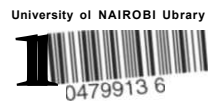J. PHARM, University of Nairobi)

ed for the part fulfillment of the requirements for

icy (Pharmaceutical Analysis) of the University of

Nairobi.

FACULTY OF PHARMACY,

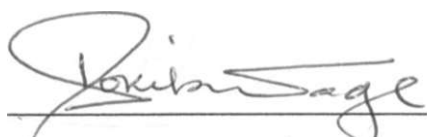)LLEGE OF HEALTH SCIENCES

UNIVERSITY OF NAIROBI

UNIVERSITY OF NAIROBI DECLARATION

I hereby declare that the contents of this thesis are my original work and have not been presented for a degree in any other university.

f-QKoWrfl-

FAITH APOLOT OKALEBO

This thesis has been submitted for examination with our approval as University Supervisors.

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Department of Pharmacology and Pharmacognosy

ACKNOWLEDGEMENTS

And so I prayed and understanding was given me,

What I learned without self-interest, I pass on without reserve,

I do not intend to hide her riches.

It was He who gave me true knowledge of all that is,

Who taught me the structure of the world and the properties of the

elements,

the alternations of the solstices and the instincts of wild animals,

the powers of spirits and mental processes of men,

the varieties of plants and medicinal properties of roots.

All that is hidden, all that is plain, I have come to know,

Instructed by Wisdom who designed them all.

The Book of Wisdom 7: 7, 13, 17-21.

DEDICATION

This work is a dedication to my parents, Prof. J. R. Okalebo and Mrs. M. T. Okalebo, and I. K. Mutai on whose constant prayers I know I can rely on.

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ABBREVIATIONS

AR: Analytical grade

COSY: Correlation Spectroscopy

CMS: Complete Medium with Serum

CNS: Central Nervous System

DARU   Drug Analysis and Research Unit

DEPT: Distortionless Enhancement by Polarization Transfer

DF: Degree of freedom

FT-IR: Fourier Transform Infra Red

GIT: Gastro Intestinal Tract

GR: General Reagent

IC_{50}:   Concentration that inhibits growth of parasites by 50%

id: Intradermally

INEPT: Insensitive Nucleus Enhanced Polarization Transfer

ip: Intraperitoneally

IR: Infra Red

KEMRI   Kenya Medical Research Institute

LD_{50}:   Concentration that kills 50% of the larvae

MIC: Minimum Inhibitory Concentration

NMR: Nuclear Magnetic Resonance

NSAIAs: Non Steroidal Anti Inflammatory Agents

ppm: parts per million

S^2:   Variance

SD: Standard Deviation

SE: Standard Error

SP:	Sulphadoxine-Pyrimethamine
TLC:	Thin Layer Chromatography
TMS:	Tetra Methyl Silane
WM:	Wash Medium
USA:	United States of America
UV:	Ultraviolet spectroscopy
WHO:	World Health Organization
wt.:	Weight
X:	Mean

Codes of the various plant extracts

SMS:	soxhlet methanol extract of the stem
SML:	soxhlet methanol extract of the leaf
MR:	cold methanol extract of the root
FPS:	petroleum ether fraction of the stem
FCS:	chloroform fraction of the stem
FES:	ethyl acetate fraction of the stem
AW:	aqueous residue of the stem

ABSTRACT

The leaves, stem and roots of *Clematis brachiata* Thunberg (Ranunculaceae) tested positive for anthraquinones, alkaloids, saponins, coumarins, sterols, carotenoids and flavanoids and cardenolides. Only the stem and leaves had tannins. The root had the highest amounts of alkaloids and anthraquinones

The stem Soxhlet methanol extract yielded 13.2 mg (0.029 % of the dried stem powder) of quercetrin (3-0-beta-L- rhamnosyl, 3', 4', 5, 7 tetrahydroxyl flavone). In addition the extract yielded 6400 mg (1.3 % of dried stem powder) of a precipitate, FAO-FRS. It was composed of a mixture of non-aromatic compounds.

