, (1992) reported that monocrotaline alkaloid isolated from seeds of C. paleda in a spore germination assay proved to be fungistatic. McLeans, (1970) reviewed the toxicity of PAs as summarised in the Table below:

Table 6: LD_{50} In Male Rats at Three Days after Dose for Some Toxic PAs

Alkaloid	LD_{50} mg/kg Body weight	Empirical Formula	Molecular Weight	Reference
Fulvine	40	$C_{16}H_{23}O_5N$	309	Scoental, 1968
Heliotrine	300	$C_{16}H_{27}O_5N$	313	Bull, *et.al.*, 1968
Lasiocarpine	72	$C_{21}H_{33}O_7N$	411	Bull, *et.al.*, 1968
Monocrotaline	175	$C_{16}H_{23}O_6N$	325	Bull, *et.al.*, 1968
Retrorsine	35	$C_{18}H_{25}O_6N$	351	Schoental, 1968
Senecionine	85	$C_{18}H_{25}O_5N$	335	Bull, *et.al.*, 1968
Seneciphylline	77	$C_{18}H_{23}O_5N$	333	Bull, *et.al.*, 1968

2.4.0 Importance of Crotalaria Species

2.4.1 As Food

Some Crotalaria species are deliberately eaten as food. In Northern Nigeria leaves of C. aschreck Forssk are eaten, made by pounding with honey or sweet stuff "alewa" and the leaves are also useful fodder for goats. C. astrubens, leaves and flowers are eaten by goat (Dalziel, 1955). In East Indies the leaves of C. juncea L. are used as vegetable, the seed as food and in India the seed has been used as stock feed. The roasted seed of C. mucronata Desv., has been used in Netherlands and East Indies as a substitute for coffee. C. retusa is used in Tanzania as vegetable (Watt and Breyer-Brandwijk, 1962).

2.4.2 Medicinal Uses

Crotalaria species as bush teas are taken mainly as a remedy for colds and other illness. The Zulus take an infusion of the root of crotalaria plants for relief of chronic cough (Watt and Breyer – Brandwijk, 1962; McLeans, 1970). Crotalaria species have found many uses in folk medicine:

1. **C. arenaria Benth: (Hausa; 'manta uwa')**

   The plant has been used as an occasional ingredient in Nigeria to wean a child or make a captured slave forget his kinsmen (doubtless for superstitious reasons). In Katagun the plant is a component of fish poison. (Dalziel, 1955).

2. **C. aschrek Forssk (C. obovata G. Don):** (Hausa farar birarria)

   The plant is used as an eye medicine either as a lotion or sometimes rubbed in the palms which are passed over the eyes. It is also mixed with similar Crotalaria species and used as a remedy for syphilis. The prescription is known as "rigya

Kafi" and is used as a love portion to make a voluntary return of a runaway wife if she drinks it without knowing (Dalziel, 1955).

3. **C. astrorubens Hochst (Hausa "biranar awaki")**

The Hausas apply it locally for treatment of scorpion bite and it is also used for treatment of lumbago if pounded with native natron (Kanwa) and rubbed on the back (Dalziel, 1955).

4. **C. axillaris Ait.**

Is used by the Dare people for amenorrhea (Watt and Breyer-Brandwijk, 1962)

5. **C. burkeana Benth**

In Zimbabwe it is used as remedies for malaria, black water fever, blood poisoning, anthrax and dysentery (Watt and Breyer-Brandwijk, 1962).

6. **C. distans Benth**

The Sotho people in South Africa mix it with Erythrina Zeyheri as a medicinal charm.

7. **C. glauca Willd-Holl**

In Sierra Leone the Sherbro tribe squeeze it on a dogs nose to aid its sense of smell in hunting (Dalziel, 1955).

8. **C. intermedia Kotschy**

The leaves are used as an application to sore feet (Dalziel, 1955; Watt and Breyer-Brandwijk, 1962).

9. **C. juncea or Sunnhemp and C. retusa**

Roots are crushed and used for haemoptysis remedy – in East Indies and India respectively.

10. **C. microcarpa Hochst (Hausa, "birana zomo", Yoruba, "eni-horo")**

In Southern Nigeria it is said to be used to kill lice. The plant has apparently some magical association if it is unwisely mixed with other medicines and given to an infant, the child's hair will turn grey before puberty (Dalziel, 1955).

11. **C. natalita**

The Lobedu people take a cold-water infusion of the pounded root for heart conditions (Watt and Breyer-Brandwijk, 1962).

12. **C. retusa Linn-Holl (Wedge-leaved Crotalaria or Devil beans)**

In West Africa the root is crushed and mixed with spices as a colic remedy and a decoction of the leaves or sometimes the free juice of the plant is taken for the relief of fever. In India a local application has been used in skin disease (Watt and Breyer-Brandwiji, 1962).

Allowing for toxicity of PAs the crotalaria species could be used with discretion as herbal teas as shown in Table 7.

Table 7: Crotalaria species used as Herbal Teas and Medicines which are known to Contain Hepatotoxic PAs

Species	Use or Disease Treated	Country	Reference
C. fulva	Colds	West Indies	McLean, 1970
C. incana	Purgative	East Africa	Watt and Breyer–Brandwijk, 1962
C. juncea	Vegetable, haemoptysis	East Indies	Watt and Breyer-Brandwijk 1962
C. laburnifolia	Medicinal	Tanzania	Watt and Breyer-Brandwijk 1962
C. mucronata	Coffee substitute	East Indies	Watt and Breyer-Brandwijk, 1962
C. recta	Childhood malaria	Tanzania	Watt and Breyer-Brandwijk 1962
C. retusa	Haemoptysis, fever, colic	West Africa	Watt and Breyer-Brandwijk 1962
C. verrucosa	Fever	West Africa	Dalziel, 1955

2.4.3 **Other Uses**

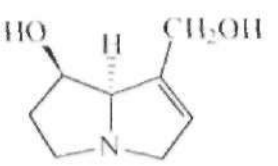
The genus Crotalaria provides a wide range of species suitable for different conditions and purposes, due to its vigorous early growth, good nodulation, reasonable pest resistance and set.

C. juncea (Sunnhemp) has been the most extensively cultivated of the crotalaria species, may be due to the high concentration of δ-hydroxynorleucine (Pilbeam, *et.al.*, 1979). The bark of C. juncea provides a high quality bast fibre for cordage and fine paper. In East Africa C. juncea, C. retusa and C. mucronata Desv. are cultivated for their high fibre and used for making cords, bags, sackings, canvas and nets. In India the fibre of C. juncea has been tried as a substitute for jute, in the making of cordage, C. retusa, C. pallida and C. mucronata have provided dyes (Watt and Breyer-Brandwijk, 1962; Polhill, 1982). C. juncea has recently been cultivated for green fertilization; and about fifteen other species including C. anagyroides, C. intermedia kotschy have been widely tried as green manure and cover crops. They are excellent soil-making crop for poor sandy land, adding much nitrogen to the soil.

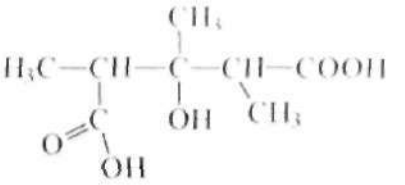
A few of the large-flowered shrubby species, C. agatiflora, C. capensis, C. laburnifolia and C. lunata have been grown for ornament in the high lands in South Africa.

2.5.0 Biosynthesis

PAs are esters of amino alcohols called necines and carboxylic acids called necic acids. Retronecine is the necine of the monocrotaline while its necic acid is the monocrotalic acid.

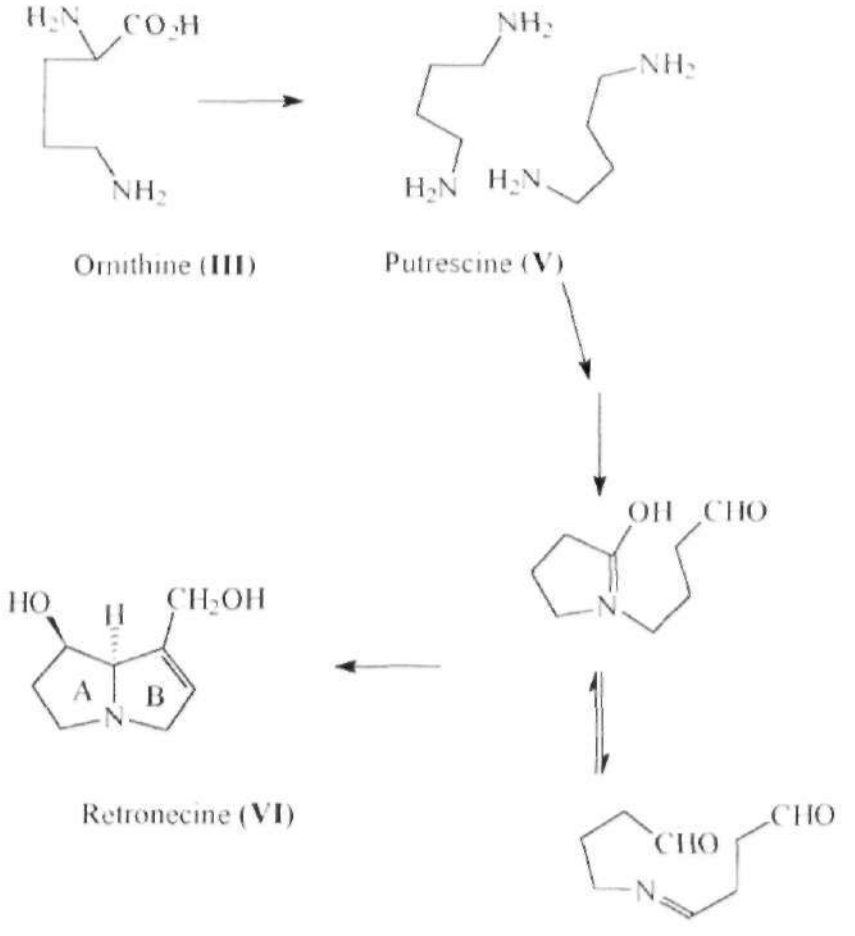


Retronecine (**I**)



Monocrotalic acid (**II**)

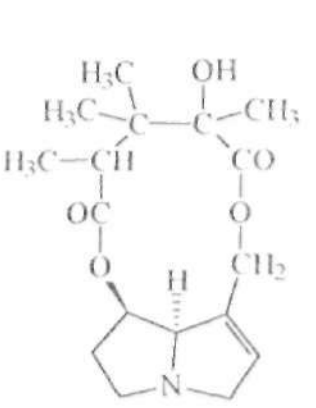
The origin of the pyrrolizidine ring system (**VI**), present in the alkaloids was reported by Robins, *et. al.*, (1979), that ornithine (**III**) is built into ring A via a symmetrical intermediate, putrescine (**V**) – Figure 3, which is a better intermediate for retronecine than Ornithine (**III**).



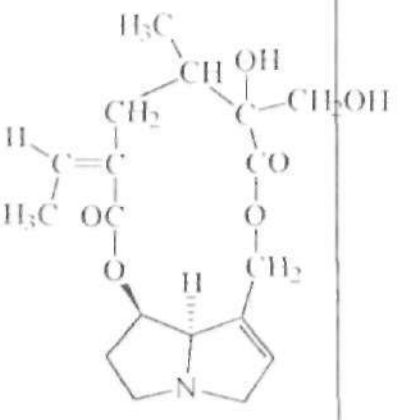
Ornithine (**III**) Putrescine (**V**)

Retronecine (**VI**)

Figure 3 : Sketch of a symmetrical intermediate Putrescine (**V**)



Monocrotaline (**VII**)



Retrorsine (**VIII**)

This and the symmetrization of the Ornithine label indicate that retronecine is formed from two molecules of Ornithine (**III**) via putrescine which is then converted into PAs via hypothetical intermediate.

Rao, *et. al.*, (1979) studied the incorporation of C-14 labelled precursors in monocrotaline. Their work revealed that Acetate-U-^{14}C, when feed to C. retusa aged between four and six months proved to be the necic acid part of monocrotaline (**VII**), while Ornithine was the best precursor of the retronecine moiety. The intake of the precursor in the stem as well as in the plant was maximium at the onset of fruiting. As the seeds mature their percentage of alkaloids and their radioactivity increased. Most probably seeds are the seat of storage of pyrrolizidine alkaloids.

2.6.0 Investigating Plants for Active Constituents.

The extraction of bio-active agents from plants is one of the most intensive areas of natural product research. When the active agent is isolated it is possible to demonstrate whether the reported physiological activity of the plant is real. The fact that

the activity has been shown to be due to a particular chemical compound makes detailed pharmacological and other academic studies possible. It is not always possible to isolate the bio-active agent in plants, cases are known where attempts at such isolation have proved fruitless even though the extract of the plant may be active. The isolation of bio-active agents from plants can be grouped into two broad groups viz.

1. Phytochemical screening: involves extraction, isolation and characterization of natural products from plants.

2. Biological screening: involves bio-activity tests of the plant extract in search of the physiological effects which it produces (Sofowora, 1993).

2.6.1.0 Extraction of Plant Material

Extraction may be performed by repeated maceration with agitation and percolation for fresh plant samples or by continuous extraction using soxhlet extractor for dried powdered plant materials. With the exception of fixed oils where light petroleum is used, alcohol is a general solvent for many plant constituents (Trease and Evans, 1992). The oil of the leaves (Martins and Olayiwole, 1990) and the seeds (Martins and Sotunde, 1990) of Crotalaria retusa L. was extracted using petroleum ether (40-60°C).

The extraction of organic bases, example alkaloids, usually necessitates basification of the plant material if a water-immiscible solvent such as trichloromethane is to be used. The pyrrolizidine alkaloid N-oxides are incompletely extracted from aqueous solutions using organic solvents example trichloromethane. However, the N-oxides in dilute hydrochloric acids are readily reduced by zinc-dust to give the corresponding basic alkaloids in quantitative yields (Koekemoer *et. al.*, 1951).

2.6.1.1 Phytochemical Screening

Plant constituents are so heterogenous chemically that most of them cannot be identified in plant extracts using a single criterion. A general phytochemical screening described by Sofowora (1993) involves use of standard chemical tests to detect the presence of various classes of natural products, examples are alkaloids, saponins, glycosides, tannins, etc.

Other specific qualitative tests may be carried out to detect the presence or absence of a suspected group of compounds. Mattocks, *et. al.*, (1987) described an improved field tests for toxic PAs on both fresh and dried plant materials.

2.6.1.2 Isolation of Constituents

The development of new and improved separation techniques such as thin-layer Chromatography is widely used to separate the constituents into its various components. It provides a rapid method of separation and purification of plant extracts for further investigation. With suitable apparatus Chalmers, *et. al.*, (1965) have found that PAs including macrocyclic diesters of melting point 200°C can be chromotagraphed readily and with reproducible retention times. A data was given for the characterization for fifty-eight alkaloids and basic derivatives using gas chromatography and supplement with thin-layer and paper chromatography to the point where for the first time identity of alkaloids in the pyrrolizidine group may be established by chromatographic comparison with reference compounds.

Mattocks, (1967) described a lengthy and inconvenient method for the detection of PAs on thin-layer chromatographs. This involves conversion of the alkaloids on the chromatographic plates to N-oxides using hydrogen peroxide followed by heating. Treatment with ethanolic anhydride then converts the N-oxides to pyrroles which with Ehrlich reagent give blue or mauve spots. The method is more sensitive and specific for alkaloids having an unsaturated pyrrolizidine ring.

A specific and rapid method for specific detection of PAs on thin-layer chromatograms was given by Molyneux, *et. al.*, (1980). It provides a rapid method of separation and monitoring of PAs in biological extracts.

2.6.1.3 Characterization of Isolated Constituents

The characterization of the active agent enables structure-related activity studies to be carried out, leading to the synthesis of a more potent drug with reduced toxicity. The active anti-malarial constituent of cinchona bark which was identified as alkaloid quinine was found to produce toxic side effects as impaired hearing on prolonged use. Attempts to modify the structure of quinine showed that not all parts of the molecule of quinine were necessary for anti-malarial activity. Synthetic anti-malarias were developed in which the activity of quinine was retained but toxicity reduced e.g. is the well-known chloroquine (Trease and Evans, 1992, Sofowora, 1993).

The use of spectroscopic techniques such as ultraviolet, infrared, nuclear magnetic resonance, mass spectroscopy and X-ray diffraction makes the characterization of organic molecules from the plant extracts a lot easier.

Constituents containing chromophoric groups as well as aromatic systems exhibit a characteristic absorption in the ultraviolet visible region. The spectra of such samples

when correlated with data from the literature for known compounds can be a valuable aid in determining the functional groups present in the sample.

Evidence for the structures of PAs of Crotalaria retusa was provided by Culvenor, *et. al.*, (1957) using the infrared absorption spectrum in supplement to other chemical analytical procedures.

Schoental, (1962) and Culvenor, *et. al.*, (1962) used infrared spectroscopy and nuclear magnetic resonance spectroscopy to elucidate the structure of alkaloids present in C. fulva Roxb. and Crotalaria crispata F. Muell respectively.

Culvenor, *et. al.*, (1965) tabulated the chemical shifts and coupling constants for the protons of the pyrrolizidine nucleus in forty PAs and derivatives.

Molyneux, *et. al.*, (1979) using nuclear magnetic resonance spectroscopy gave a quantitative evaluation of PAs and their corresponding N-oxides contents of samples of Senecio species. This technique provides a rapid method for the examination of plant samples in order to evaluate the hepatotoxicity hazard to animals and humans and for the regulation of PAs in animal feeding experiments.

2.6.2.0 Biological Screening

For long phytochemists characterized a lot of compounds and very few of such compounds were tested for any biological activity. This becomes a waste of time if the characterized drugs are found in future not to be useful to mankind. The new trend of using bio-assay guided screening and purification of plant extracts ensures that the isolated compound will have biological activity and therefore stands a good chance for

drug discovery through subsequent structure-related activity studies (Sofowora, 1993). Such bio-assay tests, include pharmacological screening, toxicity tests and anti-microbial screening.

2.6.2.1 Pharmacological Screening

A valid scheme for the study of medicinal plants and their products and one which emphasizes pharmaceutical use, can be based on pharmacological action.

McLeans, (1970) reviewed the toxicity of PAs in the various organs of the animal and the pathogenesis of their toxicity. The long-lasting effect of single doses is possibly related to cell division in some tissues. The effect led to the study of the action of PAs as anti-tumuor substances. It may also be the basis of their disputed action as carcinogens. It has long been known that liver cells suffering the long-term effects of PAs poisoning accumulate an excess nuclear chromatin (Mattocks, 1986). Therefore the studies of cell cycle have encouraged the development of techniques that could be used to look at the effect of the alkaloid upon cell division.

Mattocks, (1967) developed a colourimetric assay for the PAs and their metabolites which has made their determination in blood, urine, and tissues relatively easy. This colour reaction is quantitative when used with appropriate controls, it is specific for pyrrolizidine alkaloids.

2.6.2.2 Toxicity Tests

Awareness as to the toxicity of African medicinal plants appears to have increased as a result of the knowledge of their usefulness. Hamnouda, *et.al.*,(1988) and Fourie, *et.al.*,

(1990) reported on the acute toxicity of Lantana camara on cattle.

The median lethal dose (LD_{50}) from the number of survivors can be found by plotting a graph of log dose of the extract against number of deaths. The LD_{50} of some toxic PAs were given by McLeans, (1970).

The brine shrimp lethality test originally described by MacLaughlin, (1991) as a general test for detecting toxic constituents in plants which would have biological or pharmacological activity at lower doses is a new trend in screening of plants extracts which is becoming popular with the phytochemist. This method utilizes Brine shrimp (Artemia Salina Leach) as a simple bio-assay for natural product research. The procedure determines LC_{50} (median lethal concentration) values in $\mu g/cm^3$ of active compounds and extracts in the brine medium. Meyer, *et. al.*, (1982) reported screening results with seed extracts of forty-one species of Euphorbiacae. Fatope, *et. al.*, (1993) screened ten medicinal plants for bio-activity using Brine Shrimp lethality bio-assay and reported LC_{50} values of the fractions of the extracts. They also reported significant lethality (LC_{50} < 1.0 μgcm^{-3}) in the brine shrimp test of Annona seneglensis.

2.6.2.3 Anti-microbial Screening

This rank the highest among the biological tests carried on plants and extracts to determine their potency against micro-organisms

Traditionally, anti-microbial agents have been assayed by microbiological assay. It is a measure of the response of a growing population of micro-organisms to anti-microbial agent (Hugo and Rusell, 1993). One of the micro-biological assay techniques

is the Agar diffusion assay. In this method the samples to be assayed are applied in some form of reservoir (well) to a thin-layer of agar seeded with indicator organism.

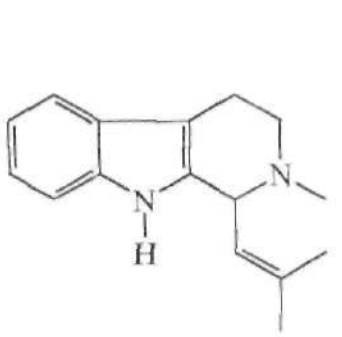
The drug diffuses into the medium and after incubation a zone of growth inhibition forms as a circle around the reservoir, that in the case where the drug contains active principle, which inhibits the growth of indicator organism. The diameter of the zone of inhibition is related to the concentration of the anti-microbial agent in the reservoir.

Bulhoes, *et. al.*, (1977) reported that Crotalaria retusa leaves were found to contain anti-biotic and Kalaichelvan, *et. al.*, (1992) showed that an alkaloid, monocrotaline from seeds of C. paleda is fungistatic.

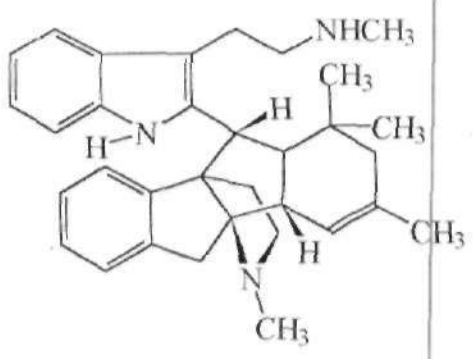
2.7.0 Anti-microbial Agents of Natural Origin

An antimicrobial agent is a chemical compound derived from or produced by living organism which is capable in small concentrations of inhibiting the life process of micro-organisms. Most of the clinically used antibiotics are produced by soil micro-organisms. Moulds or fungi and streptomyces are major sources (Trease and Evans, 1992). The examples discussed here are derived from higher plants.

Many higher plants possess constituents having antibacterial properties, but none has been utilized clinically because of high toxicity. The root of Borreria verticillata L. is used in Nigeria for many skin diseases, the leaves are used in Senegal internally and externally for treatment of leprosy. The paste from the pounded leaves is mixed with extract from Carapa procera for the treatment of whitlow and boils. A study of the Senegalese specimen showed that the roots contain two new indole alkaloids; borrerine and its apparent dimer borreverine. Structures of these alkaloid were given by Pousset, *et. al.*, (1973) and Maynart, *et. al.*, (1980).

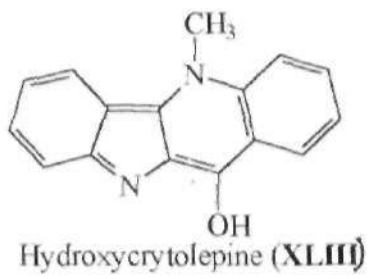


Borrerine (**XLI**)



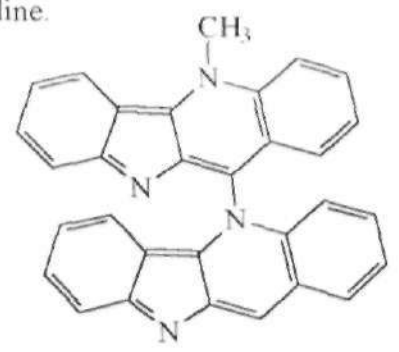
Borreverine (**XLII**)

Borreverine tartrate possesses antimicrobial activity at different concentrations for Staphylococcus aureus and Sarana lutea at minimum bactericidal concentrations of the same order as commercial antibiotics. Benjamin, (1980) reported that the leaves contain volatile oils which in turn contain sesquiterpenes, sesquiterpene-lactone, phenolic compounds and aromatic poly-carboxylic acids.

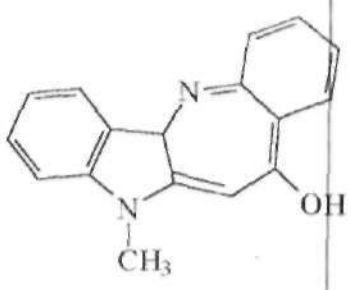


Hydroxycrytolepine (**XLIII**)

Cryptolepsis sanguinolenta (Lindl) Scht. family Periplocaceae, root extract gave three new alkaloids characterized as, hydroxycryptolepine, cryptoheptine and cryptoquinololine.

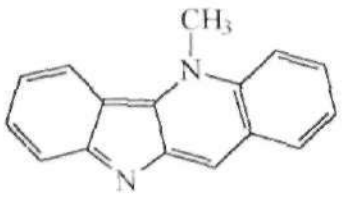


Cryptoquinololine (**XLIV**)



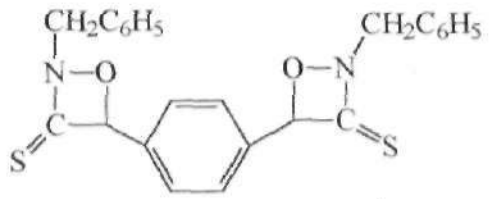
Cryptoheptine (**XLV**)

The aqueous extract of the roots were screened against four urinogenital disease organisms (the plant is used locally by Ghanaian herbalists for treatment of urinogenital diseases). The extract was active against Candidas albicans, Neisseria gonorrhoea, Echerichia coli but inactive against Pseudomonas aeruginosa. The bactericidal property of the root on Staph. aureus are probably due to cryptolepine (Boakye – Yiadom, *et. al.*, 1979).



Cryptolepine **(XLVI)**

The flowers of Moringa pterygosperma Gaertn. (family Moringaceae), on phytochemical studies showed the presence of pterygospermin an unstable low melting point substance which decomposes readily to benzyl isothiocyanate. The later showing antibacterial activity against both gram positive and gram negative organisms including Bacillus subtilis, Staph. aureus, Myobacterium phlei, Salmonella sticottmicellei, S. typhi and Shigella flexnei (Sofowora, 1993).



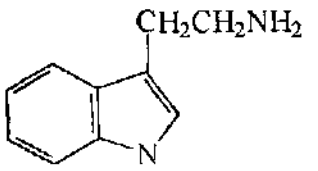
Pterygospermin **(XLVII)**



Benzylisothiocyanate

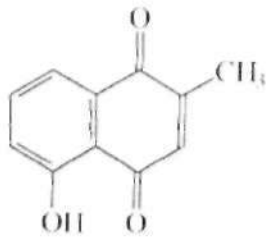
The extracts of Euphorbia hirta L. (family Euphorbiaceae) inhibits the growth of Entamoeba histolytica (Dhar, *et. al.*, 1968). Similar anti-dysentric effect have been reported by Pousset, (1981), ad Ajo, *et.al.*, (1985) in support of the use of this plant in treatment of amoebic dysentry.

Momordica charantia L. (Cucurbitacea) have been shown to have appreciable anti-bacterial activity against E. coli and Staph. aureus (Georges, *et. al.*, 1949). Results of the screening of the entire plant of Congolese origin showed the presence of trace amounts of alkaloids–5-hydroxytryptamine and saponins but the absence of flavonoids, tannins, quinones, steroids and terpenes (Sofowora, 1993).



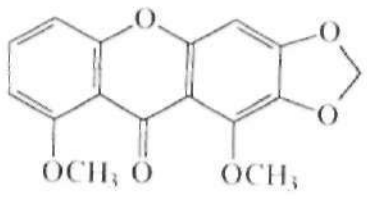
5-Hydroxytryptamine (**XLVIII**)

The plant Plumbago zeylanica L. is popular throughout Africa and Asia as a remedy for parasitic skin diseases such as leprosy, scabies and ulcers (Dalziel, 1955). Various reports have supported the anti-microbial and anti-fungal activity of the leaves and roots extracts. The root has been shown to contain plumbagin, a yellow naphthaquinone which is responsible for its anti-microbial activity (Sofowora, 1993).

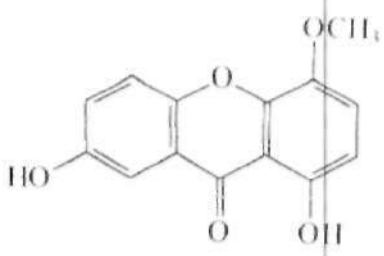


Plumbagin (**XLIX**)

Polygala nyikensis is used in Malawi for various skin diseases of fungal origin. The root of the plant has been shown to exert its antifungal activity due to the presence of xanthones. The few xanthones shown below which are present in this plant provide scientific evidence for its use in traditional medicine in Malawi for skin diseases of fungal origin (Marston, *et. al.*, (1993), Sofowora, (1993).



1,8-Dimethoxy-2,3-methylene-dioxyxanthone (**L**)



1,7-Dihydroxy-4-methoxy-xanthone (**LI**)

CHAPTER THREE

3.0 EXPERIMENTAL

3.1.0 Plant Materials

The seeds of C. retusa L. were collected at the last quarter of 1996 and the leaves were collected at the second quarter of 1997; at the Institute of Agricultural Research (IAR), farm The plant was identified and a voucher of the specimen was deposited at the herbarium in the department of Biological Sciences, Ahmadu Bello University, Zaria.

The seeds and leaves samples were washed and air-dried by exposure to sunlight for 3 days and 7 days respectively. Separate samples of seeds and leaves were grounded in a clean dry mortar into powdered form and passed through 1.00mm mesh size sieve.

3.2.0 Materials and Reagents

3.2.1 Reagents for Extraction of Crude Mixture of Pyrrolizidine Alkaloids (PAs)

(i) 95% ethanol (B.D.H)

(ii) Petroleum ether (60 – 80°C)

(iii) 2M hydrochloric acid (A.R)

(iv) Zinc dust (B.D.H).

(v) Concentrated ammonia solution (B.D.H)

(vi) Trichloromethane (B.D.H)

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3.2.2 Solvents for Crystallization of PAs

(i) Ethanol (B.D.H)

(ii) Methanol (A.R)

(iii) Propan-2-one (A.R)

(iv) Ethylethanoate (A.R)

3.2.3 Purification of Solvents

(1) Ethanol

95% Ethanol was purified by the method described by Lund and Bjerrum (1931). Magnesium turnings (5.0g) and iodine (0.5g) were warmed with ethanol ($75cm^3$) in a 2 litre round bottomed flask until the colour of iodine disappeared. The volume of ethanol was made up to $1000cm^3$ and the mixture was refluxed for 3 hours. It was then distilled to obtain ethanol devoid of water and impurities and stored in the cupboard.

(2) Trichloromethane

Trichloromethane is usually stabilized by addition of a little amount of ethanol. This was removed by shaking trichloromethane ($250cm^3$) with several portions (3 x $10cm^3$) of concentrated tetraoxosulphate(VI) acid, washed thoroughly with water (4 x $10cm^3$) in a separating funnel and dried over

anhydrous calcium chloride overnight. It was then filtered, distilled, stored in a brown bottle wrapped with black polythene bag, which was kept in the dark to avoid photochemical formation of phosgene.

(3) Ethoxyethane

This was made free from peroxide impurities by shaking 1000cm^3 of it with a solution made by dissolving Iron (II) tetraoxosulphate (VI) (6.0g), and concentrated tetraoxosulphate (VI) acid (6.0cm^3) in distilled water (110cm^3). The mixture was washed with distilled water (3 x 20cm^3) dried over anhydrous calcium chloride for 24 hours. It was then filtered and further dried in sodium metal chips until a clear colourless solvent is obtained. Ethoxyethane was then stored in a dark cool place and distilled from sodium chips just before use.

Other organic solvents were distilled before use

3.2.4 Reagents for Phytochemical Tests

(i) 1% hydrochloric acid (A.R)

(ii) 28% and 10% ammonia solutions

(iii) Trichloromethane (B.D.H).

(iv) Benzene (B.D.H).

(v) 1% Iron (II) chloride solution

(vi) Pyridine (A.R)

(vii) 2% sodium nitropruside solution

(viii) 20% sodium hydroxide solution

(ix) 2% methanolic 3,5-dinitrobenzoic acid.

(x) 5.7% aqueous sodium hydroxide.

(xi) Methanol (A.R)

(xii) Concentrated tetraoxosulphate (VI) acid (A.R)

(xiii) Ethanolic anhydride (B.D.H)

(xiv) Ammonical silver nitrate solution: Silver nitrate (3.0g) was dissolved in water ($50cm^3$), ammonia solution was added dropwise until initial precipitate of silver oxide was dissolved. The solution was made up to $100cm^3$ with distilled water.

(xv) Gelatine – Salt reagent: 1% solution of gelatine containing 10% sodium chloride.

(xvi) Meyers reagent: mercuric chloride (1.36g) was added to distilled water ($60cm^3$) to give solution A. Potassium iodide was added to distilled water ($20cm^3$) to make solution B. The two solutions (A and B) were mixed and made up to $100cm^3$ with distilled water.

(xvii) Dragendorff's reagent Bismuth nitrate (0.85g) was dissolved in ethanoic acid (10cm^3) and distilled water (40cm^3) was added to give a stock solution (A). Potassium iodide (8.0g) was dissolved in distilled water (20cm^3) to give a stock solution (B). Five cubic centimetres of each stock solution A and B were mixed with 33% ethanoic acid (20cm^3) and distilled water (100cm^3) was added to give the Dragendorff's reagent.

(xviii) 1% Picric acid Solution Picric acid (1.0g) was dissolved in distilled water (95cm^3).

3.2.5 Reagents Used for Specific Tests for Unsaturated Toxic Pyrrolizidine Alkaloids and Their N-oxides

(i) a) Ascorbic acid (ASC) – 5% aqueous solution

b) Ascorbic acid – saturated solution of ascorbic acid

(ii) Nitropruside (NP) reagent – to a 5% w/v aqueous solution of sodium nitropruside (10cm^3) was added 0.1M sodium hydroxide (0.1cm^3) - (shelf life 24 hours).

(iii) Ehrlich reagent A - 4-dimethylaminobenzaldehyde (5.0g) was dissolved in a mixture of ethanoic acid (60cm^3), distilled water (30cm^3) and 60% perchloric acid (10cm^3) – (shelf life 1 week when kept in the dark).

(iv) Ehrlich reagent B - 4-dimethylaminobenzaldehyde (5.0g) was dissolved in a mixture of ethanol (75cm^3), ethanoic acid (25cm^3) and 60% perchloric acid (1cm^3) – (shelf life 1 week when kept in the dark).

(v) 10% Potassium trioxocarbonate (IV) solution

(vi) Trichloromethane (B.D.H)

(vii) 20% Sodium chloride solution

(viii) *o*-Chloranil solution 0.5% *o*-chloranil w/v in acetonitrile (shelf life 8hours)

(ix) Methanol containing ethanediol (5% v/v)

(x) Petroleum ether (boiling point 80 – 100°c)

(xi) Iron (II) tetraoxosulphate (VI) 20% w/v in methanol

3.2.6 Materials for Thin-Layer Chromatography

(i) Silica G-6 (Merck) with fluorescent indicator

(ii) Gallenkemp spreader and mounting board

(iii) Fifteen 20 x 20cm glass plates and ten 10 x 20cm glass plates

(iv) Microscopic slides (50)

(v) Template

(vi) Sprayer

(vii) Capillary tubes

(viii) Glass developing tank for plates up to and including 20 x 20cm size

(ix) Small glass developing tank for microscopic slides up to and including 2.5 x 7.5cm size.

3.2.7 Preparation of Plates

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

A slurry was made by stirring silica gel (30g) in distilled water (65cm^3) which was allowed to stand for 15 minutes and the contents shaken to give a slurry of a uniform paste. This was spread as 250μm thickness on a previously arranged ten 10 x 20cm glass plates which were cleaned with propan-2-one. The plates were air dried overnight and activated at 120°C for 30 minutes in the oven and used as soon as they cooled to room temperature.

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

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

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

3.2.8 Visualizing Reagents for Thin-Layer Chromatography

(i) 0.5% w/v Iron(III) tetraoxosulphate(VI) in 0.5M tetraoxosulphate(VI) acid

(ii) 0.2% w/v potassium ferricyanide

(iii) 10% w/v 1,3-dinitrobenzene in benzene.

(iv) Sodium hydroxide (6.0g) in water (25cm^3) added to methanol (45cm^3).

(v) Concentrated ammonia solution (B.D.H).

(vi) 1% w/v ethanolic aluminium chloride

(vii) 1% w/v *o*-chloranil solution – (tetrachloro-*o*-benzoquinone) (1.0g) dissolved in benzene (9.9cm^3)

(viii) Ehrlich reagent: 4-dimethylaminobenzaldehyde (2.6g) dissolved in absolute ethanol (100cm^3)

(ix) Ethanoic anhydride reagent: 10% v/v solution in benzene.

3.2.9 Materials for Brine Shrimp Lethality Bio-Assay

(i) Small tank with perforated dividing dam.

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

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

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

3.2.10 Reagents for Brine Shrimp Lethality Bio-Assay

(i) Artermia saline Leach (brine shrimp eggs) from store

(ii) Sea water (from Bar beach Lagos)

(iii) Dimethyl sulphoxide – DMSO, (B.D.H).

3.2.11 Materials For Antimicrobial Screening

(i) Microbiological media (nutrient broth): nutrient agar and Sabouraud dextrose agar.

(ii) Test organisms.

- Staphylococcus aureus (staph. aureus)
- Pseudomonas aeruginosa (Ps. Aeruginosa)
- Bacillus subtilis (B. subtilis)
- Escherichia coli (E. coli)
- Candidas albicans (C. albicans)

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

(iv) Test tubes

(v) Sterile pipette teats

3.2.12 Materials And Equipment For Spectroscopic Studies

(i) Nujol – a mulling agent

(ii) ATI Mattson Genesis Series IR – spectrophotometer

(iii) Deuteurated – dimethylsulphoxide ($Me_2S0 - d_6$).

(iv) Tetramethylsilane

(v) EM – 360, 60MHz NMR spectrophotometer.

(vi) ATI Unicam UV-Visible Vision Software V3. 32 spectrophotometer

3.3.0 Methods

3.3.1 Phytochemical Screening

3.3.1.1 Preliminary Extraction

This was carried out using the following solvents:- Petroleum ether (60-80°C), Ethoxyethane, Trichloromethane and Ethylethanoate

Grounded leaves (100g), grounded seeds (48g) were respectively packed into a Soxhlet extractor and continuously extracted using 95% ethanol (400cm^3 for the

powdered leaves and 250cm^3 in the case of powdered seeds). Each extraction was carried out for 72 hours that is when the solvent was colourless (an indication that ethanol extractable materials have been completely extracted). The extracts were respectively concentrated *in vacuo* to give thick dark green syrup from leaves (11.85g) and dark brown syrup in the case of seeds extract (6.81g).

Each extract was shaken successively using the following solvents

(i) Petroleum ether (60 – 80°C)

(ii) Ethoxyethane

(iii) Trichloromethane and

(iv) Ethylethanoate

In order of increasing polarity. Different fractions obtained are shown in the Figure 4 below.

Powdered leaves (100.0g) and seeds (48.0g) each was also extracted with water (400cm^3). The extracts were concentrated on a hot plate and the yield of each extract was calculated with reference to the air-dried powdered plant material

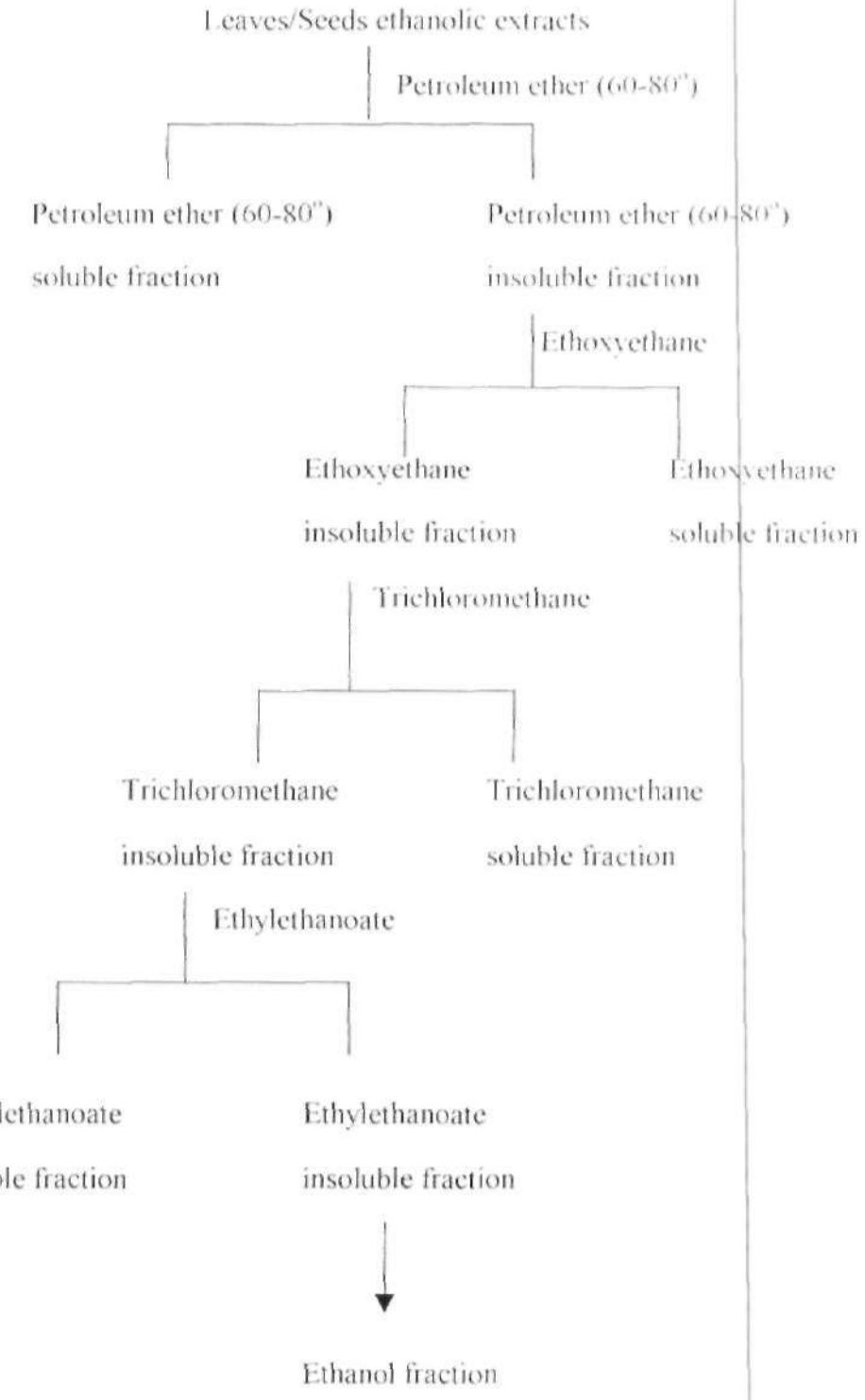


Figure 4: Successive Extraction of Crude Ethanol Extracts of Leaves and Seeds

3.3.1.2.0 Phytochemical Tests

The crude ethanol and water extracts were screened to determine the classes of natural products present

3.3.1.2.1 Test for Alkaloid

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

The crude extract (0.5g) was dissolved in 1% dilute hydrochloric acid (5.0cm^3) on a steam bath and filtered. The filtrate (1.0cm^3) was made alkaline with 28% ammonia solution (pH 10) and then extracted with trichloromethane (3 x 5cm^3). The combined trichloromethane extracts were concentrated *in vacuo* and then treated with equal volume of 1% hydrochloric acid. This was divided into three equal portions and each was respectively treated with few drops of Meyer's, Dragendorff's reagents and 1% Picric acid solution. A creamy white precipitate was produced by Meyer's reagent while reddish brown and yellow precipitate were respectively given by Dragendoff's reagent and 1% Picric acid solution which showed the presence of alkaloids. The water extract did not produce any colour change with any of the reagents.

3.3.1.2.2 Test for Tannins

Each extract (0.5g) was dissolved in distilled water (10cm^3) boiled and filtered. The filtrate was divided into two equal portions.

Few drops of 1% Iron (II) chloride solution on addition to the first portion gave a blue-black precipitate which showed the presence of condensed tannins. Water extract produced greenish brown precipitate. To the second portion was added gelatine – salt reagent. There was no formation of white precipitate hence the absence of hydrolysable tannins.

3.3.1.2.3 Test for Anthraquinones

Borntrager's test - This was used to test the presence of free and combined anthraquinones. Each extract (0.5g) was shaken with benzene ($10cm^3$) and filtered. Ammonia solution ($10cm^3$) was added to the filtrate and shaken. There was no red or violet colour formed in the lower phase in all the solutions of the extracts tested. This showed the absence of free anthraquinones.

Also each extract (0.5g) was boiled with tetraoxosulphate (VI) acid ($10cm^3$) and filtered hot. Benzene ($5cm^3$) was added to the filtrate followed by 10% ammonia solution ($2.5cm^3$). Neither pink, red or violet colouration was observed in the ammonia lower phase which showed that the combined anthraquinones were absent.

3.3.1.2.4 Test for Cardiac Glycosides:

(1) Legal Test

Each extract (0.5g) was dissolved in pyridine and a few drops of 2% sodium nitropruside with a few drops of 20% sodium hydroxide solution were added. All the extracts gave a deep red colour which faded to a brownish yellow. This showed the presence of Cardenolides in the extracts.

2. Kedde Test

8% Methanolic solution of each extract was made and 1cm^3 was mixed with 2% methanolic 3, 5 – dinitrobenzoic acid (1cm^3) and 5.7% aqueous sodium hydroxide. A violet colour that faded through reddish-brown to brownish yellow was produced for each extract. In addition a precipitation of crystalline solid was observed in the case of ethanol and water leaves extract which showed the presence of lactone ring in the cardenolides. No precipitate was seen in the case of ethanol and water seed extracts.

3. Lieberman Burchardt Test

Each extract (0.5g) was dissolved in ethanoic anhydride (2cm^3) and cooled in an ice bath. Tetraoxosulphate (VI) acid (1cm^3) was carefully added. The ethanol seed extract produced a colour change from dark pink to greenish pink that showed the presence of a steroidal nucleus as the aglycone portion of the cardiac glycoside. The dark pink colour was an evidence of the presence of terpenes. This test was negative for water and ethanol leaves extract showing the absence of steroidal nucleus in the cardiac glycosides of these extracts.

4. Salkowski Test

Each extract (0.5g) was dissolved in trichloromethane (1cm^3) and concentrated tetraoxosulphate (vi) acid (1cm^3) was carefully added to form a lower layer. The ethanol seed extract gave a reddish-brown colour at the interface that showed the

presence of a steroidal ring as the aglycone portion of the cardiac glycoside. No colour change was seen at the interface in the case of the water and ethanol leaves extract hence the absence of steroidal ring in the cardiac glycoside.

3.3.1.2.5 Tests for Flavonoids

1. Ammonical Silver Nitrate Test

Each extract (0.2g) was dissolved in distilled water (1cm^3) and a few drops of ammonical silver nitrate solution was added. The following colour changes were observed before and after heating for 5 minutes.

The solution of ethanol seed extract was light brown in colour before heating, which changed to dark brown after heating. The solution of ethanol leaves extract was yellowish brown before heating and changed to dark brown after heating. While the solution of water leaves extract was black before heating and remainekd black after heating. The colour changes before and after heating showed the presence of flavonol glycosides.

A brown, blue or black colour before heating with the brown or blue turning black or brownish black and black still remains black shows the presence of flavanol glycosides. No colour change before and after heating shows the presence of flavanone glycosides. The only exception in this class of flavonoid glycoside is hesperidine, which turned black after heating. A reddish brown before heating which remains the same colour after heating shows the presence of flavone aglycones. Chalcones do not form

colour before heating except 2, 3, 4 – trihydroxychalcones; it forms a reddish brown or black colour after heating.

2. Untreated Test

This implies non-use of reagent. Each extract (0.2g) was dissolved in 50% ethylethanoate solution (1cm^3). All the extracts produced pale yellow colour in the visible light, which showed the presence of flavonol glycosides.

3.3.1.2.6 Test for Saponins

Each extract (0.5g) was shaken with distilled water (1cm^3) in a test-tube and kept aside. There was persistent frothing which lasted for more than two minutes in all the extracts that showed the presence of saponins.

The solutions of each extract were tested with blood sample (from rat). All the tested solutions haemolysed the blood sample hence the presence of toxic saponins.

3.3.1.2.7 Specific Tests for Unsaturated Pyrrolizidine Alkaloids and their N-oxides

A simple field tests which is adequate to indicate most potentially toxic unsaturated pyrrolizidine alkaloid containing plants described by Mattocks *et. al.*, (1987) were carried out on fresh samples of leaves, seeds flowers and roots of C. retusa L.

Two tests are described below the first test (Test A) is sensitive to PA N-oxide and the second test (Test B) shows presence of unsaturated basic PAs.

1. Test for Unsaturated PA N-oxide (Test A)

Each plant material was ground in a mortar with ascorbic acid solution ($8cm^3$). The mixture was filtered and divided into two equal portions (sample and blank). Nitropruside reagent ($0.2cm^3$) was then added to the sample and heated to 70°C for one minute. Ehrlich reagent A ($1cm^3$) was then added to both sample and blank and both test tubes were heated for one minute. Magenta colour developed in all the test samples in comparison with the blank; which showed the presence of PA N-oxide in the leaves, seeds, flowers and roots of C. retusa L.

2. Test for Unsaturated Basic PAs (Test B)

The filtered plant extract from (1) above was diluted with its own volume of 10% potassium trioxocarbonate (IV) solution and shaken with trichloromethane ($2cm^3$) in a separating funnel. The lower trichloromethane layer was run off. *o*-Chloranil reagent ($0.1 - 0.2cm^3$) was added to it and the solution heated for about 30 seconds, then shaken with a few drops of ascrobic acid solution to remove the orange *o*-chloranil colour.

Ehrlich reagent, B ($1.0cm^3$) was added and heated for one minute with shaking to mix the phases. All the samples tested except the root formed a magenta colour in the upper aqueous phase, which showed the presence of free basic PA in all the plant parts tested with the exception of the root.

3.3.2.0 Bulk Extraction

Plant materials grounded to 1.0mm mesh size (Leaves 210g, seeds 78g) were extracted continuously (Soxhlet) with 95% ethanol (1dm^3 for leaves and 300cm^3 for the seeds). Each extract was concentrated *in vacuo* to give leaves extract 25.02g, seeds extract 11.21g.

3.3.2.1 Extraction and Isolation of Crude Mixture of Pyrrolizidine Alkaloids (PAs)

The concentrated crude ethanol extracts of the leaves and seeds obtained above were respectively dissolved in dilute hydrochloric acid (50cm^3 for leaves extract and 20cm^3 in the case of seeds extract). Each solution was defatted with petroleum ether (60 – 80°C). The aqueous phase was reduced with excess zinc dust (3.0g for the leaves extract and 2.0g for the seeds extract) and kept aside for one hour. It was then filtered and the filtrate was basified with ammonia solution (pH 10). The alkaloids were extracted with trichloromethane (3 x 10cm^3 for the leaves extract and 3 x 5cm^3 for the seeds extract). Fractions obtained from each sample were pooled and evaporated over the water bath to obtain a crude mixture of PAs , as light brown gummy substance from the leaves extract and dark brown gummy substance in the case of the seeds extract. The isolation procedure is illustrated in Figure 5.

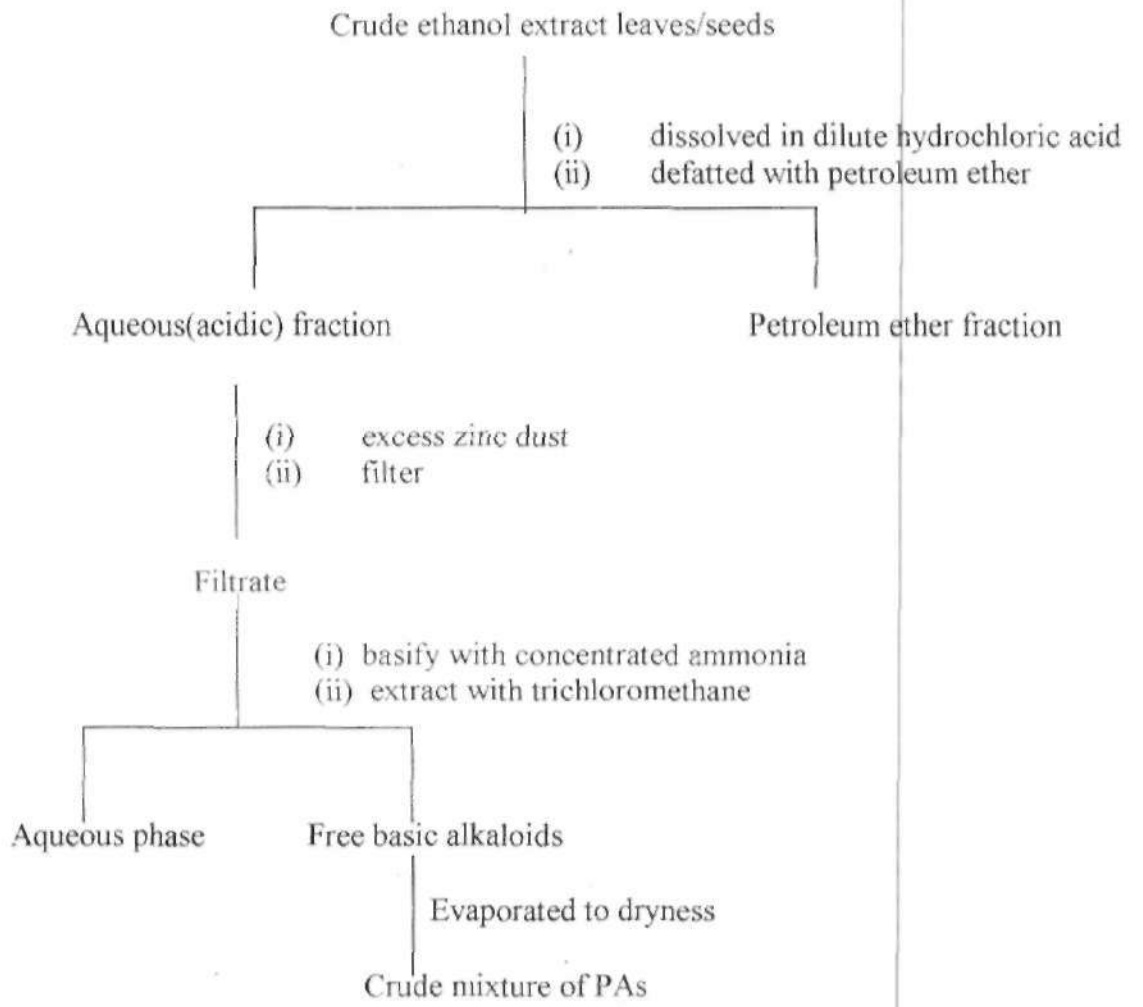


Figure 5 : Isolation of Crude Mixture of PAs

3.3.2.2 Cleaning-Up of Crude Mixture of PAs

Crude mixture of PAs (0.4g) from the leaves was dissolved in 2M hydrochloric acid ($10cm^3$) then shaken with trichloromethane (2 x $2cm^3$) and removed as the lower layer. The aqueous upper layer was basified with concentrated ammonia solution ($15.0cm^3$) to pH 10. PAs were extracted with trichloromethane (2 x $2cm^3$) and the combined fractions were evaporated to dryness in air, to give dirty white crystals (0.02g), which was washed with methanol to obtain a neater white crystals. The procedure is outlined in Figure 6.

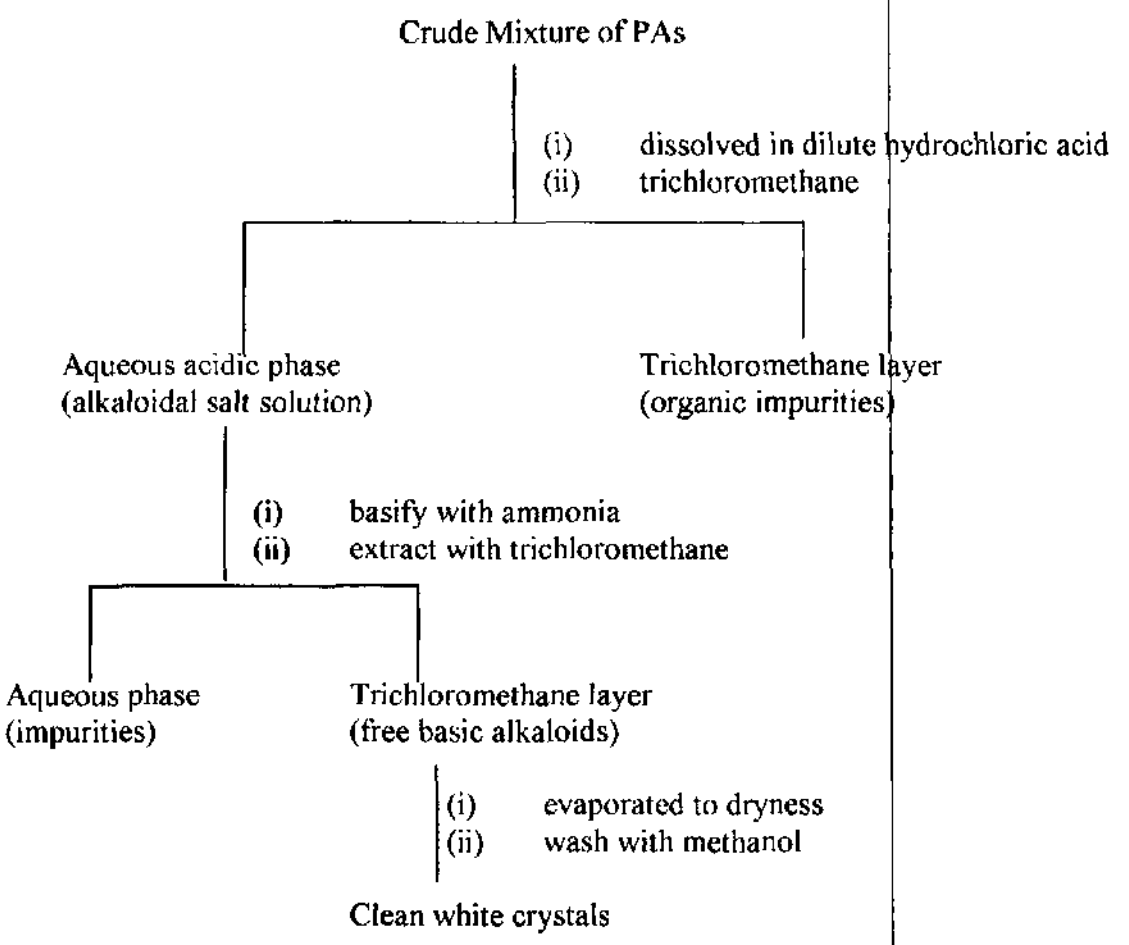


Figure 6 : Cleaning-up of Crude Mixture of PAs

3.3.2.3 Crystallization of Crude Mixture of PAs

Various solvents such as ethanol, propan-2-one, ethylethanoate, and methanol were tried as well as solvent combinations like ethanol-propan-2-one, methanol-ethylethanoate.

White needle-like crystals (melting point 205 – 220°C) were formed in each case at the upper part of the test tube, the solvent was removed by evaporation at room temperature to obtain the crystals which was light-brown in colour due to retention of solvent of crystallization after several recrystallizations.

3.3.3.0 Thin-Layer Chromatography (TLC)

The methanolic solutions of the samples listed below were spotted on kieselgel G. (Merck) TLC plates (0.25mm thickness) using drawn out capillary tubes.

$CAPL_1$ Ethanol leaves extract

$CAPS_1$ Ethanol seeds extract

$CAPL_2$ Trichloromethane leaves extract

CAPL Crude mixture of PAs from leaves

CAPS Crude mixture of PAs from seeds

$CCAPL_1$ cleaned-up CAPL

$CCAPL_3$ crystals from CAPL

The plates were then developed using different solvent systems (see 3.3.3.2) in tank (for plates up to and including 20 x 20cm size) which was allowed to saturate for about ten minutes at room temperature.

Plates were air-dried and viewed under visible and ultraviolet light (254nm and 366nm) respectively to locate the spots or zones corresponding to the resolved components. Measurements were made from the centre point of the spot at the origin to the centre point of the resolved spot, and from the centre point of the origin spot to the solvent front.

3.3.3.1 Selection of Mobile Phase (Solvent System)

The choice of mobile phase was based on the nature of the two broad groups of substances chromatographed, viz:

Group A Samples: consists of $CAPL_1$, $CAPS_1$, $CAPL_2$. These are mainly ethanol soluble components, hence they have the same polarity with ethanol. These components will move with the solvent front, if ethanol alone was used as the mobile phase.

Group B Samples: consists of CAPL, CAPS, $CCAPL_1$, $CCAPL_3$, which are either crude mixture or pure form of pyrrolizidine alkaloids.

The choice of mobile phase for Group A samples was made using the guidelines of solvent combinations for TLC separation of different classes of chemical compounds as described by Kirchner (1966) and Touchstone (1992). In trying to obtain a less polar solvent system for Group A samples the following ratios: 4:1; 2:1; 3:1; of solvent systems:-

i) Petroleum ether (60 – 80°C): ethylethanoate.

ii) Benzene: ethylethanoate were used.

For best resolution of the components the ratio 3:1 was selected for each of the above solvent system. Although $CAPL_2$ is the trichloromethane extract the components were well separated using the aforementioned solvent systems.

The solvent systems used by Mattocks (1967b) and Molyneux *et. al.*, (1980) for TLC of PAs were chosen for Group B samples. These solvent systems chosen for comparable results are as follows:

i) Trichloromethane: propan-2-one: ethanol: ammonia solution (5:3:1:1)

ii) Trichloromethane: methanol:17% ammonia solution (82.5:15.5:2)

iii) Butanol: ethanoic acid: water (4:1:5) - for PAs N-oxides.

3.3.3.2 Isolation of the Components of $CAPL_1$ and $CAPL_2$ Extracts Using Preparative TLC

Ten 20 x 20cm plates (1.00mm thickness) were prepared for each extract. On each plate was loaded 0.10g of a given extract. Separations were accomplished by two developments (drying between developments) using benzene: ethylethanoate (3:1) solvent system; at an average time run of 45 minutes. Developed plates were air-dried and viewed under visible light and ultraviolet light (254nm and 366nm) respectively. Eight bands were observed for each extract. The distances moved by the resolved bands were measured and their R_F values determined (Section 4.4.1). Each band was scraped off with spatula, this was dissolved in methanol, allowed to stand for five minutes and filtered. The fractions obtained from each extract were concentrated *in vacuo*, and then spotted on the same plate (0.25mm thickness), developed with benzene: ethylethanoate (3:1). Those fractions from a given extract with same R_F values were combined; and evaporated to dryness. The results are recorded and discussed in section 4.4.1 and their chromatograms are shown in Figures 11 and 12.

3.3.3.3 Identification of Class of Compounds Using Visualizing Reagents

The isolated fractions obtained after preparative TLC of $CAPL_1$ and $CAPL_2$ were spotted on TLC plates and sprayed with any of the visualizing reagents listed below for identification of various classes of compounds.

i) Phenolic Compounds: Iron (III) tetraoxosulphate (VI) and potassium ferricyanide.

ii) Cardiac glycosides: 1, 3 – dinitrobenzene and methanolic sodium hydroxide

iii) Flavonoids: (a) ammonia vapour (b) aluminium chloride.

iv) Pyrrolizidine akaloids: *o*–chloranil and Ehrlich reagents.

1. Test for Phenolic Compounds

Equal volumes of 0.5% Iron (III) tetraoxosulphate (VI) in 0.5 M tetraoxosulphate (VI) acid and 0.2% potassium ferricyanide were mixed and sprayed on the isolated fractions from CAPL and $CAPL_2$ which were spotted on TLC plates. Colour changes were observed before and after heating in the oven at 110°C for ten minutes (Kirchner, 1967). The results of the colour reaction are recorded and discussed in section 4.4.2.1.

2. Test for Steroid Glycosides

The isolated components from $CAPL_1$ and $CAPL_2$ were spotted on TLC plates and sprayed with 10% - 1, 3–dinitrobenzene in benzene; and heated in the oven at 60°C then sprayed with a solution containing sodium hydroxide (6.0g) in water ($25cm^3$) and methanol ($45cm^3$) – i.e. methanolic sodium hydroxide before observing the colour changes (Touchstone, 1992).

3. Test for Flavonoid Compounds

The isolated fractions obtained from $CAPL_1$ and $CAPL_2$ were spotted on TLC plates and exposed to concentrated ammonia vapour for about ten minutes, and any colour changes were observed and recorded.

Also the spotted isolated fractions obtained from $CAPL_1$ and $CAPL_2$ were sprayed with 1% ethanolic aluminium chloride and the colour changes were observed.

3.3.3.4 Detection of Pyrrolizidine Alkaloids (PAs) on Thin-Layer Chromatograms

Specific method of detection of PAs on thin-layer chromatograms as described by Molyneux *et. al.*, (1980) was used. This involves the following procedure:

1. Detection of Basic Pyrrolizidine Alkaloids

The chromatograms containing spotted samples of CAPL, CAPS, $CCAPL_1$ and $CCAPL_3$ were air-dried then sprayed with *o*-chloranil solution and dried on a steam bath for about one minute. This was re-sprayed with Ehrlich reagent and again heated on a steam bath for one minute and the colour of the spots were observed. There was development of stable purple colour in all the chromatograms, which showed the presence of PAs.

2. Detection of PAs N-oxide

The chromatograms containing spotted samples of CAPL, CAPS, $CCAPL_1$ and $CCAPL_3$ were air-dried; and sprayed with ethanoic anhydride and subsequently sprayed with Ehrlich reagent; and the colour of the spots were observed. Stable purple colour developed only in the immobile components.

3.3.4.0 Biological Activity of the Extracts

Two different bioassay screening methods were used. First was the Brine shrimp lethality bioassay, which is a general test for cytotoxicity. The active extracts obtained were thereafter subjected to a bioassay for specific activity, which in this ease is the antimicrobial activity test.

3.3.4.1.0 Brine Shrimp Lethality Bioassay

Sample extracts used were as follows:

$CAPS_1$ ethanol seeds extract
$CAPL_1$ ethanol leaves extract
$CAPL_2$ trichloromethane leaves extract
$CAPL_3$ ethylethanoate leaves extract
CAPL crude mixture of PAs from leaves
CAPS crude mixture of PAs from seeds.

3.3.4.1.1. Sample Preparation

Test tubes were prepared for testing as follows: For each sample of the extract test tubes were labeled at concentrations 1000 (A), 500 (B), 250 (C), 125 (D) and 62.5 (E) ug/cm^3. Three test tubes were prepared for each concentration, (a total of fifteen test tubes for each sample and one test tube for the control).

Seawater 0.5cm^3 was placed in each test tube except in test tube labeled A. Samples were prepared by dissolving each extract (30mg) in the solvent-dimethylsulphoxide (3cm^3). This gave a stock solution A (containing 10mgcm^{-3} DMSO). 0.5cm^3 of this was placed each in test tubes labeled A and B (a total of six test tubes). From test tube labeled B the stock solution (0.5cm^3) was transferred to test tubes labeled C, D, and E to obtain the corresponding concentrations by dilution (500, 250, 125, and 62.5 ug/cm^3) respectively. 0.5cm^3 Removed from test tube labeled E was discarded. Each solution was made up to 5.0cm^3 after addition of the shrimps with the seawater. The control contains 5cm^3 of seawater and the shrimps.

3.3.4.1.2 Hatching the Shrimps

Brine shrimp eggs were hatched in small tank with perforated dividing dam which was clamped in the dish to make two unequal compartments. The eggs (30mg) were sprinkled into the larger compartment which was covered while the smaller compartment was illuminated under the fluorescent lamp; so as to attract shrimps through the perforations in the dam. After 48 hours the phototropic nauplii (shrimp larvae) were ready for use.

3.3.4.1.3 Bioassay

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

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

3.3.4.1.4 Determination of LC_{50}

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

3.3.4.2.0 Antimicrobial Screening Using The Agar Diffusion Method

Plant Extracts Used

$CAPL_1$	-ethanol leaves extract
$CAPS_1$	-ethanol seeds extract
$CAPL_2$	-trichloromethane leaves extract
$CAPL_3$	-ethylethanoate leaves extract
CAPL	-crude mixture of PAs from leaves
CAPS	-crude mixture of PAs from seeds
$CCAPL_1$	-cleaned-up CAPL
$CCAPL_3$	-crystals from CAPL

3.3.4.2.1 Preparation of media

All dehydrated microbiological media were reconstituted in freshly distilled water according to the manufacturer's instructions and specifications. Agar media, Sabouraud dextrose agar and nutrient agar were dissolved with the aid of heat and dispensed in aliquot of $20cm^3$ in glass bottles. They were sterilized in an Adalphi bench autoclave at pressure of 15psi and temperature of 121°C for fifteen minutes. After cooling they were stored in a refrigerator until ready for use.

3.3.4.2.2 Preparation of microbial culture

Typed or clinical bacteria isolates of the following organisms: Staph.aureus, Ps.aeruginosa, B. subtilis and E.coli. were sub-cultured on sterile nutrient broth, incubated overnight at a temperature of 37°C. The resulting cultures were diluted with sterile normal saline such that the final dilution contains approximately 10^8 cfu/ml.

Generally 1:1000 dilution of overnight cultures of staph.aureus and B. subtilis gave approximately this value of 10^8 cfu/ml. Whereas a 1:5000 dilution gave the same population of bacteria density in the case of E.coli and Ps.aeruginosa. The final microbial level were determined using pour plate method.

Candidas albicans was sub-cultured on the slant agar of sabouraud dextrose agar and incubated at 22-25°C for forty eight hours. The resulting colonies were harvested into sterile nutrient broth and de-aggregation was achieved with the aid of sterile glass beads. The resulting broth suspension of this organism was then diluted with sterile normal saline such that one millilitre contains 10^7cfu/ml.

3.3.4.2.3. Preparation of Solution of Extracts for Antimicrobial Screening Tests

Solutions of the extract were prepared at 10%, 5% and 1% concentrations using dimethylsulphoxide (DMSO) as solvent.

One gramme powder or one cubic centimetre of the syrup of each extract was separately dissolved in DMSO ($9.0cm^3$) to give a stock solution – 10%. From this 5% and 1% concentrations were prepared by diluting with the appropriate volume of sterile water. The control was 10% DMSO only.

Preparation of Petridishes

Petridishes were washed and sterilized before use. They were labeled indicating the concentrations below and test organisms and extracts on top.

3.3.4.2.4.1 Determination of Antimicrobial Activity of the Extracts from the Leaves and Seeds of C.retusa L.

The Agar diffusion method was employed in this test and it basically involved the following procedure:- The previously prepared sterile nutrient agar and sabourand dextrose agar were melted in a water bath thermostated at 45°C. Thereafter the standardized suspension of the organisms ($0.2cm^3$) were mixed with the molten cooled agar media and poured on sterile petridishes and allowed to set.

Using a 8.0mm cork-borer four holes were bored on each of the molten cooled agar media ($20cm^3$) contained in the petridish. The bottom of each well was sealed with about two drops of the molten agar. The test solution ($0.2cm^3$ or 5 drops) was filled in the holes.

A pre-incubation time of two hours was allowed to make the drug diffuse into the agar. Thereafter the petridishes were taken and incubated in Bayer and Tactlock incubator thermostated at 30°C for twenty-four hours in the case of bacteria organisms.

The inoculated petridishes containing C. albicans were incubated at temperature of 22°C for forty-eight hours. At the end of incubation period the petridishes were examined and the diameter of inhibition zones of the extracts were measured using vernier calipers and transparent ruler.

3.3.5.0 Spectroscopic Studies

3.3.5.1 Infra-red Spectroscopy

The infrared spectra of CAPS, CAPL, $CAPL_2$ and $CCAPL_3$ extracts were run in nujol using ATI Mattson Genesis series IR-Spectrophotometer.

3.3.5.2 Nuclear Magnetic Resonance Spectroscopy

The samples CAPL and $CCAPL_3$ each was run in EM-360, 60MHz nuclear magnetic resonance spectrometer, as solution in deuterated-dimethylsulphoxide (Me_2SO-d_6) to dissolve all materials in the sample.

3.3.5.3 Ultraviolet Visible Spectroscopy

The Ultraviolet (UV) spectra of samples of CAPS, CAPL, $CAPL_2$ and $CCAPL_3$ were run on ATI Unicam UV-Visible vision software V3.32 spectrophotometer. The spectra were taken using two silica cells – a blank cell containing the solvent and the sample cell that contains a solution of the sample. The base line error was corrected by automatic subtractive method. The sample solutions used were in the concentration range of 19.8-23.4 g/litre. The extracts, CAPS, CAPL, $CCAPL_3$ and $CAPL_2$ were all soluble in methanol and ethanol. However $CAPL_2$ in addition was found to be soluble in five other solvents viz: dichloromethane, trichloromethane, tetrahydrofuran, ethoxyethane and dioxan. The UV spectra of $CAPL_2$ were taken in the aforementioned solvents, while those of CAPS, CAPL, and $CCAPL_3$ were run in methanol and ethanol only. The instrument was calibrated using potassium dichromate solution.

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

4.1.0 PHYTOCHEMICAL SCREENING

4.1.1 Preliminary Extraction

The extractive values depicted in Table 8 showed the percentage of a material, which is soluble in a particular solvent. The solvents such as petroleum ether (60–80°C), ethoxyethane, trichloromethane and ethylethanoate extracted larger amount of substances from the leaves compared with the seeds. Conversely solvents such as ethanol and water extracted larger amount of materials from the seed compared with the leaves.

Trichloromethane extracted larger amount of materials from the seeds than ethylethanoate. May be the seeds contain larger amount of trichloromethane soluble substances. Ethanol extracted the largest amount of materials and petroleum ether (60–80°C) extracted the least amount of materials. Water extracted less amounts of material than ethanol. Probably the leaves and seeds of C. retusa L. contain materials, which are less soluble in water.

With the exception of fixed oils where light petroleum is used for extraction, alcohol is a general solvent for many plant constituents (Brain *et. al.*, 1975, Trease and Evans, 1992). The decoction of the leaves of C. retusa is used locally as a remedy for treatment of fever (Sofowora, 1986) hence the use of water as a solvent for extraction.

The leaves and seeds samples contain very large amount of ethanol soluble materials as a result this solvent was employed for bulk extraction. Further investigations on the extracts as depicted in Table 8, were limited by small amounts available and their cytotoxic properties.

Table 8 Percentage Yield of the Extracts using Different Solvents

S/No	Solvent	Leaves Extract		Seeds Extracts	
		Mass/100g of powered leaves	Percentage Yield	Mass/48g of powered seeds	Percentage Yield
i)	Petroleum ether (60 – 80°C)	0.025	0.025	0.008	0.017
ii)	Ethoxyethane	0.021	0.021	0.008	0.017
iii)	Trichloromethane	0.361	0.361	0.080	0.167
iv)	Ethylethanoate	0.468	0.468	0.030	0.063
v)	Ethanol	10.720	10.720	6.680	13.917
vi)	Water	6.200	6.200	3.936	8.200

4.1.2.0 Phytochemical Tests

4.1.2.1 Alkaloid

General tests for alkaloids using three different reagents gave positive results for ethanol leaves and seeds extracts as depicted in Table 9. Water extracts from leaves and seeds were devoid of alkaloids, since all the alkaloidal reagents used gave negative response. It shows that water could not extract the alkaloids in the leaves and seeds of C. retusa.

Table 9: Results of General Identification Tests for Alkaloids

Sample	Observations			Inference
	Meyer's reagent	Dragendoff's reagent	1% Picric acid	
Ethanol leaves Extract	Creamy white Precipitate	Reddish-brown Precipitate	Yellow Precipitate	Alkaloid Present
Ethanol seeds Extract	Creamy white precipitate	Reddish-brown Precipitate	Yellow Precipitate	Alkaloid Present
Water leaves extract	No colour change	No colour change	No colour change	Alkaloid absent
Water seeds extract	No colour change	No colour change	No colour change	Alkaloid absent

4.1.2.2 Tannins

The ethanol and water extracts of leaves and seeds all gave colour changes, which revealed the presence of condensed tannins when tested with 1% iron (II) chloride solution. The results are depicted in Table 10. Hydrolysable tannins were absent in all the samples tested, as they failed to give white precipitate with gelatin salt reagent.

Table 10: Response of the Extracts to Tannins Tests

Samples	Observations		Inference
	Iron(ii) Chloride	Gelatin-salt reagent	
Ethanol leaves extract	Blue-black Precipitation	No white precipitate	Condensed tannins present
Ethanol seeds extract	Blue-black precipitation	No white precipitate	Condensed tannins present
Water leaves extract	Greenish brown precipitate	No white precipitate	Condensed tannins present
Water seeds extract	Brown precipitation	No white precipitate	Condensed tannins present

4.1.2.2 Anthraquinones

None of the extracts responded positively to Borntrager's test for both free and combined anthraquinones as shown in Table 11.

Table 11: Result of Borntrager's Test on the Extracts

Sample	Observations	
	Free	Combined
Ethanol leaves extract	No colour change	No colour change
Ethanol seeds extract	No colour change	No colour change
Water leaves extract	No colour change	No colour change
Water seeds extract	No colour change	No colour change

4.1.2.3 Cardiac Glycosides

The results of the various identification tests of cardiac glycosides on the extracts are shown in Table 12.

The legal test on the extracts produced a deep red colour, which faded to brownish yellow, an evidence of the presence of cardenolides. Yadava *et. al.*, (1995) isolated a novel cardenolide from the seeds of C. retusa. The precipitate formed in the case of ethanol and water leaves extracts as shown in Kedde test revealed the presence of lactone ring in these extracts. However the ethanol and water seeds extracts gave a positive response to Salkowski test by formation of reddish brown colour at the interface hence showed the presence of steroidal ring as the aglycone portion of the cardiac glycoside. Also the dark pink colour which faded to greenish pink in the case of Lieberman-Burchardt test for ethanol and water seed extracts; revealed the presence of steroidal nucleus. This is in support of the result obtained from Salkowski's test for the

ethanol and water seed extracts. In addition the dark pink colour which faded to greenish pink showed that terpenes are also present in the ethanol and water seed extracts.

The leaves of C. retusa therefore contain cardiac glycosides with lactone-ring while the seeds of this plant contain cardiac glycosides without lactone-ring, rather it has steroidal nucleus as its aglycone. Terpenes are also present in the seeds.

Table 12: Results of Identification Tests for Cardiac Glycosides

Sample	Legal Test	Kedde Test	Salkowski Test	Lieberman Burchardt Test	Inference
Ethanol leaves extract	Deep red faded to brownish yellow	Brownish-yellow with precipitate	No colour change at the interface	Dark green	Cardenolide present
Ethanol seeds extract	Deep red faded to brownish-yellow	Brownish yellow without precipitate	Reddish brown colour at the interface	Dark pink which faded to greenish pink	Cardenolide present
Water leaves extract	Deep red faded to brownish yellow with precipitate	Reddish brown with precipitate	No colour change	No colour change	Cardenolide present
Water seed extract	Deep red faded to brownish yellow with precipitate	Brownish yellow without precipitate	Reddish brown colour at the interface	Dark pink which faded to greenish pink	Cardenolide present

4.1.2.4 Flavonoids

The results of the tests of flavonoids are depicted in Table 13. The light brown and yellowish brown solutions of the ethanol extracts of leaves and seeds respectively became darker on heating, which showed the presence of flavonol glycosides. The water leaves and seeds extracts, which remained black after heating was also an evidence of the presence of flavonol glycosides. All the solutions of the extracts produced a pale yellow colour under visible light, which also revealed the presence ot flavonol glycosides.

Flavonoids are classified as condensed tannins. All the extracts gave positive response for condensed tannins test. The result of the test for flavonoids is in support of the result of the test for condensed tannins in these extracts. Also the family Leguminosae to which C. retusa belongs are known to have flavonoids in abundant (Trease and Evans, 1992). Free quercitin, β-sitosterol and flavonol-3-O-glycosides were identified from C. retusa, by Subramanian *et. al.*, (1968).

Table 13: Results of the Identification Tests of Flavonoid in the Extracts

Sample	Observations			Inference
	Ammonical Silver Nitrate Solution			
	Before heating	After heating	Untreated	
Ethanol extract leaves	Yellowish brown	Dark brown	Pale yellow in visible light	Presence of flavonol glycoside
Ethanol extract seeds	Light brown	Dark brown	Pale yellow in visible light	Presence of flavonol glycoside
Water extract leaves	Black	Black	Pale yellow in visible light	Presence of flavonol glycoside
Water extract seeds	Black	Black	Pale yellow in visible light	Presence of flavonol glycoside

4.1.2.6.Saponins

All the solutions of the extracts produced stable foams, which persisted for more than two minutes on warming. This was an evidence of the presence of saponins in these extracts. The blood sample from rat was haemolysed by all the solutions of the extracts, which showed that the saponins are toxic to blood. This result is in support with the reported toxicity of C. retusa (Rose *et.al.*, 1957, Hooper and Scalan, 1977, Sofowora, 1986).

4.1.2.7 Specific Tests for Unsaturated Pyrrolizidine Alkaloids (PAs) in Fresh Plant Samples

The results of the detection of PAs as described by Mattocks *et.al.*, (1987) are depicted in Table 14. The formation of magenta colour in the sample solutions of all the parts of the plant tested revealed the presence of PA-N-oxide (Test A). The absence of magenta colour in the upper aqueous layer for the test of PAs (Test B) in the case of the root sample showed that the roots of C. retusa are devoid of unsaturated PAs. This is an evidence that the PAs in the roots of this plant occur mainly as N-oxides. In many plants PAs occur largely as N-oxides which are not bitter to taste and are not rejected in feed by animals (Bull. *et.al.*, 1968).

The strong magenta colour observed in the case of flowers showed that this part of the plant is rich in PAs and their N-oxides, probably this is why livestock are not attracted to feed on C. retusa, inspite of the lush growth of the plant (puts on fresh green leaves and flowers) in dry season which should attract grazing animals.

Both leaves and seeds have similar unsaturated PAs while roots had mainly PA-N-oxide and the flowers are rich in both PAs and their N-oxides.

Table 14 Results of detection of PA-N-oxides (Test A) and Unsaturated PAs (Test B) in Fresh Plant Samples

Plant Part	Observations		Inference
	Test A	Test B	
Leaves	Magenta colour	Magenta colour in aqueous phase	PA-N-oxide + PAs present
Seeds	Magenta colour	Magenta colour in the aqueous phase	PA N-oxide + PAs present
Flowers	Strong magenta colour	Strong magenta colour in the aqueous phase	PA N-oxide + PAs present
Roots	Magenta colour	No magenta colour in the aqueous phase	PA N-oxide pesent

4.2.0 Bulk Extraction and Isolation of PAs

The amount of crude mixture of PAs isolated from the ethanolic leaves and seeds extracts are shown in Table 15. It was necessary to reduce the extracts with zinc-dust (Koekemoer *et. al*., 1951, Mattocks and Nwude, 1988), due to occurrence of PAs as N-oxides in the samples used (section 4.1.2.7). The zinc dust converted N-oxide to free basic alkaloids before they were isolated. The alkaloidal content of the seed 2.6% is higher than that of the leaves 2.2% which is in agreement with that reported by Culvenor *et.al*., (1957) seeds 5% and leaves 2.1%.

Table 15: Percentage Alkaloidal Content of the leaves and seeds of C. retusa L.

Plant Materials	Mass of powered plant material	Mass of crude mixture of PAs	Percentage yield of crude mixture of PAs
Leaves	210g	4.60g	2.1905
Seeds	78g	2.03g	2.6026

The cleaned up solid crude mixture of PAs as well as the crystals both melted with decomposition

i) Cleaned-up crude mixture of PAs from leaves, $CCAPL_1$ melted at 168°C – 180°C

ii) Crystals of PAs, $CCAPL_3$ melted at 205 – 220°C

Both samples have wide range of melting points an evidence that they require further purification, monocrotaline a PAs has a melting point of 200°C – 202°C (Culvenor and Smith, 1957)

4.2.0 Thin-Layer Chromatography (TLC)

4.2.1 Evaluating the Thin-Layer Chromatograms

The R_F (retardation factor) value is a convenient way to express the position of a substance on a developed chromatogram. It is the distance moved by the spot from the origin expressed as a ratio to the distance moved by the solvent front. Qualitative separation can be achieved with a solvent front run of 10cm. The R_F of a compound in a given system is a physical characteristic which is independent of the presence of other substances applied simultaneously hence it is used in identification of the components of an extract (Brain *et.al*., 1975).

The number of components present in Group A samples ($CAPL_1$, $CAPS_1$, $CAPL_2$) using two different solvent systems, their R_F values as well as their colour in visible light (254nm) and ultraviolet light (336nm) are depicted in Tables 16 and 17. The chromatograms are shown in Figures 7 and 8. More spots and better separations were obtained from Group A samples using solvent system; benzene: ethylethanoate (3:1) compared with petroleum ether (60-80°C); ethylethanoate (3:1) solvent system. Also the

R_F values of the components of Group A samples varied slightly with different batches of solvents used.

The results obtained are not absolute but should be regarded as relative. The yellow colour both in visible light and ultraviolet light observed for $CAPS_1$ showed the presence of flavonoid compounds (Haslam, 1966). The component whose R_F value was 0.32 was obtained for sample $CAPL_1$ and $CAPS_1$ using the same solvent system. This component (R_F 0.32) must be present in both leaves and seeds of this plant.

Table 16: R_F values of Group A, samples using the Solvent System, Benzene: ethylethanoate (3:1)

Sample	Distance moved by solvent front 16.7cm. Time run: 32 mins.				
	No. of spots	Distance moved by solute front (cm)	R_F	Colour in	
				Visible light (254nm)	Ultraviolet (366nm)
$CAPL_1$	4	i) 5.4	0.32	-	Weak purple
		ii) 8.9	0.53	green	Yellowish brown
		iii) 11.0	0.66	faint green	Greenish brown
		iv) 15.2	0.90	-	Orange
$CAPS_1$	4	i) 1.9	0.11	-	Weak purple
		ii) 4.1	0.25	yellow	Yellow
		iii) 5.4	0.32	-	Weak purple
		iv) 8.1	0.49	yellow	Yellow
$CAPL_2$	5	i) 3.3	0.20	Brown	Orange
		ii) 11.9	0.71	Green	Green
		iii) 14.3	0.86	-	Orange
		iv) 15.6	0.93	-	Green
		v) 16.0	0.96	-	Orange

Table 17. R_f Values of Group A Samples using Solvent System Petroleum ether (60 80°C): Ethylethanoate (3:1)

Sample	Distance moved by solvent front 16.2cm. Time run: 30 mins				
	No of spots	Distance moved by solute front (cm)	R_f	Colour in	
				Visible light (254nm)	Ultraviolet (366nm)
$CAPL_1$	3	i) 6.0	0.37	-	Weak purple
		ii 12.2	0.75	Greenish brown	Orange
		iii) 13.8	0.85	Yellow	Orange
$CAPS_1$	2	i) 1.9	0.12	Faint yellow	Yellow
		ii) 4.6	0.28	Faint yellow	Yellow
$CAPL_2$	5	i) 2.7	0.17	Greenish brown	Orange
		ii) 6.8	0.42	-	Green
		iii) 9.5	0.59	-	Orange
		iv) 11.3	0.70	-	Green
		v) 13.0	0.80	-	Orange

(Key – no visible spot)

The R_f values of the components of Group B samples (CAPL, CAPS, $CCAPL_1$, $CCAPL_3$) using three different solvent systems are depicted in Tables 18 and 19. Their chromatograms are shown in Figures 9 and 10.

The number of components for all the samples was the same with the different solvent systems used. The unmoved components in CAPL and $CCAPL_1$ were moved using the solvent system for PA N-oxides that is butanol:ethanoic acid:water (4:1:5). More spots were obtained with this solvent system which showed the presence of PA N-oxide.

The R_f values varied slightly with different solvent systems used. The relative immobile components (R_f 0.23 and 0.06) showed the high polarity of these components which may be the same component moved further (R_f 0.32 and 0.21) when a more polar solvent system (Butanol:ethanoic acid:water (4:1:5) was used.

The R_F value of 0.64 was obtained for all the Group B samples using the same solvent system (trichloromethane:propan-2-one:ethanol:ammonia solution 5:3:1:1). This is attributed to the presence of this component (R_F 0.64) in both leaves and seeds of this plant. It is also the component present in the crystals, $CCAPL_3$. Most probably the same component has R_F 0.41 in the solvent system:- trichloromethane:methanol:17% ammonia solution (82.5:15.5:2). Molyneux *et. al.*, (1980) reported that monocrotaline has R_F value of 0.63 and 0.39 respectively in these solvent systems. The samples were not run on the same chromatogram with known standard compounds of PAs (since these were not available) hence the components could not be identified by name.

The cleaned-up extract, $CCAPL_1$, produced less number of spots compared with the crude-mixture of PAs extract from the leaves, CAPL, when three different solvent systems were used. It was evident from this that the cleaned-up extract had lost some of the components during the process of cleaning which were originally present in CAPL.

Using two different solvent systems $CCAPL_3$ produced only one resolved spot while $CCAPL_1$ gave one resolved spot and an unmoved component (Table 18). Moreover the immobile components of $CCAPL_1$ were moved further to produce three resolved spots (Table 19), using a different solvent system. The presence of one component in $CCAPL_3$ in contrast to three components in $CCAPL_1$ obviously revealed that the cleaning-up method did not purify CAPL as the crystallization method. However both methods of purification retained the same component, which was present in the original crude extract, CAPL and whose R_F values are respectively 0.64, 0.41, and 0.47 in the three different solvent systems employed.

Table 18: R_F Values of the Group B Samples Using Two Different Solvent Systems

Solvent System: trichloromethane:propan-2-one: ethanol:ammonia solution (5:3:1:1) Distance moved by solvent front = 18.0cm				Solvent System: trichloromethane:17%-methanol: ammonia solution (82:5:15.5:2). Solvent front = 18.0cm.			
Sample	No. of Spots	Solute Front (cm)	R_F	Sample	No. of Spots	Solute Front	R_F
CAPL	i)	4.2	0.23	CAPL	i)	1.1	0.06
	ii)	11.5	0.64		ii)	7.3	0.41
	iii)	12.5	0.69		iii)	8.2	0.46
	*	0.0	0.00		*	0.0	0.00
CAPS	i)	4.3	0.24	CAPS	i)	1.0	0.06
	ii)	11.5	0.64		ii)	7.3	0.41
$CCAPL_1$	i)	11.5	0.64	$CCAPL_1$	i)	7.3	0.41
	*	0.0	0.00		*	0.0	0.00
$CCAPL_3$	i)	11.5	0.64	$CCAPL_3$	i)	7.3	0.41

Key * - Unmoved component

Table 19: R_F Values of CAPL and $CCAPL_1$ Using Solvent System: Butanol:ethanoic acid:water (4:1:5)

Distance moved by solvent Front = 17.5cm.

Sample	No. of Spots	Distance moved by Solute Front (cm)	R_F
CAPL	i)	3.6	0.21
	ii)	5.6	0.32
	iii)	7.0	0.40
	iv)	8.3	0.47
	v)	11.4	0.65
$CCAPL_1$	i)	3.6	0.21
	ii)	8.3	0.47
	iii)	11.4	0.65

4.2.2 Isolation of the Components of $CAPL_1$ and $CAPL_2$ Using Preparative TLC

The resolution of the components of a mixture, using preparative TLC is not as good as analytical TLC. However it has the following advantages over column chromatography

i) The separated zones are easily removed from the plate and eluted

ii) Less amount of solvent system is required and hence it is more economical.

iii) The smaller particle size (5 – 40μ) of the preparative thin-layers gives a sharper distinct separations (Touchstone, 1992)

The extracts $CAPL_1$ and $CAPL_2$ were chosen due to their biological activity and little literature on the leaves of this plant. In separating their components using benzene:ethylethanoate (3:1) as the solvent system eight bands were obtained for each extract, their R_f values are shown in Tables 20 and 21.

Table 20. R_f Values of the Bands from Preparative TLC of $CAPL_1$

No. of bands	Movement of Solvent Front = 18.0cm Time run = 45 minutes			
	Distance moved by solute (cm)	R_f	Colour in	
			Visible light (254nm)	Ultraviolet light (366nm)
1	0.5	0.03	Dark green	Orange brown
2	1.8	0.10	Faint green	Yellowish brown
3	5.5	0.31	Faint green	Claret maroon
4	7.6	0.42	Very faint green	Faint purple
5	14.0	0.78	Faint green	Light brown
6	15.2	0.84	Weak yellow	Very light brown
7	16.5	0.92	Faint yellow	Light orange
8	17.0	0.94	-	Light brown

Table 21 R_f Values of the Bands from Preparative TLC of CAPL$_2$

No. of bands	Movement of Solvent Front = 18.0cm Time run = 45 minutes			
	Distance moved by solute (cm)	R_f	Colour in	
			Visible light (254nm)	Ultraviolet light (366nm)
1	0.5	0.03	Dark brown	Orange brown
2	1.9	0.12	Green	Claret maroon
3	5.4	0.30	Light green	Orange
4	7.8	0.43	-	Faint purple
5	14.2	0.79	Faint yellowish green	Claret maroon
6	15.5	0.86	Faint yellowish green	Brown
7	16.7	0.93	Faint yellow	Light orange
8	17.2	0.96	-	Light brown

Further to the spotting of the fractions of CAPL$_1$ and CAPL$_2$ on the same plate (0.25mm thickness) and developing with the same solvent system (benzene:ethylethanoate 3:1), some of the fractions were observed to have moved the same distance from point of application and hence have the same R_f values, the results are depicted in Table 22 and the chromatograms are shown in Figures 11 and 12.

Table 22: R_f Values of the Fractions of CAPL$_1$ and CAPL$_2$

CAPL$_1$ Fractions	Solvent front = 18.0cm		CAPL$_2$ Fractions	Solvent front = 18.0cm	
	Distance moved by solute (cm)	R_F		Distance moved by solute (cm)	R_f
1 and 2	4.0	0.22	1 and 3	5.0	0.28
3 and 4	9.0	0.50	4	9.4	0.52
5,6,7 and 8	15.5	0.86	2 and 5 6,7 and 8	13.3 14.4	0.74 0.90

Fractions of the same extract with the same R_f values were combined, giving three fractions from $CAPL_1$ and four fractions from $CAPL_2$. The combined fractions were available in very small quantities hence could not be used for spectroscopic studies.

4.2.3 Characterization of Classes of Compounds Using Visualizing Reagents

4.2.3.1 Phenolic Compounds and Glycosides

Colour reaction tests of the fractions from $CAPL_1$ and $CAPL_2$ (obtained after preparative TLC) when sprayed with visualizing reagents gave the results depicted in Table 23.

The appearance of brown colours in the fractions of the two extracts when sprayed with Iron (III) tetraoxoxsulphate (VI) and potassium ferricyanide reagents showed the presence of phenolic compounds in the extracts. Also the violet colour which faded rapidly after spraying with 1,3-dinitrobenzene and methanolic sodium hydroxide was an evidence of the presence of steroid glycosides in these extracts Touchstone (1992).

Table 23: Results of the Colour Reaction Test for Phenolic Compounds and Steroid Glycosides

Sample	Fractions as combined from Prep. TLC	R_f	Colour with	
			Iron (III) tetraoxosulphate (VI) and Potassium *ferricyanide*	1,3-dinitrobenzene and methanolic sodium hydroxide
$CAPL_1$	1 and 2	0.22	Reddish brown	Brownish green
	3 and 4	0.50	Brown	Faint brownish green
	5,6,7 and 8	0.86	Brown	Weak purple
$CAPL_2$	1 and 3	0.28	Greenish brown	Green
	4	0.52	Faint greenish brown	Purple
	2 and 5	0.74	Faint brown	Faint green
	6,7 and 8	0.90	Faint brown	Faint purple

4.2.3.2 Flavonoid Compounds

The results of colour reaction exhibited by the fractions of $CAPL_1$ and $CAPL_2$ (as combined after preparative TLC) when sprayed with visualizing reagents are shown in Table 24.

The appearance of orange colour on exposure to ammonia vapour as well as green colour on spraying with aluminium chloride was an evidence of the presence of flavonone in fractions of $CAPL_1$. Also the appearance of weak purple when the fractions of $CAPL_1$ were exposed to ammonia vapour and yellow colour when sprayed with aluminium chloride showed the presence of isoflavonone in $CAPL_1$. The fractions of $CAPL_2$ exhibited colour reaction with the visualizing reagents which showed the presence of isoflavonone and chalcone, that is light dull red after exposure to ammonia vapour and orange when sprayed wth aluminium chloride (Haslam, 1966).

These results from use of visualizing agents are in agreement with those of phytochemical screening of these extracts. The presence of flavonoid and particularly isoflavones is common with the family Leguminosae (Trease and Evans, 1992).

Table 24: Results of Colour Reaction Test of Flavonoid Compounds with Visualizing Reagents

Sample	Fractions as combined from Prep. TLC	R_f	Colour with	
			Ammonia vapour	1% Ethanolic aluminium chloride
$CAPL_1$	1 and 2	0.22	Orange	Green
	3 and 4	0.50	Light brown	Yellow
	5,6,7 and 8	0.86	Faint purple	Faint yellow
$CAPL_2$	1 and 2	0.28	Orange brown	Orange
	4	0.52	Weak purple	Yellow
	2 and 5	0.74	Orange	Greenish orange
	6,7 and 8	0.90	Light brown	Faint yellow

4.2.4 Specific Detection of Pyrrolizidine Alkaloids (PAs) on Thin-Layer Chromatograms

Several reagents have been used for the detection of PAs on thin-layer chromatograms. These vary in sensitivity and specificity viz:

i) Exposure to iodine vapour: It is the least specific reagent, giving brown spots with many classes of compounds.

ii) Use of Dragendorff's Reagent: This is general for alkaloids, non-specific for PAs and its sensitivity is limited by the pale colour of its spots (Mattocks, 1967).

The method used in this work was that developed by Molyneux *et.al*., (1980). In this method use is made of the pyrrole specific Ehrlich's reagent, and oxidation of PAs to pyrrole was achieved rapidly by treatment with *o*-chloranil reagent.

On spraying thin-layer chromatograms containing spotted samples of CAPL, CAPS, $CCAPL_1$ and $CCAPL_3$ (Figures 9.0 and 10.0) with 1% solution of *o*-chloranil all the spots with the exception of the immobile components (*) developed to blue-colour which faded within one minute of heating the plates on a steam bath. The results are depicted on Table 25.

Subsequent spraying with Ehrlich's reagent and further heating on a steam bath gave pyrrole characteristic stable purple spots. On the other hand when the chromatograms containing spotted samples of CAPL, CAPS, $CCAPL_1$ and $CCAPL_3$ were sprayed with ethanoic anhydride followed with Ehrlich's reagent purple spots developed only in the immobile components (*), which was typical of unsaturated PA N-oxide (Mattocks, 1967, Molyneux *et.al*., 1980).

The N-oxides failed to react with *o*-chloranil and could not be converted to pyrroles. However, they were readily converted to pyrroles by spraying with ethanoic anhydride and subsequently detected by Ehrlich's reagent. The relative immobile components made it possible for the alkaloid and their N-oxides to be analysed by both R_F values and the different methods of detection on the thin-layer chromatograms.

Table 25: Results of Specific Detection of PAs on Thin-layer Chromatograms

Solvent system: trichloromethane:propan-2-one:ethanol:ammonia solution (5:3:2:1)			Colour with		Inference
Sample	No. of Spots	R_F	*o*-Chloranil and Ehrlich's reagent	Ethanoic anhydride and Ehrlich's reagent	
CAPL	i)	0.23	Blue→purple	No positive color change	PAs present
	ii)	0.64	Blue→purple	No positive color change	PAs present
	iii)	0.69	Blue→purple	No positive color change	PAs present
	*	immobile spot	No positive color change	Purple	PA N-oxide present
CAPS	i)	0.24	Blue→purple	No positive color change	PAs present
	ii)	0.64	Blue→purple	No positive color change	PAs present
$CCAPL_1$	i)	0.64	Blue→purple	No positive color change	PAs present
	*	immobile spot	No positive color change	Purple	PA N-oxide present
$CCAPL_3$	i)	0.64	Blue→purple	No positive color change	PAs present

4.3.0 Biological Activity

4.3.1 Brine Shrimp Lethality Bioassay

The results of the percentage death per 30 brine shrimp (Artemia Salina Leach) are shown in Tables (26 – 31). The median lethal concentration – LC_{50} (that is the

concentration of the extracts that kills 50% of the brine shrimp) values in $\mu g cm^{-1}$ of the active principles in the six extracts were determined by calculations at 95% confidence intervals using the values of probits and log dose for each extract. The results are depicted in Table 32. The equations used to calculate LC_{50} values are shown in Appendices 1A – 1F. The statistical tables employed include

i) Probit values shown in Appendix IIA

ii) Theoretical values of the correlation coefficient shown in Appendix IIB

iii) The t-values were obtained from Appendix IIC

The results suggest that $CAPL_1$, $CAPS_1$, $CAPL_2$ and $CAPL_3$ are moderately toxic at median concentration. The toxicity could be compared with those of caffeine and thymol reported by Meyer *et. al.*, (1982). It also shows the presence of toxic constituents such as saponins, tannins and alkaloids. The extracts CAPL, and CAPS exhibited high toxicity at low concentrations. Their LC_{50} values fall below the minimum concentration used for this bioassay. Cases of Crotalaria species poisoning in humans, livestock and experimental animals has been reported by Culvenor *et.al.*, (1962), Watt and Breyer-Brandwijk, (1962), McLeans (1970), Tandon *et.al.*, (1976), Lyford, *et.al.*, (1976). CAPL and CAPS are crude mixtures of PAs and it is evident from this bioassay that the most active principles responsible for the cytotoxicity of C. retusa are PAs (Table 32).

Table 26: Percentage Death Response Caused by Ethanol Leaves Extract - $CAPL_1$

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp per 30	Percentage response
1000	3.0000	24	80
500	2.6990	13	43
250	2.3970	9	30
125	2.0969	4	13
62.5	1.7959	0	0

Table 27: Percentage Death Response Shown by Ethanol Seed Extracts – $CAPS_1$

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp per 30	Percentage response
1000	3.0000	30	100
500	2.6990	17	57
250	2.3970	10	33
125	2.0969	6	20
62.5	1.7959	0	0

Table 28: Percentage Death response Caused by Trichloromethane leaves Extract $CAPI_2$

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp per 30	Percentage response
1000	3.0000	30	100
500	2.6990	13	43
250	2.3970	6	20
125	2.0969	4	13
62.5	1.7959	0	0

Table 29: Percentage death Response Inflicted by Ethylethanoate leaves extract $CAPI_3$

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp per 30	Percentage response
1000	3.0000	27	90
500	2.6990	12	40
250	2.3970	7	23
125	2.0969	4	13
62.5	1.7959	0	0

Table 30: Percentage Death Response Caused by Crude Mixture of PAs from Leaves Extract – CAPL

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp per 30	Percentage response
1000	3.0000	30	100
500	2.6990	28	93
250	2.3970	25	83
125	2.0969	22	73
62.5	1.7959	20	66

Table 31: Percentage Death Response Caused by Crude Mixture of PAs from Seeds Extract – CAPS

Dose $\mu g/cm^3$	Log dose	No. of dead shrimp Per 30	Percentage response
1000	3.0000	30	100
500	2.6990	29	96
250	2.3970	23	76
125	2.0969	22	73
62.5	1.7959	19	63

Table 32: LC_{50} Values in Brine Shrimps at 24 Hour after Dose of Extracts from C. retusa

Sample	Percentage death per 30					LC_{50} $\mu g/cm^3$	95% confidence interval
	Dose ($\mu g/ml$) 1000	500	250	125	62.5		
$CAPL_1$	80	43	30	13	0	483	473 – 493
$CAPS_1$	100	57	33	20	0	316	311 – 321
$CAPL_2$	100	43	20	13	0	347	341 – 353
$CAPL_3$	90	40	23	13	0	457	450 – 464
CAPL	100	93	83	73	66	59	50 – 68
CAPS	100	96	76	73	63	65	57 – 73

LC_{50} - median lethal concentration

472326

Samples

$CAPL_1$	-ethanol leaves extract
$CAPS_1$	-ethanol seeds extract
$CAPL_2$	-trichloromethane leaves extract
$CAPL_3$	-ethylethanoate leaves extract
CAPL	-crude mixture of PAs from leaves
CAPS	-crude mixture of PAs from seeds

4.3.2 Antimicrobial Activity Test

There were some inhibitory activities against some given test organisms as shown in Tables (33 and 34).

The leaves extracts, particularly the trichloromethane $CAPL_2$ exhibited significant antimicrobial activities against Bacillus subtilis (B. subtilis), Escherichia coli (E. coli) and Pseudomonas aeruginosa (P. aeruginosa). Generally, no activity was exhibited against staphylococcus aureus except ethylethanoate leaves extract – $CAPL_3$ and ethanol seed extract CAPS, which showed inhibition at higher concentrations.

The crude mixtures of PAs – CAPL and CAPS from leaves and seeds respectively were found to possess greater antimicrobial activity against Ps.aeruginosa and Candidas albicans (C. albicans). Their activity against Ps.aeruginosa was greatest compared with other test organisms – B. subtilis, E. coli and Staph. aureus. Similarly only these extracts exerted activity against C. albicans. All other extracts of leaves and seeds were devoid of any antimicrobial activity at the concentrations used in this test.

The possession of antimicrobial activity by the extracts of this plant tends to support the local use of the plant in treatment of colic and scabies (Sofowora, 1986). Although scabies is not a bacterial infection it is in most cases aggravated due to secondary infections by bacterial organisms in which the application of antibacterial agents are indicated. The selective inhibition of Ps. aeruginosa and E. coli showed that

$CAPL_2$ has components that are selectively inhibitory to gram negative organisms. The ethanolic extract $CAPL_1$ and $CAPS_1$ may contain plant phenolics (-tannins, glycosides, flavonoids) whose effectiveness was limited to B. subtilis. Phenolic compounds have long been known to be toxic to micro-organisms (Harborne, 1964, 1967). It is evident from this study that CAPL and CAPS contain components that were not present in the other extracts and which could only be isolated by the method of extraction of PAs.

Table 33: Antimicrobial Zones of Inhibition by Extracts from C. retusa L.

Extract	Concentration mg/ml	Zone of Inhibition in (mm)				
		Staph. aureus	B. subtilis	Ps. aeruginosa	E. coli	C. albicans
$CAPL_1$	10.00	-	-	-	-	-
	50.00	-	4.0	-	-	-
	110.00	-	6.0	-	-	-
$CAPS_1$	10.00	-	-	-	-	-
	50.00	-	3.0	-	-	-
	110.00	2.0	4.0	-	-	-
$CAPL_2$	10.00	-	-	-	-	-
	50.00	-	6.0	4.0	3.0	-
	110.00	-	8.0	7.0	6.0	-
$CAPL_3$	10.00	-	-	-	-	-
	50.00	2.0	-	-	-	-
	110.00	4.0	-	-	-	-
CAPL	10.00	-	-	-	-	10.0
	50.00	-	-	3.0	-	18.0
	110.00	-	-	8.0	-	23.0
CAPS	10.00	-	-	-	-	12.0
	50.00	-	-	4.0	-	20.0
	110.00	-	-	10.0	-	24.0
*Control DMSO	110.00	-	-	-	-	-

Table 34: Zones of Inhibition of P. aeruginosa and C. albicans by $CCAPL_1$ and $CCAPL_3$

Sample	Organism	Zones of Inhibition in (mm)			
		Concentration (mg/ml)			
		110 mg/ml	50 mg/ml	10 mg/ml	*Control
$CCAPL_1$	C. albicans	21.0	18.0	12.0	-
	P. aeruginosa	9.8	6.3	3.8	-
$CCAPL_3$	C. albicans	28.0	22.0	15.0	-
	P. aeruginosa	10.2	8.6	2.5	-

Key
* controls were run in all organisms using 10% DMSO
- no inhibition
$CCAPL_1$ – cleaned up CAPL
$CCAPL_3$ – crystals from CAPL

The pronounced antifungal activity of CAPL (plate 1), CAPS, $CCAPL_1$ and $CCAPL_3$ confirms the report of Kalaichelvan *et. al.* (1992), that an alkaloid monocrotoline in a spore germinated assay from seeds of C. paleda proved to be fungistatic. This alkaloid has been reported to be the major alkaloid present in C. retusa L. (Adams *et. al.*, 1939; Culvenor *et. al.*, 1957).

Generally the leaves and seeds of this plant contain most likely the same secondary metabolites which account for their similar antimicrobial activity profiles. The possession of antibacterial activity of the plant also tends to support an earlier report made by Bulhoes *et. al.*, (1977) that aerial parts of C. retusa L. contained antibiotic properties.

4.5.0 Spectroscopic Studies

4.5.1 Infrared Spectroscopy

The infrared spectra of CAPS, CAPL, $CAPL_2$ and $CCAPL_3$ (the active extracts) are shown in Figures 13-16.

The infrared spectrum of CAPL showed hydroxyl absorption at 3497.73cm^{-1} (broad) and carbonyl absorption at 1734.08cm^{-1} (medium). CAPS showed hydroxyl absorption at 3150-3400cm^{-1} (broad) and carbonyl absorption at 1733.36 cm^{-1} (medium). The hydroxyl absorption of CAPL $_2$ was shown at 3383.85 cm^{-1} (broad) and carbonyl absorption at 1740.47 cm^{-1} (medium). CCAPL $_1$ showed hydroxyl absorption at 3498.41cm^{-1} (medium) with shoulder at 3125.08cm^{-1} and carbonyl absorption at 1735.20 cm^{-1}.

The broad bands due to –OH in the extracts showed that each sample contains at least one –OH group. It was also evident that each sample has saturated ester carbonyl, (*ester stretch vibration for saturated cyclic d-lactone and large rings is between 1750-1735*cm^{-1}). The absorption of CAPS at 1601.62cm^{-1} compare very well to the absorption of retusamine at 1603cm^{-1} in nujol reported by Culvenor *et. al.*, (1967).

4.5.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectra of PAs are rather complex, most of the signals due to pyrrolizidine nucleus being obscured by signals derived from the esterifying necic acids and demonstrating quite considerable shifts with variation in the solvent (Culvenor *et. al.*, 1965). The only signal of the basic ring which can be easily identified is the vinyl H-2 proton which is seen as broadened singlet (actually multiplet) centered at δ 6.2 in the macrocyclic diester when the spectra was run in $CDCL_3$. This signal is the only one which is a structural feature due to all toxic PAs. It was used by Molyneux *et. al.*, (1979) for estimation of total alkaloid.

The proton nuclear magnetic resonance spectra of CAPL and CCAPL $_3$ are shown in Figures 17.0 and 18.0, and their signals are depicted in Table 35.0. The signals are too

weak in intensity and very poor resolution to allow detailed interpretation. CAPL had a signal at δ 6.0 (spectra was run in DMSO-D_6). This signal compare very well with the signal of vinyl H-2 proton, which is a characteristic feature of PAs (Molyneux *et. al.*, 1979). The δ 6.0 signal was absent in the spectrum of $CCAPL_3$ probably due to small amount of crystals available for the NMR. Most signals of CAPL are in agreement with those given by Culvenor *et. al.*, (1962) for monocrotaline. This alkaloid is almost likely the major constituent of PAs in C. retusa. The low intensity of most signals is likely due to low concentration available for the spectroscopic measurement.

Table 35: NMR Signals Shown by CAPL and $CCAPL_3$.

Sample	δ-value	Intensity	Remarks
CAPL	1.0 - 1.5	Weak	Multiplet
	1.8 - 2.1	Very weak	Singlet (broad)
	2.4 - 2.7	Very weak	Singlet (broad)
	3.3 - 3.9	Weak	Singlet
	4.5 - 4.7	Weak	Singlet
	6.0	Very weak	Singlet
$CCAPL_3$	1.0 - 1.6	Weak	Multiplet
	2.5 - 2.7	Weak	Singlet
	3.2 - 6.5	Medium	Very broad

4.5.3 Ultraviolet-Visible Spectroscopy

The experimental results of ultraviolet-visible spectroscopy are usually reported in terms of molar extinction coefficient, ε or its logarithm, log ε. A plot of logε versus wavelengths of absorption of the active extracts, CAPS, CAPL, $CCAPL_3$ and $CAPL_2$ in the various solvents employed for this UV study are shown in Figures 19-22.

The UV absorption spectra of these extracts displayed different wavelengths of maximum absorption when different solvents were used as depicted in Tables 36 and 37. The polar solvents methanol and ethanol showed very close absorption maxima in all the extracts. Also each extract exhibited a single absorption peak, in addition CAPL in methanol showed a shoulder (λ_{max} 328nm, log ε 32.70).

The UV spectra of CAPS, CAPL, $CCAPL_3$ and $CAPL_2$ (Figures 19-22) showed a low intensity wavelength bands in the range 228-272nm (log ε 52.69-17.35) which may be attributed to $n \rightarrow \pi^*$ transitions due to the presence of carbonyl groups. Absorption of carbonyl groups in the region 275-295nm is caused by $n \rightarrow \pi^*$ transitions. However polar solvents generally shift $n \rightarrow \pi^*$ transition band to shorter wavelength (Dyer, 1980). Also α,β-unsaturated esters generally show a high intensity absorption peak in the region 205-225nm (log ε 10.0-20.0). Since the absorptions are near 200nm the spectra will frequently show no maximum and only intense end absorption will be observed. This is the case with the end absorption maxima of all the extracts in the various solvents used. None has absorption maximum in the region 205-225nm but displayed absorption maxima very close to 225nm such as 228nm, 232nm and 236nm.

The series of broad bands of low intensity shown by $CAPL_2$ in all the solvents employed absorbed in the region between 230 and 270nm may be due to the presence of conjugated or aromatic systems, which are associated with vibrational effects of the $\pi \rightarrow \pi^*$ transitions. The spectra of simple heterocyclic aromatic compounds resemble spectra of benzenoid compounds in that they consist of series of bands of lower intensity at longer wavelengths (λ_{max} 240-300nm) (Dyer, 1980). $CAPL_2$ displayed several low to medium intensity broad absorption peaks with bands extending well into the visible

region. This extract showed intense absorption in the region 400-450nm in all the solvents employed. This may be attributed to the presence of coloured compounds.

The results in Table 37, suggest that the absorption maxima of CAPL $_2$ are solvent dependent. Absorption maxima in dichloromethane and dioxane are however the same inspite of their different polarities. The absorptions in the less polar solvents such as dichloromethane, trichloromethane, tetrahydrofuran (THF), ethoxyethane and dioxane, were generally shifted to shorter wavelengths when compared with absorption displayed by polar solvents, methanol and ethanol.

Table 36 Ultraviolet-visible absorption spectra data of some extracts from Crotalaria retusa L.

Extract	Solvent Used			
	Methanol		Ethanol	
	λ_{max} (nm)	Log ε	λ_{max} (nm)	Log ε
CAPS	272.00	17.35	272.00	13.89
CAPL	236.00	38.31	232.00	38.82
	328.00(shoulder)	32.70	-	-
CCAPL$_{-3}$	228.00	52.69	232.00	51.91
CAPL$_{-2}$	232.00	44.14	280.00	49.40

Table 37 Ultraviolet-visible absorption spectra data of CAPL$_{-2}$ in different solvents

Solvent	λ_{max} (nm)	Log ε
Methanol	232.00	44.14
Ethanol	280.00	49.40
Dichloromethane	228.00	48.10
Trichloromethane	346.80	38.80
Tetrahydrofuran	336.00	37.72
Ethoxyethane	272.00	59.97
Dioxane	228.00	44.44

4.6.0 CONCLUSION AND RECOMMENDATIONS

4.6.1 CONCLUSION

The phytochemical investigation of C. retusa L. revealed that the seeds and leaves contain similar classes of natural products – alkaloids, tannins, cardiac glycosides and saponins. Specific identification tests of unsaturated PAs showed that this group of alkaloids, are not restricted to Australian Crotalaria retusa. It also showed that the roots contain mainly the PA-N-oxide and the flowers-assay was particularly rich in the alkaloids, while the seeds however has higher alkaloidal content than the leaves. The detection of classes of compounds on thin-layer chromatograms gave results which are consistent with those of the phytochemical screening test of this plant.

LC_{50} values showed that all the extracts were toxic, however differences were found in intensity of toxicity. The low LC_{50} values of crude mixtures of PAs is a proven evidence that the major toxic principle of this plant is due to the presence of PAs.

The microbiological aspect of this study also revealed that the crude mixture of PAs have significant antibacterial and antifungal activities with larger zones of inhibition being recorded for crystals and cleaned-up samples of PAs. This is in support of the low LC_{50} values of samples of crude mixture of PAs from leaves and seeds. It also tends to confirm the local application of the plant as a remedy for skin diseases.

With the use of *infrared, proton nuclear magnetic resonance and ultraviolet-visible* spectroscopic techniques the presence of carbonyl, hydroxyl groups and aromatic systems were confirmed in the active extracts of this plant. The phenolic glycosides, tannins and PAs are known to contain these groups.

4.6.2 RECOMMENDATIONS

Although some works on the toxicity of Crotalaria retusa L. have been published, there are still more areas of investigation; these include -

(i) More work should be carried out on the phytochemistry of the roots and flowers using well established analytical method to determine the quantity of PAs and PA-N-oxide present. In line with this a comparative study on toxicity of PAs and its N-oxides could be studied to ascertain which of them is a major active principle in C. retusa.

(ii) Isolation in a larger quantity of other bioactive components such as flavonoids and saponins which are present in this plant. This will motivate detailed phytochemical investigation of these classes of compounds in the plant. Moreso it will enable the toxicity of saponins of C. retusa L. to be compared with those of PAs.

(iii) Testing of the active principles in CAPL and CAPS on scabies condition and other skin infections in animals, as well as being tried on other species of fungi. This is to ascertain its local use as a remedy for skin infection.

Its is hereby suggested that further investigation should be carried out on $CAPL_2$. The bioactive agents of $CAPL_2$ could be isolated and characterized. This will enable structure-related activity studies to be carried out, leading to synthesis of a more potent drug with reduced toxicity. Also further antimicrobial activity tests could be carried out using other types of bacteria as well as the establishment of a minimum inhibition concentration (MIC) for each organism.

It is also necessary to complete the characterization of the crystals as well as the crude mixture of the active extracts using high resolution GC-MS, this will also identify and quantify the amount of each component available. In addition the use of high resolution ^{1}H NMR and ^{13}C NMR are necessary for the elucidation of the structures of the compounds present in the various components.

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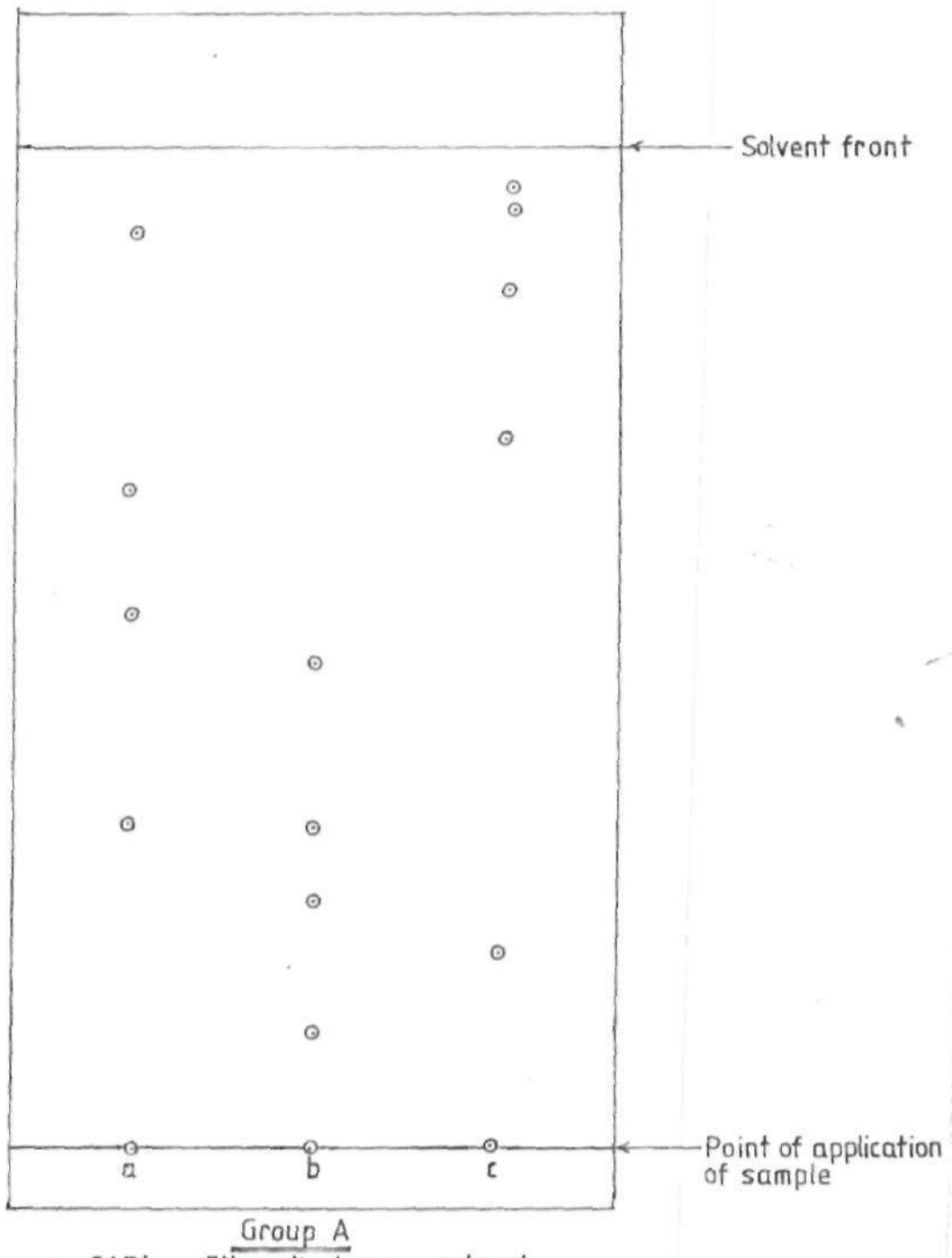
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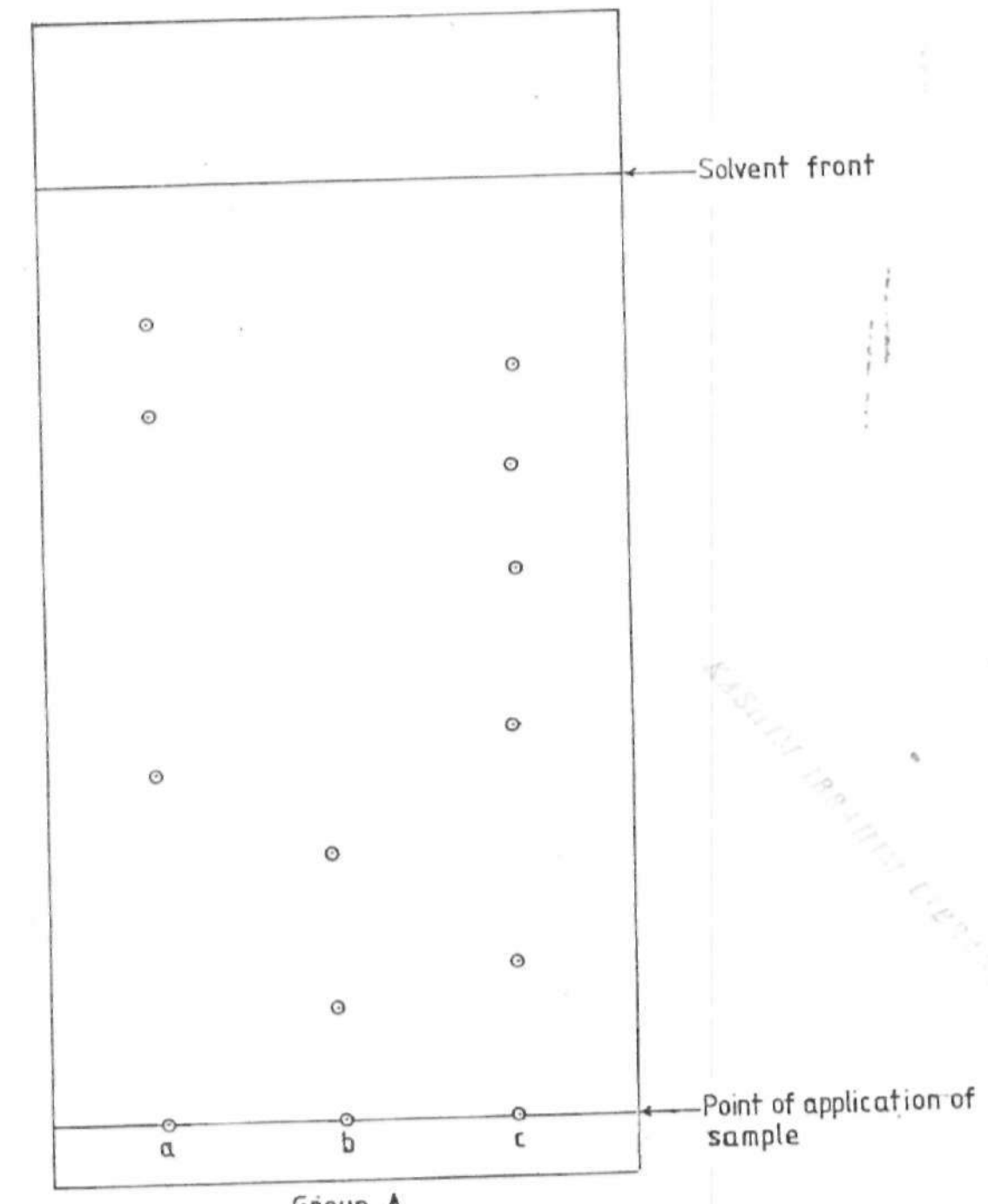


Group A

Key :— a—CAPL$_1$ —Ethanolic leaves extract
b—CAPS$_1$— Ethanolic seeds extract
c—CAPL$_2$—Trichloromethane leaves extract

Scale:— 1cm on paper = 1cm on Chromatogram

Figure 7. Thin-Layer Chromatogram of Group A extracts of C. retusa using Benzene;Ethylethanoate (3:1)

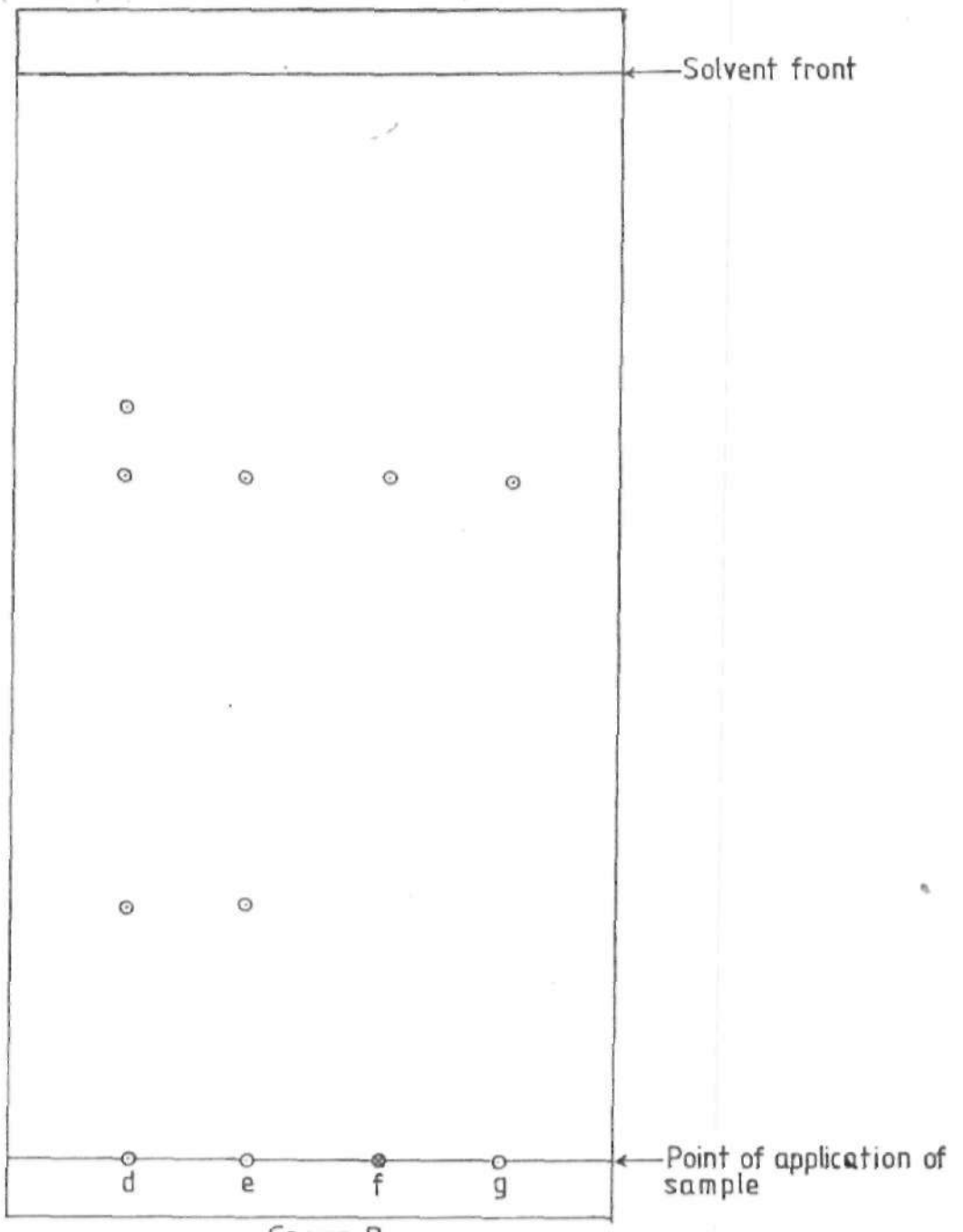


Group A

Key:— a—CAPL$_1$ —Ethanolic leaves extract
b—CAPS$_1$ —Ethanolic seeds extract
c – CAPL$_2$ —Trichloromethane leaves extract

Scale :—1 cm on paper = 1 cm on the Chromatogram

Figure 8. Thin—Layer Chromatogram of Group A extracts of C. retusa using Petroleum ether (60 – 80°C), ethylethanoate (3:1)

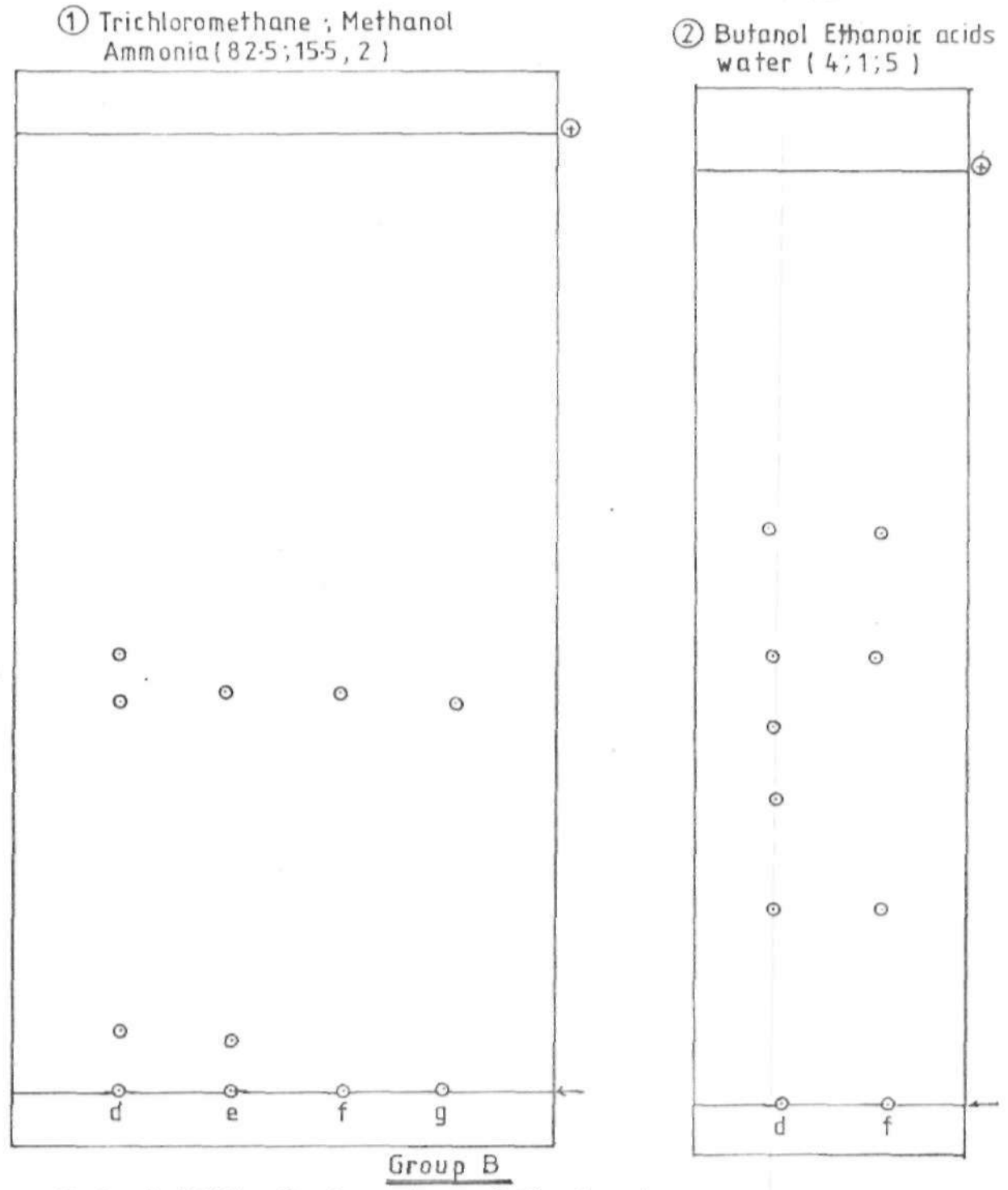


Group B

Key:— d – CAPL – Crude mixture of PAs from leaves
e – CAPS — Crude mixture of PAs from seeds
f – CCAPL1 – Cleaned-up CAPL
g – CCAPL – Crystals from CAPL
× – Unmoved compenents

Scale:— 1cm on paper = 1cm on the Chromatogram

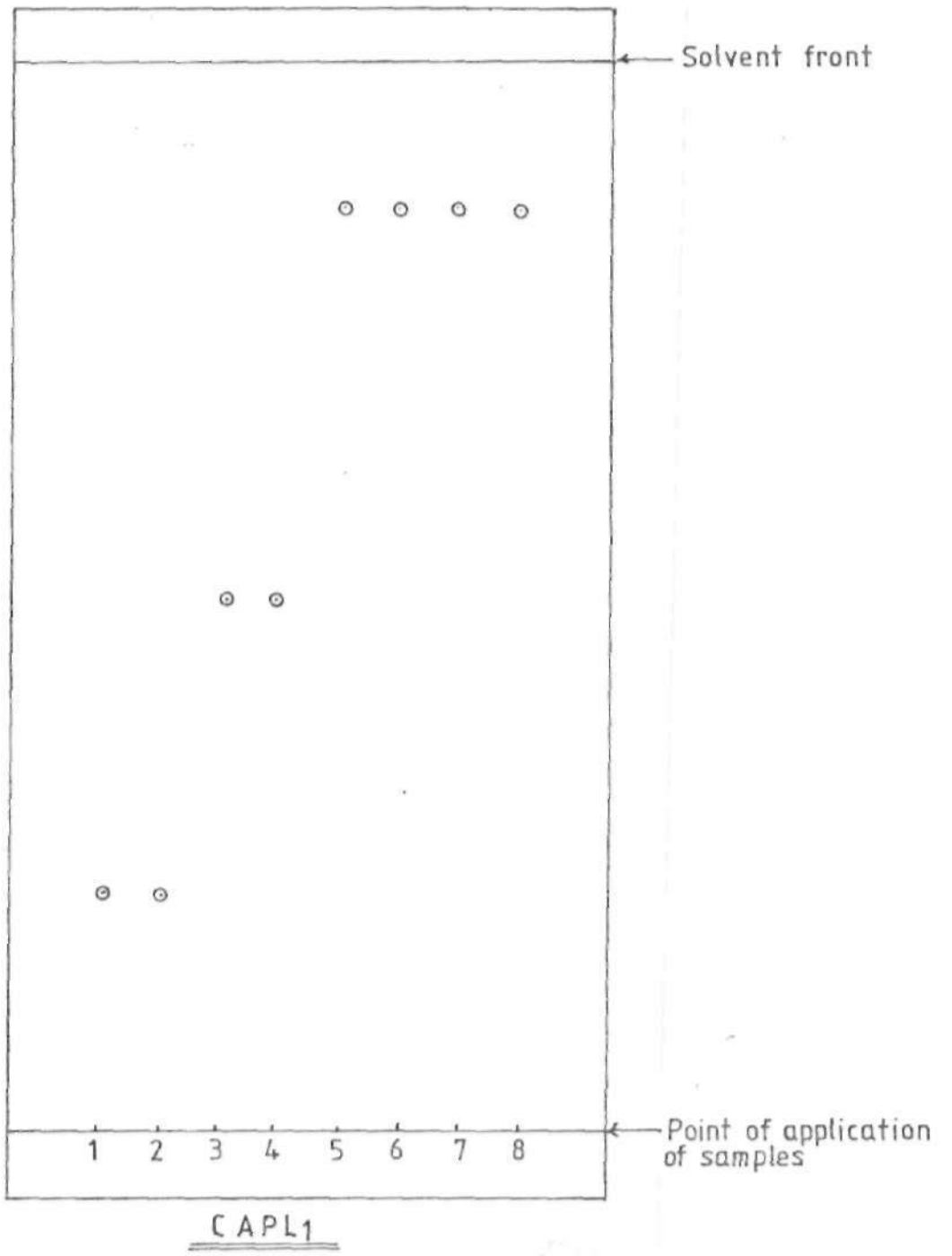
Figure 9. Thin – Layer Chromatogram of Group B extracts from C. retuse L. – using Trichloromethane, Propan-2- one, Ethanol, Ammonia solution (5:3:1:1)



Group B

Key:— d—CAPL — Crude mixture of PAs from leaves
e- CAPS — Crude mixture of PAs from seeds
f - $CCAPL_1$ - Cleaned-up CAPL
g - $CCAPL_3$ - Crystals from CAPL
⊗ - Unmoved components
⊕ - Solvent front
① and ② - Solvent systems
→ — Point of application of the samples
Scale:— 1cm on paper = 1cm on Chromatogram

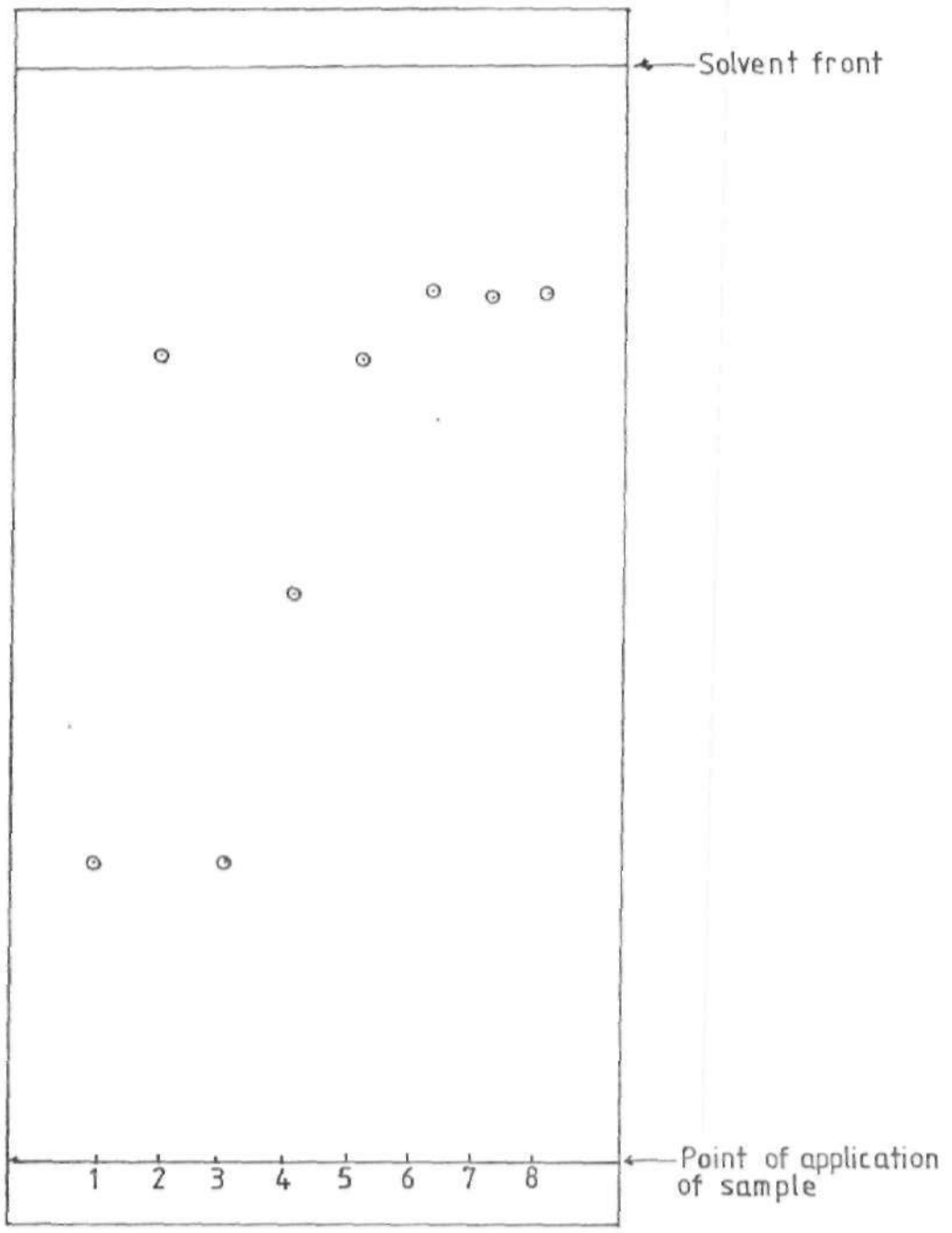
Figure 10 - Thin-Layer Chromatogram of Group B extracts using two different solvent systems



Key:— Nos 1-8 corresponds to numbers of bands (separated compenents)

Scale:— 1cm on paper = 1cm on Chromatogram

Figure 11·Thin-Layer Chromatogram of fractions from Ethanolic leaves extract-using Benzene, Ethylethanoate (3:1)



CAPL2

Key:— Nos 1-8 correspond to fractions obtained after preparative TLC

Scale:— 1cm on paper = 1cm on Chromatogram

Figure 12. Thin - Layer Chromatogram of fractions from Trichloromethane leaves extract - using Benzene : Ethylethanoate (3:1)

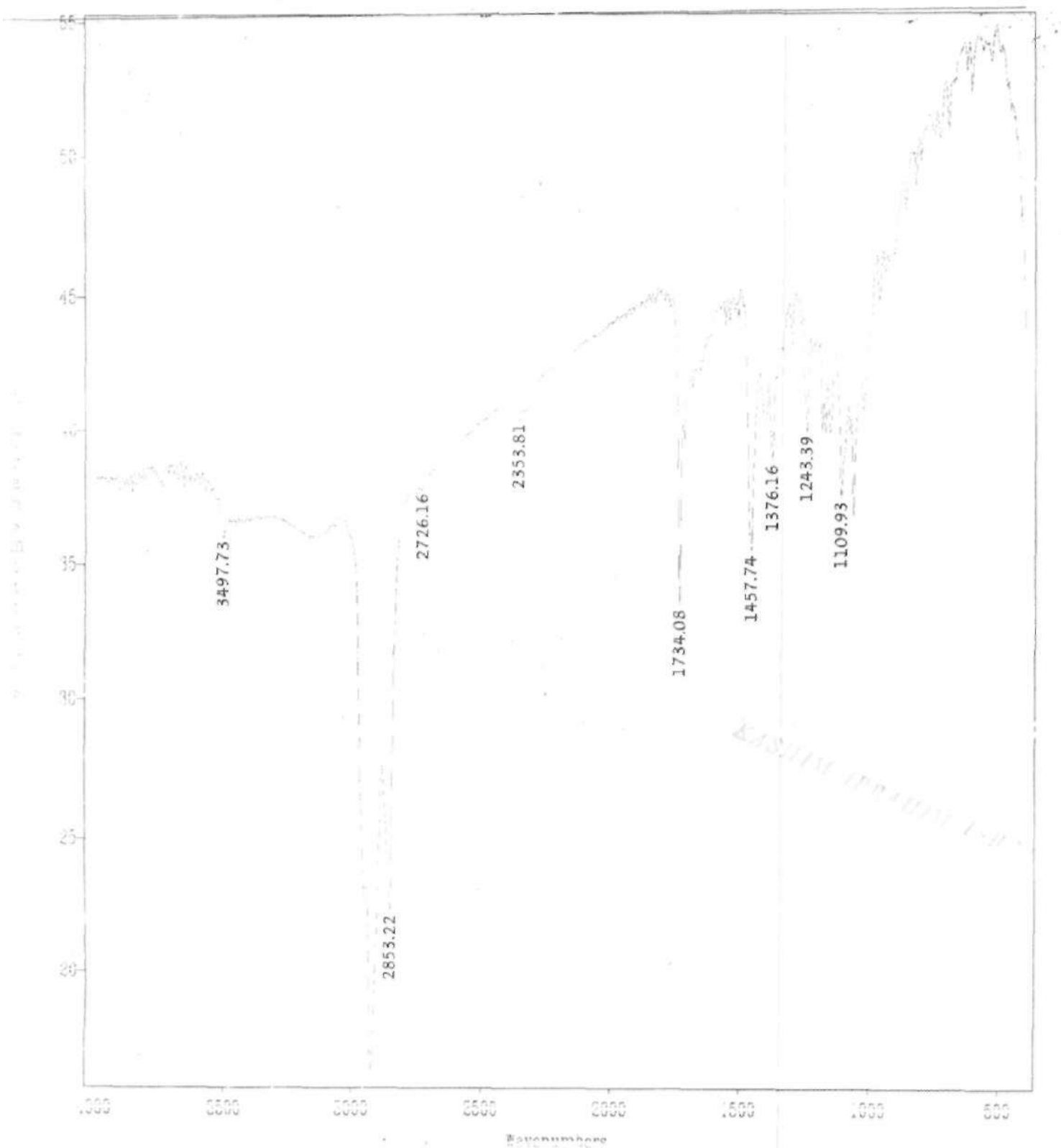


Figure 13: Infrared Spectrum of Crude Mixture of PAs from Leaves Extract in Nujol.

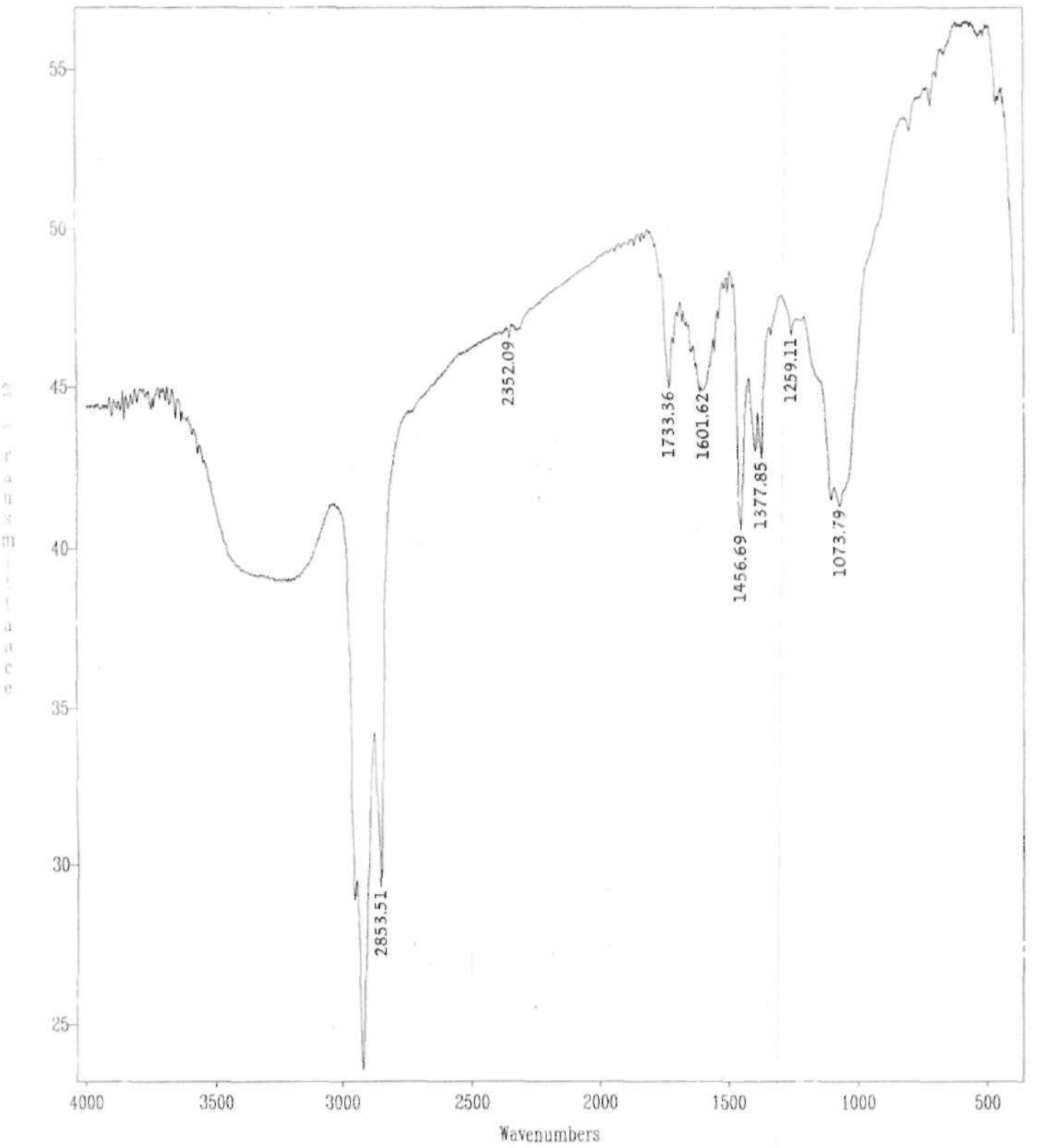


Figure 14: Infrared Spectrum of Crude Mixture of PAs from Seeds Extract in Nujol.

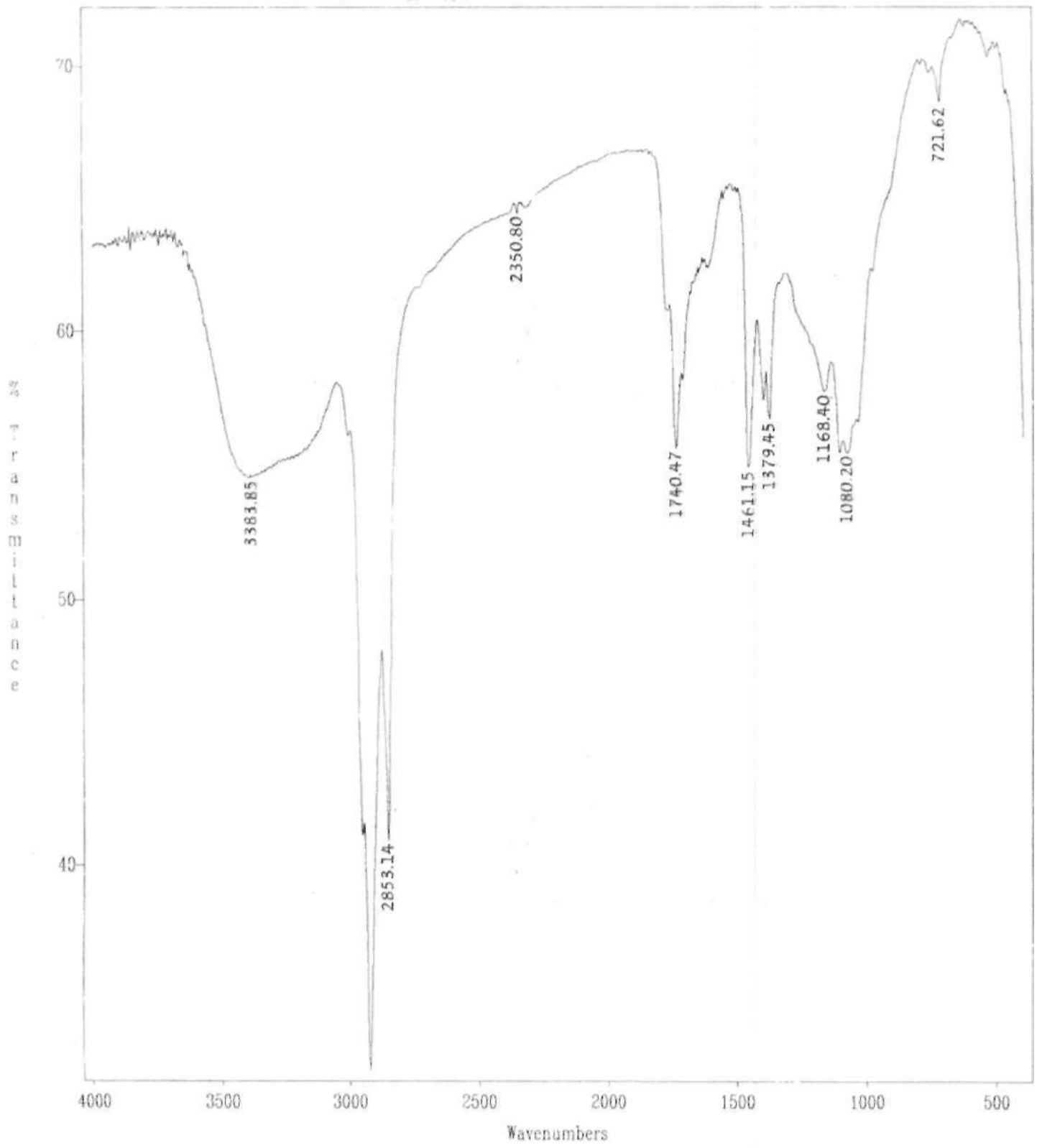


Figure 15: Infrared Spectrum of Trichloromethane Leaves Extract in Nujol

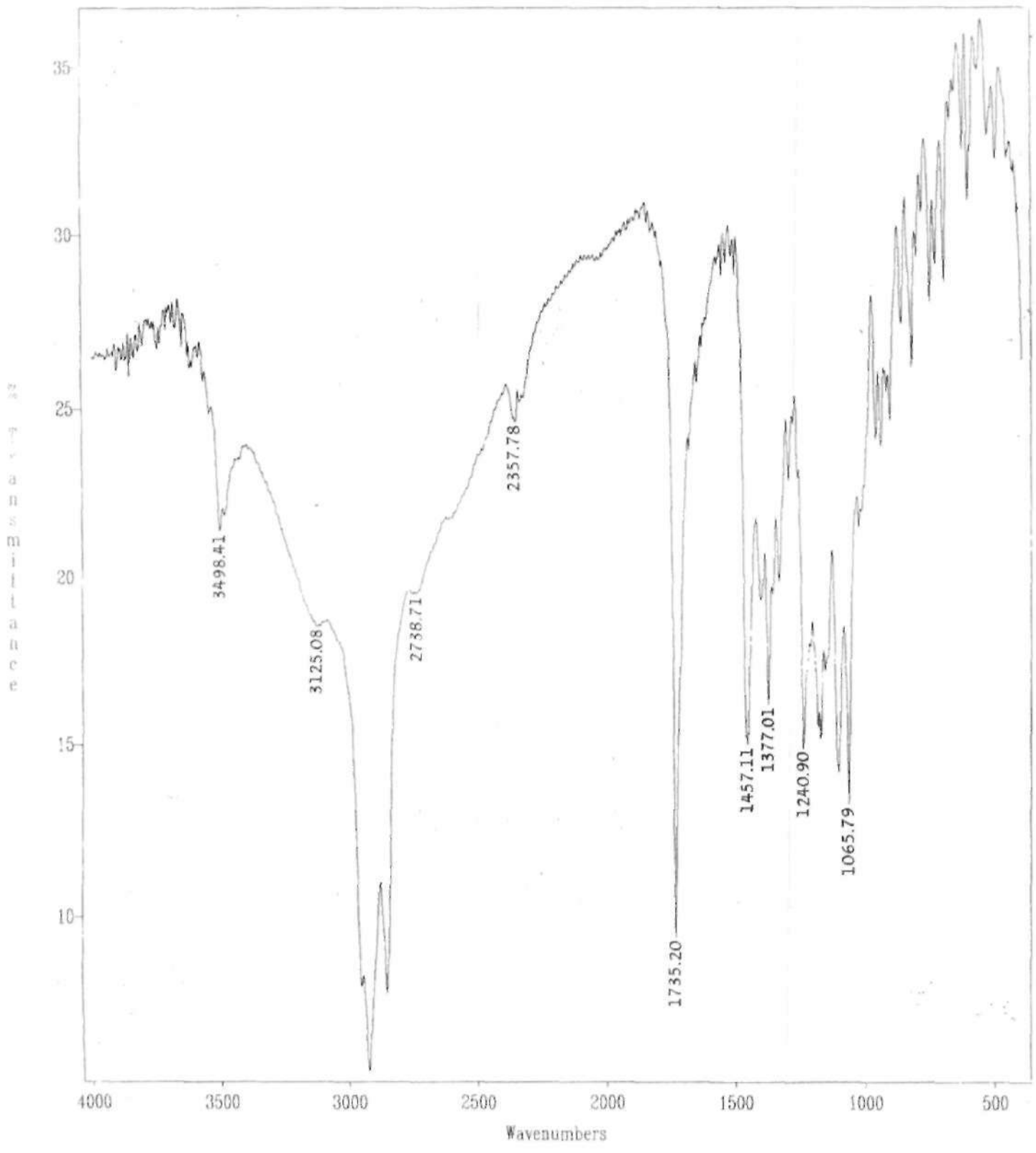


Figure 16: Infrared Spectrum of Crystals from Crude Mixture of PAs Leaves Extract in Nujol

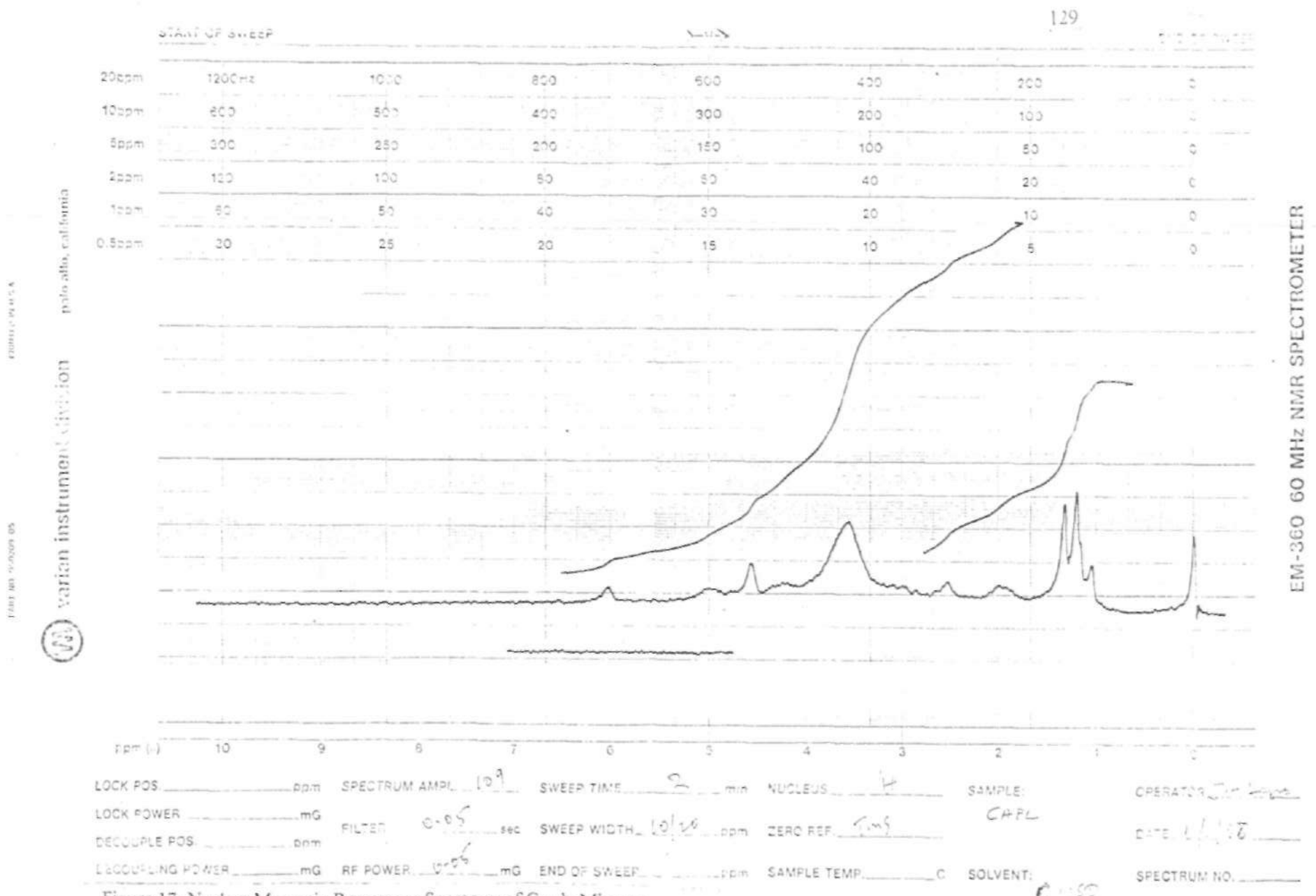


Figure 17: Nuclear Magnetic Resonance Spectrum of Crude Mixture of PAs from Leaves Extract

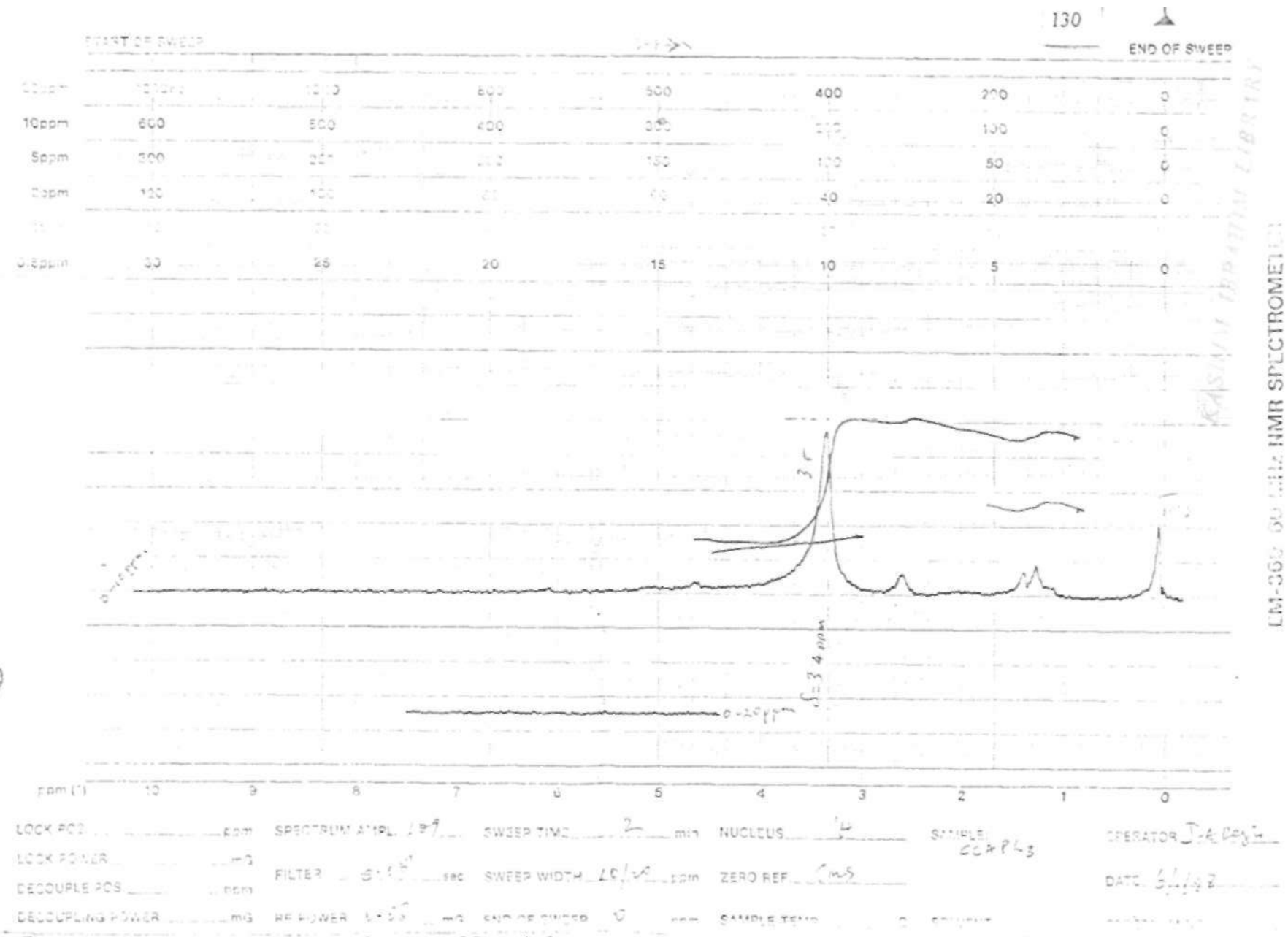


Figure 18: Nuclear Magnetic Resonance Spectrum of Crystals from Crude Mixture of PAs Leaves Extract

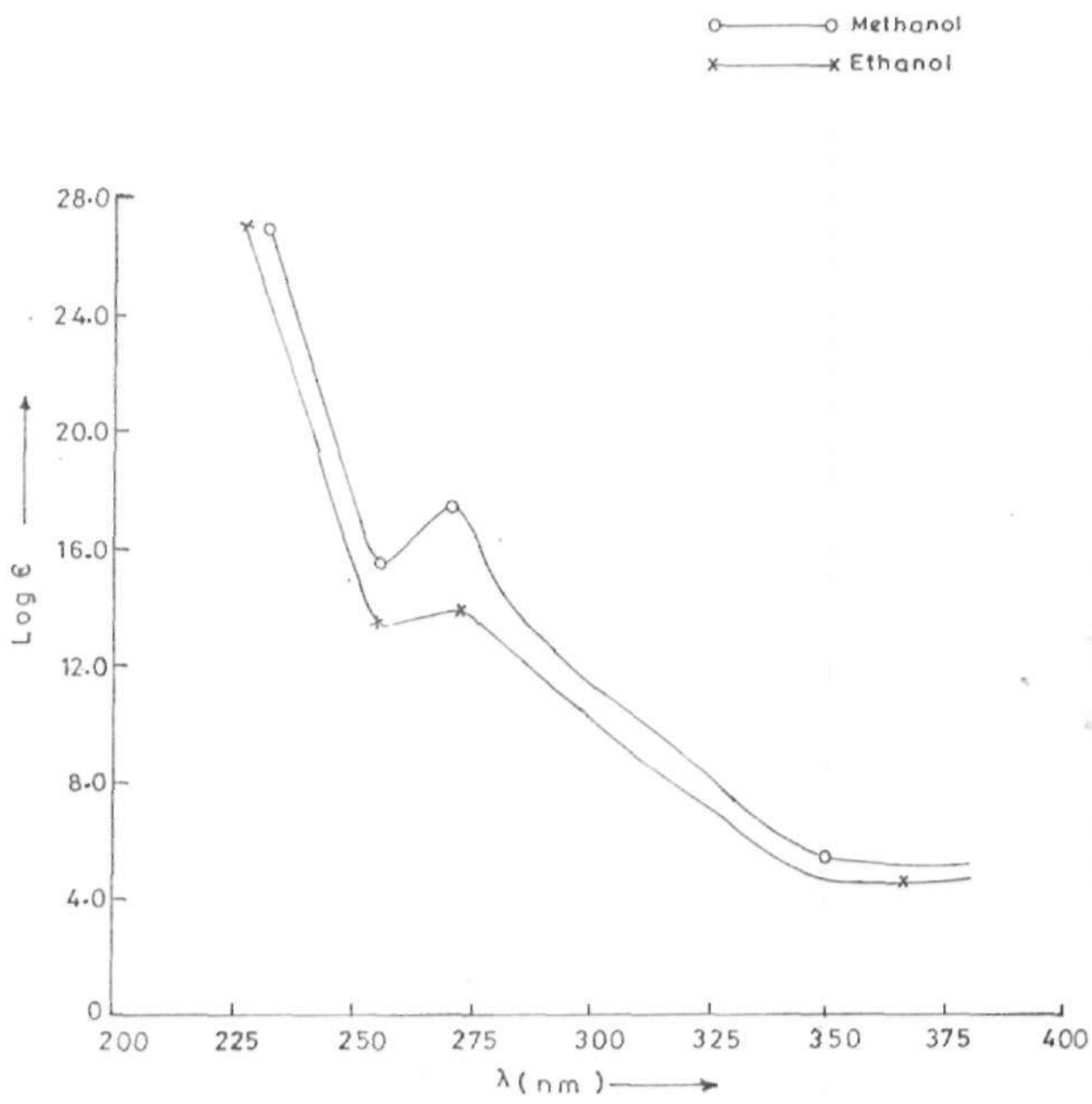


Figure 19· Ultraviolet spectrum of Crude mixture of PAs from seeds in Methanol and Ethanol

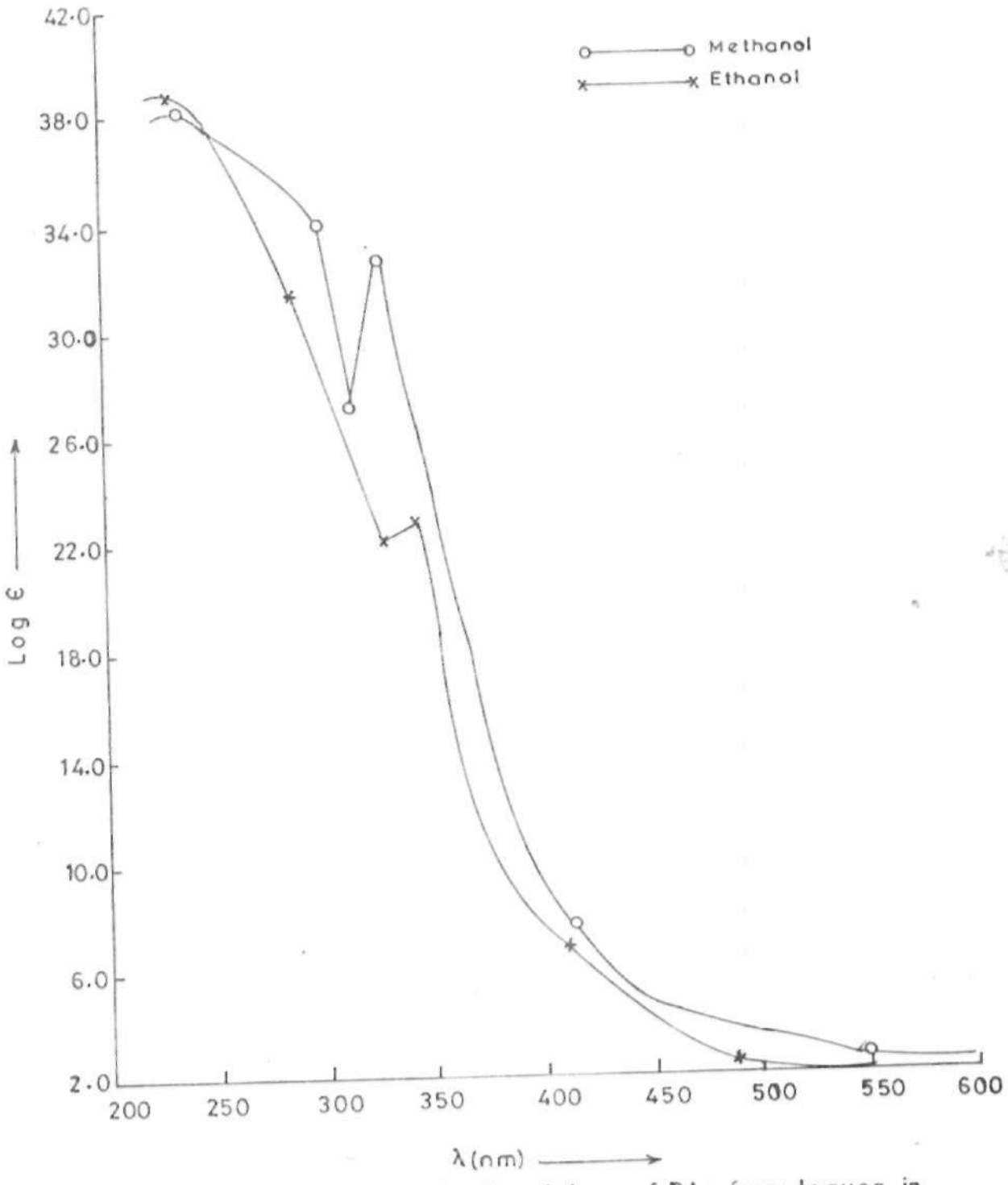


Figure 20. Ultraviolet spectrum of Crude mixture of PAs from leaves in Methanol and Ethanol

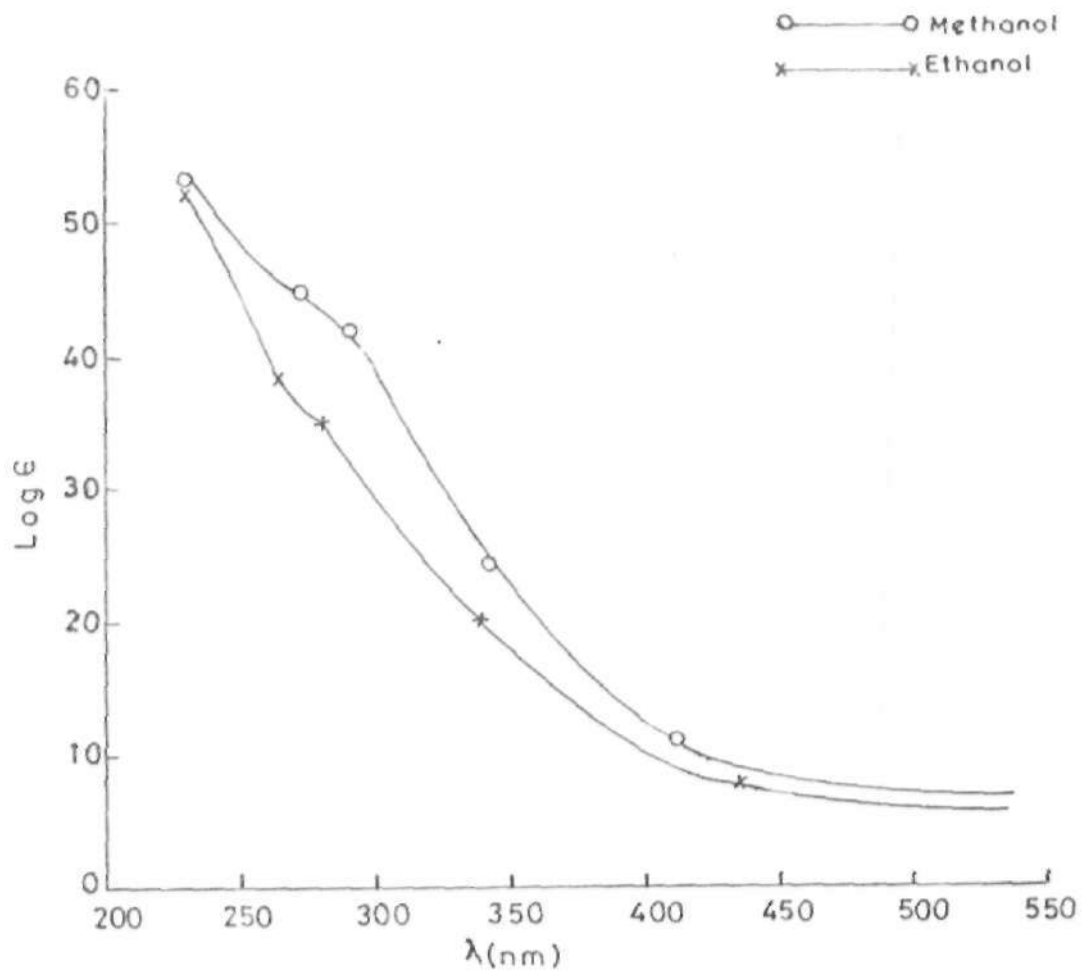


Figure 21. Ultraviolet spectrum of Crystals of PAs (from Crude mixture of PAs leaves extract) in Methanol and Ethanol

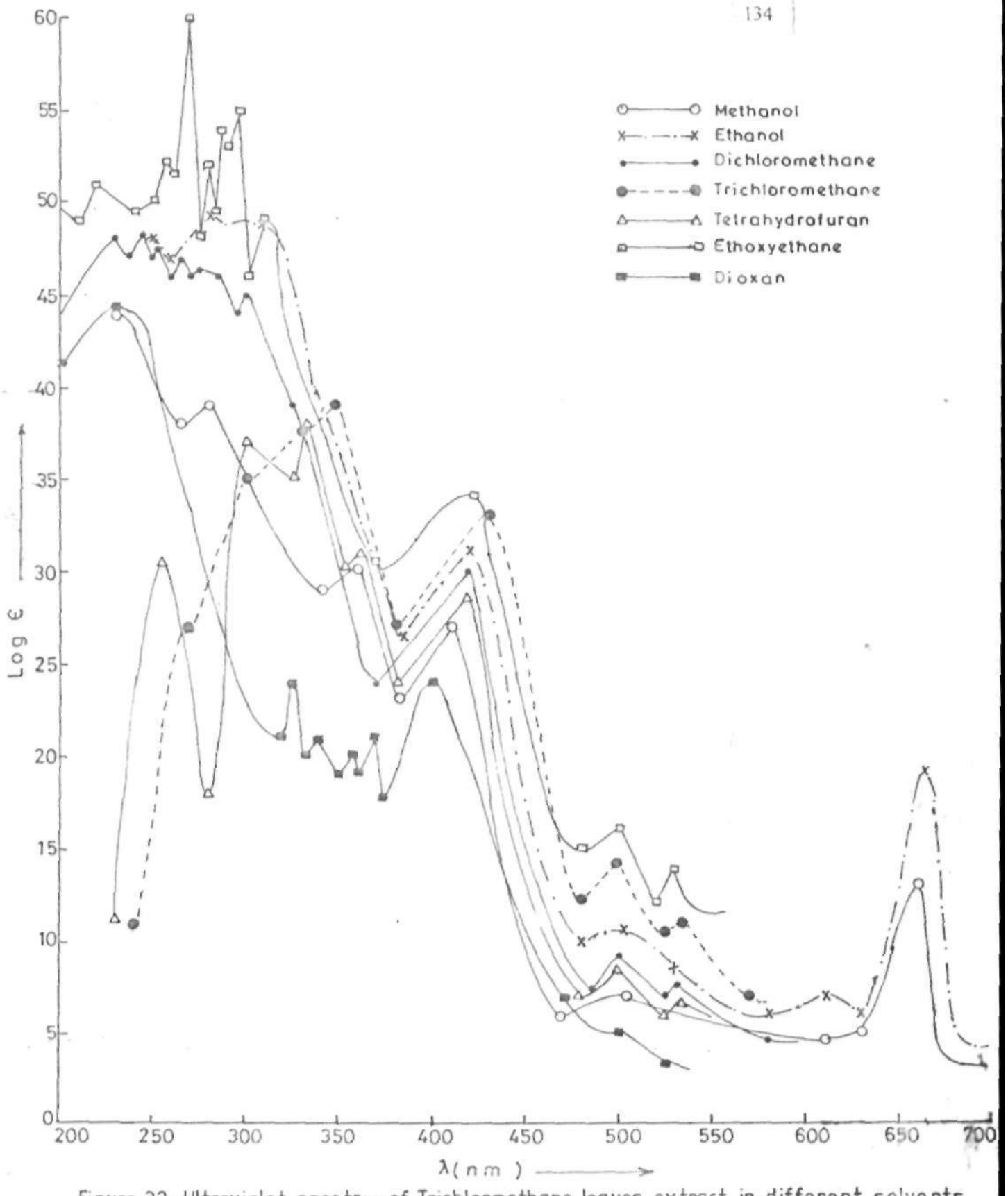


Figure 22. Ultraviolet spectrum of Trichlormethane leaves extract in different solvents

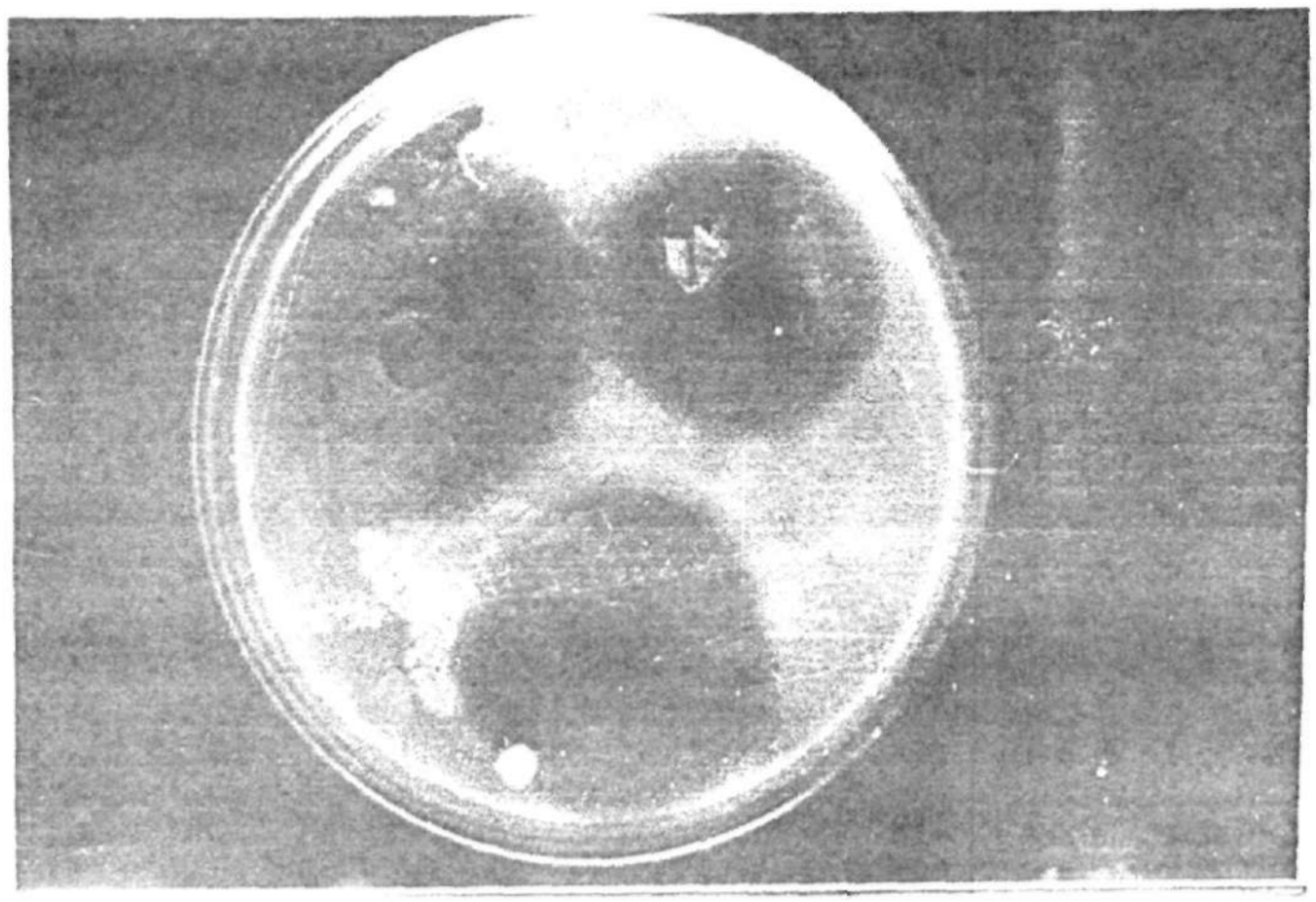


Plate 1 – Zones of Inhibition on Candidas albicans based on the Concentration

Of Crude Mixture of PAs from Leaves Extract

Appendix I

Calculation of LC_{50} values of the extracts of C. retusa L.

These were carried out at 95% confidence intervals using the method of linear regression analysis (Saunders and Fleming, 1971) based on the values of log.dose (X) and probits (Y) which were obtained from Table 26-31 and appendix IIA respectively.

Appendix 1A

Calculation of LC_{50} values of ethanol leaves extract $CAPL_1$

In order to calculate the regression line equation the following summations are made

X	X^2	Y	Y^2	XY
3.00	9.00	5.84	34.11	17.52
2.70	7.29	4.82	23.23	13.01
2.40	5.76	4.48	20.07	10.75
2.10	4.41	3.87	14.98	8.13
1.80	3.24	0.00	0.00	0.00

$$\sum(X)=12..\sum(X^2)=29.7..\sum(Y)=19.01..\sum Y^2=92.39..\sum(XY)=49.41$$
$$number.of.\deg rees.of.freedom,...N=5,...\bar{X}=2.4,...\bar{Y}=3.80$$

(i) Correlation Coefficient, 'r': Is first calculated to test whether there is significant linear correlation between X and Y values. 'r' is defined by the equation

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

$$\sum(X)\sum(Y)/N = 12 \times \frac{19.01}{5} = 45.62$$

$$\sum(X^2) - \sum {(X)^2}/{N} = 29.7 - \frac{12^2}{5} = 0.9$$

$$\sum(Y^2) - \sum \frac{(Y)^2}{N} = 92.39 - \frac{19.01^2}{5} = 20.11$$

$$r = \frac{49.41 - 45.62}{\sqrt{0.9 \times 20.11}} \approx \frac{3.79}{4.25}$$

$r = 0.892$

This value of 'r' calculated is greater than the theoretical value of 'r', for P' = 0.05 and degrees of freedom $\phi = 3$; which is 0.88. (see appendix IIB). There is therefore significant correlation between X and Y and so it is worth proceeding with regression analysis.

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

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

$$b = \frac{49.41 - 45.62}{0.9}$$

$b = 4.21$

(iii) Regression line equation:- is given by

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

Using Y=5 (since 5 is probit corresponding to 50% death response)

Therefore 5-3.80 = 4.21(X-2.4)

$1.2 = 4.21(X\text{-}10.10) \Rightarrow X = 2.68$

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

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

$$\sum dy^2 = \sum (y^2) - \sum \frac{(y)^2}{N} = 92.39 - \frac{19.01^2}{5} = 20.11$$

$$\sum dx^2 = \sum (x^2) - \sum \frac{(x)^2}{N} = 0.9$$

$$V_y = \frac{1}{3}(20.11 - 4.21^2 \times 0.9) = \frac{1}{3}(20.1 - 15.95) = 1.39$$

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

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

$$\sum (X - \bar{X})^2 = \sum (X^2) - \sum (X)^2 / N = 0.9$$

$$V_x = \frac{1.39}{(4.21)^2}[1 + \frac{1}{5} + \frac{(5 - 3.80)^2}{(4.21)^2} \bullet \frac{1}{0.9}]$$

$$V_x = 0.078(1.2 + \frac{1.44}{15.95}) = 0.1006$$

$$\therefore \sqrt{V_x} = 0.317$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and $P = 0.95$; these limits are $\pm 3.18 \times 0.317 = \pm 1.009$. The value of X calculated from the regression line equation for Y = 5 is 2.68.

Therefore the concentration of the solution of $CAPL_1$ which will cause 50% death is given by

$X = 2.68 \pm 1.0$

Antilog of $X = 483.15 \pm 10$

Therefore LC_{50} ($CAPL_1$) = 483µg/ml

95% confidence interval = 473 - 493µg/ml

Appendix IB

Calculation of LC_{50} values of ethanol seeds extract $CAPS_1$

In order to calculate the regression line equation the following summations are made

X	X^2	Y	Y^2	XY
3.00	9.00	8.09	65.45	24.27
2.70	7.29	5.18	26.83	13.99
2.40	5.76	4.56	20.79	10.94
2.10	4.41	4.16	17.31	8.74
1.80	3.24	0.00	0.00	0.00

$$\sum(X) = 12..\sum(X^2) = 29.7..\sum(Y) = 21.99..\sum Y^2 = 130.38..\sum(XY) = 57.94$$
$$number.of.\deg rees.of.freedom,..N = 5,...\overline{X} = 2.40,..\overline{Y} = 4.40$$

(i) Correlation Coefficient, 'r': Is first calculated to test whether there is significant linear correlation between X and Y values. 'r' is defined by the equation

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

$$\sum(X)\sum(Y)/N = 12 \times \frac{21.99}{5} = 52.78$$

$$\sum(X^2) - \sum {(X)^2}/{N} = 29.7 - \frac{12^2}{5} = 0.90$$

$$\sum(Y^2) - \sum\frac{(Y)^2}{N} = 130.38 - \frac{21.99^2}{5} = 33.67$$

$$r = \frac{57.94 - 52.78}{\sqrt{0.9 \times 33.67}} = \frac{5.16}{5.50}$$

$$r = 0.938$$

This value of 'r' calculated is greater than the theoretical value of 'r', for P' = 0.05 and degrees of freedom ϕ = 3; which is 0.88. There is therefore significant correlation between X and Y and so it is worth proceeding with regression analysis.

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

(v)

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

$$b = \frac{57.94 - 52.78}{0.9}$$

$$b = 5.73$$

(iii) Regression line equation:- is given by

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

Using Y=5 (since 5 is probit corresponding to 50% death response)

Therefore 5-4.40 = 5.73(X-2.40)

0.6 = 5.73(X-2.40)

X = 2.50

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

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

$$\sum dy^2 = \sum (y^2) - \sum \frac{(y)^2}{N} = 130.38 - \frac{21.99^2}{5} = 33.67$$

$$\sum dx^2 = \sum (x^2) - \sum \frac{(x)^2}{N} = 0.9$$

$$V_y = \frac{1}{3}(33.67 - 5.73^2 \times 0.9) = 1.37$$

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

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

$$\sum (X-\bar{X})^2 = \sum (X^2) - \sum (X)^2 / N = 0.9$$

$$V_x = \frac{1.37}{(5.73)^2}[1 + \frac{1}{5} + \frac{(5-4.40)^2}{(5.73)^2} \bullet \frac{1}{0.9}]$$

$$V_x = 0.042(1.20 + 0.0122) = 0.051$$

$$\therefore \sqrt{V_x} = 0.23$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and $P = 0.95$, these limits are $\pm 3.18 \times 0.23 = \pm 0.731$. The value of 'X' calculated from the regression line equation for $Y = 5$ is 2.50.

Therefore the concentration of the solution of $CAPS_1$ which will cause 50% death is given by

$X = 2.50 \pm 0.731$

Antilog of $X = 316.23 \pm 5.38$

Therefore $LC_{50}\ (CAPS_1) = 316\mu g/ml$

95% confidence interval = 311 - 321µg/ml

Appendix IC

Calculation of LC_{50} values of trichloromethane leaves extract $CAPL_2$

In order to calculate the regression line equation the following summations are made

X	X^2	Y	Y^2	XY
3.00	9.00	8.09	65.45	24.27
2.70	7.29	4.82	23.23	13.01
2.40	5.76	4.16	17.31	9.98
2.10	4.41	3.87	14.98	8.13
1.80	3.24	0.00	0.00	0.00

$\sum(X) = 12.0, \sum(X^2) = 29.7, \sum(Y) = 20.94, \sum Y^2 = 120.97, \sum(XY) = 55.39$

$number\ of\ degrees\ of\ freedom, \ldots N = 5, \ldots \bar{X} = 2.4, \ldots \bar{Y} = 4.20$

(i) Correlation Coefficient, 'r': Is first calculated to test whether there is significant linear correlation between X and Y values. 'r' is defined by the equation

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

$$\sum(X)\sum(Y)/N = 12 \times \frac{20.94}{5} = 50.26$$

$$\sum(Y^2) = \sum\frac{(Y)^2}{N} = 120.97 - \frac{20.94^2}{5} = 33.27$$

$$r = \frac{55.39 - 50.26}{\sqrt{0.9 \times 33.27}} = \frac{5.13}{5.47}$$

$$r = 0.938$$

Calculated 'r' = 0.88 and = 0.95, for P' = 0.05 and ϕ = 3. There is therefore significant correlation between X and Y and so it is worth proceeding with regression analysis.

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

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

$$b = \frac{55.39 - 50.26}{0.9}$$

$$b = 5.70$$

(ii) Regression line equation - is given by

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

Using Y=5 (since 5 is probit corresponding to 50% death response)

Therefore (5-4.20) = 5.70(X-2.4)

$$0.8 = 5.70X - 13.68$$

$$X = 2.54$$

$$1.17 = 4.49X - 10.78$$

$$X = 2.66$$

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

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

$$\sum dy^2 = \sum (y^2) - \sum \frac{(y)^2}{N} = 21.71$$

$$\sum dx^2 = \sum (x^2) - \sum \frac{(x)^2}{N} = 0.9$$

$$V_y = \frac{1}{3}(21.71 - 4.49^2 \times 0.9) = 1.19$$

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

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

$$\sum (X - \bar{X})^2 = \sum (X^2) - \sum (X)^2 / N = 0.9$$

$$V_x = \frac{1.19}{(4.49)^2}[1 + \frac{1}{5} + \frac{(5 - 3.83)^2}{(4.49)^2} \bullet \frac{1}{0.9}]$$

$$V_x = 0.059(1.2 + 0.076) = 0.075$$

$$\therefore \sqrt{V_x} = 0.274$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and P = 0.95; these limits are $\pm 3.18 \times 0.274 = \pm 0.871$. The value of X calculated from the regression line equation for Y = 5 is 2.66.

Therefore the concentration of the solution of $CAPL_3$ which will cause 50% death is given by

$X = 2.66 \pm 0.871$

Antilog of $X = 457 \pm 7.4$

Therefore LC_{50} $(CAPL_3) = 457\mu g/ml$

95% confidence interval = 450 - 464µg/ml

Appendix 1E

Calculation of LC_{50} values of crude mixture of PAs from leaves extract (CAPL)

In order to calculate the regression line equation the following summations are made

X	X^2	Y	Y^2	XY
3.00	9.00	8.09	65.45	24.27
2.70	7.29	6.48	41.99	17.50
2.40	5.76	5.95	35.40	14.28
2.10	4.41	5.61	31.47	11.78
1.80	3.24	5.41	29.27	9.74

$$\sum(X) = 12.0.\sum(X^2) = 29.7.\sum(Y) = 31.54.\sum(Y^2) = 203.58.\sum(XY) = 77.57$$
$$number.of.\deg rees.of.freedom,...N = 5,...\overline{X} = 2.4,...\overline{Y} = 6.31..\Phi = N - 2 = 3$$

(i) Correlation Coefficient, 'r': Is first calculated to test whether there is significant linear correlation between X and Y values. 'r' is defined by the equation

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

$$\sum(X)\sum(Y)/N = 12 \times \frac{31.54}{5} = 75.70$$

$$\sum(X^2) - \sum^{(X)^2}\!/_{N} = 29.7 - \frac{12^2}{5} = 0.9$$

$$\sum(Y^2) - \sum\frac{(Y)^2}{N} = 203.58 - \frac{31.54^2}{5} = 4.63$$

$$r = \frac{77.57 - 75.70}{\sqrt{0.9 \times 4.63}} = \frac{1.87}{2.04}$$

$$r = 0.917$$

Value of 'r' calculated > 0.88 and < 0.95, for P' = 0.05 and ϕ = 3. There is therefore significant correlation between X and Y and so it is worth proceeding with regression analysis.

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

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

$$b = \frac{77.57 - 75.70}{0.9}$$

$$b = 2.08$$

(iii) Regression line equation:- is given by

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

Using Y=5 (since 5 is probit corresponding to 50% death response)

Therefore 5 - 6.31 = 2.08(X-2.4)

5-6.31+4.99 = 2.08X

X = 1.769

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

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

$$\sum dy^2 = \sum (y^2) - \sum \frac{(y)^2}{N} = 4.63$$

$$\sum dx^2 = \sum (x^2) - \sum \frac{(x)^2}{N} = 0.9$$

$$V_y = \frac{1}{3}(4.63 - 2.08^2 \times 0.9) = 0.243$$

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

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

$$\sum (X-\bar{X})^2 = \sum (X^2) - \sum (X)^2/N = 0.9$$

$$V_X = \frac{0.243}{(4.33)^2}\left[1 + \frac{1}{5} + \frac{(5-6.31)^2}{(4.33)^2} \bullet \frac{1}{0.9}\right]$$

$$V_X = 0.056(1.2 + 0.440) = 0.092$$

$$\therefore \sqrt{V_X} = 0.303$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and $P = 0.95$; these limits are $\pm 3.18 \times 0.303 = \pm 0.964$. The value of X calculated from the regression line equation for Y = 5 is 1.769.

Therefore the concentration of the solution of CAPL which will cause 50% death is given by

$X = 1.769 \pm 0.964$

Antilog of $X = 58.7 \pm 9.2$

Therefore LC_{50} (CAPL) = 59µg/ml

95% confidence interval = 50 - 68µg/ml

Appendix IF

Calculation of LC_{50} values of crude mixture of PAs from seeds (CAPS)

In order to calculate the regression line equation the following summations are made

X	X^2	Y	Y^2	XY
3.00	9.00	8.09	65.45	24.27
2.70	7.29	6.75	45.56	18.23
2.40	5.76	5.71	32.60	13.70
2.10	4.41	5.61	31.47	11.78
1.80	3.24	5.33	28.41	9.59

$$\sum(X) = 12.0.\sum(X^2) = 29.7.\sum(Y\ ) = 31.49.\sum(Y^2) = 203.5.\sum(XY) = 77.57$$
$$number.of\ .\deg rees.of\ .freedom,...N = 5,...\overline{X} = 2.4,...\overline{Y} = 6.30..\Phi = N - 2 = 3$$

(i) Correlation Coefficient, 'r': Is first calculated to test whether there is significant linear correlation between X and Y values. 'r' is defined by the equation

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

$$\sum(X)\sum(Y)/N = 12 \times \frac{31.49}{5} = 75.58$$

$$\sum(X^2) - \sum^{(X)^2}\!\!/_{N} = 29.7 - \frac{12^2}{5} = 0.9$$

$$\sum(Y^2) - \sum\frac{(Y)^2}{N} = 203.50 - \frac{31.49^2}{5} = 5.18$$

$$r = \frac{77.57 - 75.58}{\sqrt{0.9 \times 5.18}} = \frac{1.99}{2.16}$$

$$r = 0.921$$

This value of 'r' calculated is greater than the theoretical value of 'r' for P' = 0.05 and $\phi = 3$ which is 0.88. There is therefore significant correlation between X and Y and so it is worth proceeding with regression analysis.

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

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

$$b = \frac{77.57 - 75.58}{0.9}$$

$$b = 2.21$$

(iii) Regression line equation:- is given by

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

Using Y=5 (since 5 is probit corresponding to 50% death response)

Therefore 5 - 6.30 = 2.21(X-2.4)

5+5.3-6.30 = 2.21X

X = 1.81

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

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

$$\sum dy^2=\sum(y^2)-\sum\frac{(y)^2}{N}=5.18$$

$$\sum dx^2=\sum(x^2)-\sum\frac{(x)^2}{N}=0.9$$

$$V_y=\frac{1}{3}(5.18-2.21^2\times 0.9)=0.26$$

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

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

$$\sum(X - \bar{X})^2 = \sum(X^2) - \sum(X)^2 / N = 0.9$$

$$V_X = \frac{0.26}{(4.88)^2}[1 + \frac{1}{5} + \frac{(5 - 6.30)^2}{(4.88)^2} \bullet \frac{1}{0.9}]$$

$$V_X = 0.053(1.2 + 0.385) = 0.084$$

$$\therefore \sqrt{V_X} = 0.2898$$

Limits of error are then calculated using the value of 't' for $\phi = 3$ and P = 0.95; these limits are $\pm$ 3.18 $\times$ 0.2898 = $\pm$ 0.922. The value of X calculated from the regression line equation for Y = 5 is 1.81.

Therefore the concentration of the solution of CAPS which will cause 50% death is given by

X = 1.81 $\pm$ 0.922

Antilog of X = 64.6 $\pm$ 8.4

Therefore LC_{50} (CAPS) = 65µg/ml

95% confidence interval = 57 - 73µg/ml

Appendix IIA

Probit corresponding to percentages

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

Appendix IIB

Theoretical values of the correlation coefficient P'=0.05

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

Degree of freedom ϕ=N-3

Appendix IIC

Values of 't'

Degree of freedom ϕ	Value of 't'		Degree of freedom ϕ	Value of 't'	
	P=0.95 P'=0.05	P=0.99 P'=0.01		P=0.95 P'=0.05	P=0.99 P'=0.01
1	12.71	63.7	11	2.20	3.11
2	4.30	9.92	12	2.18	3.05
3	3.18	5.84	15	2.13	2.95
4	2.78	4.60	20	2.09	2.85
5	2.57	4.03	25	2.06	2.79
6	2.45	3.71	30	2.04	2.75
7	2.37	3.50	40	2.02	2.70
8	2.31	3.36	60	2.00	2.66
9	2.26	3.25	120	1.98	2.62
10	2.23	2.17	∞	1.96	2.58

GLOSSARY

Antiallergic- an agent that prevents a state of hypersensitivity acquired through exposure to a particular allergen

Antidiabetic – an agent that prevents or alleviates diabetes

Antigastric– an agent that prevents stomach disorder.

Antihypertensive – an agent that counteracts high blood pressure

Antispasmodic – an agent that relieves spasm.

Antithrombitic– a substance that neutralizes the action of thrombin and thus limits or restricts blood coagulation.

Antiulcerogenic – an agent that supresses ulceration

Asthma – a disease marked by recurrent attacks of paroxysmal dyspnea, with wheezing, cough, and a sense of constriction, due to spamodic contraction of the bronchi.

Astrigent – substances causing contraction and arresting discharges.

Bronchitis – inflamation of the bronchial tubes.

Cardiac arrhythmias – absence of rhythm, applied especially to any variation from normal rhythm of the heart beat.

Cirrhosis – a disease of the liver marked by progressive destruction of liver cells, accompanied by replacement of the liver substance by connective tissue.

Colic – acute abdominal pain.

Demulcent – a s, .oothing mucilaginous or oily medication or application.

Diuretic – an agent that promotes the secretion of urine.

Expectorant – a substance that promotes the ejection by spitting of mucus or other fluids from the lungs and trachea.

Haemolysis – the separation of the haemoglobin from the corpuscles and its appearance in the fluid in which the corpuscles are suspended.

Haemoptysis- the expectoration of blood or of blood stained sputum.

Haemorrhoids- a varicose dilatation of a vein of the superior haemorrdiodal plexus

Heart arrest- (heart failure): the clinical condition resulting from inability of the myocardium of the ventricles to maintain an adequate flow of blood to all the tissues of the body; a sudden fatal cesation of the heart's action.

Hepatitis- inflammation of the liver.

Hepatoxicity: the quality or property of a substance to exert a destructive or poisonous effect upon liver cells.

Hypertension- abnormally high tension especially high blood pressure.

Lamitic crotalism- inflamation of laminae of a horse's foot due to consumption of crotalaria species

LD50 - median lethal dose.

Malignant tumour- swelling, morbid enlargement. A mass of new tissue which persists and grows independently of its surrounding structures which has no physiologic use. One which is likely to progress and eventually destroy life.

Purgatives- a medicine that causes evacuation from the bowels.

Rheumatoid arthritis- a chronic disease of the joints, usually polyarticular marked by inflammatory changes in the synovial membranes and articular structures and by atrophy and rarefaction of the bones.

Sedatives- an agent that allays activity and excitement.

Styptics – astringent arresting haemorrhage by means of an astringent quality.

Syphilis – a contagious veneral disease lesions; due to microorganism the Treponema pallidum and transmitted usually by direct contact.

Veno- occlusive: characterized by obstruction of the veins.

Vermifuge- an agent that expels worms or intestinal animal parasites an anthelminthic.

*All definitions were adapted from Dorland's Illustrated Medical Dictionary (1960), 24th edition, W. B. Saunders Company.