The roots yielded 170 mg (0.068 % of dried root powder) of a non-aromatic unsaturated lactone.

The Soxhlet methanol extracts of the leaves and stem had very good activity against brine shrimps (LD_{50} 66.5 (ig/ml and 365.6 |ig/ml respectively). An ethyl acetate fraction of the stem Soxhlet extract, FES, had the greatest activity against the shrimps (LD_{50} = 23.08 (ig/ml).

The cold methanol extract of the root showed good *in vitro* antimalarial activity (LD_{50} = 39.9 jig/ml) against highly chloroquine resistant isolate, *Plasmodium falciparum* Vl/S.
The leaf and stem extracts showed low *in vitro* antimalarial activity. Quercetrin is known to have *in vivo* antimalarial activity.

None of the isolates and plant extracts showed significant antimicrobial activity.

FAO-FRS, the cold methanol extracts of the leaf and stem showed antinociceptive and local anesthetic effects.

The cold methanol extracts of the leaf, stem and roots caused relaxation of the isolated rabbit ileum. At low concentrations, FAO-FRS caused relaxation of the isolated rabbit ileum and at high concentration it had a dose dependent contractile effect.

The traditional use the leaves and stems of C. *brachiata* Thunb as analgesics, local anesthetics, antimalarial agents and spasmolytics, seems to have sound scientific rationale. The traditional use of the roots for the management of malaria and as a purgative seems to have scientific rationale.

CHAPTER ONE

INTRODUCTION AND LITERATURE SURVEY

1.1 The role of herbal medicine

Despite considerable advances in medicine there is a search for new drug molecules to manage emerging infections such as HIV/AIDS. Drug resistance has rendered many molecules ineffective and many of the drugs in current use can be improved in terms of efficacy, selectivity and toxicity. The plant kingdom offers a rich source of new molecules.

Many present day medicines are derived directly or indirectly from higher plants. The most recent additions to drugs in clinical use from higher plants are taxoids and campotherins which are used in the management of cancer and artemisinin for the management of malaria. Higher plants are rich sources new compounds with novel structures and novel mechanisms of drug action. Drugs from plants are used in a crude form or as purified substances isolated from the plant.

The sale of herbal plant products is growing in Europe and North America. Sales are driven by demands for herbal alternatives by patients (Joshi and Kaul, 2001). As patients embrace herbal products, clinicians ar^ frustrated by lack of scientific evidence to baeic therapeutic claims (Thompson, 1997).

A vast number of herbal medicines have not been subjected to vigorous scientific study for efficacy, toxicity and chemical constitution.

Natural product research is guided by ethnopharmacological knowledge. However, knowledge on medicinal use on plants is rapidly being lost, as it is no longer being passed down from the older generation to the younger generation.

The need for research on natural products cannot therefore be over emphasized. East Africa is endowed with a wide variety of flora and fauna. *Clematis brachiata* Thunberg was chosen for study because it is widely distributed and abundant in large amounts. It has a wide variety of medicinal uses and hence is likely to yield pharmacologically useful molecules. Very few pharmacological investigations have been done on C. *brachiata* and no compounds have been isolated from the plant to date.

1.2 Botanical description of *Clematis brachiata* Thunb.
Svnoms
Clematis hirsuta Guillemin *et* Perrotet
Clematis glaucescens Fresenius
Clematis petersiana Klotzsch
Clematis frisiorum Ulbr.
The vernacular names of G *brachiata* are presented in Table 1.
c

TABLE 1: THE VERNACULAR NAMES OF *CLEMATIS BRACHIATA* THUNB.

»

TRIBE	NAME	TRIBE	NAME
Kenya		**Democratic Republic of Congo**	
Maasai	*ENGISUGI-OLENA RIO,*	Zande	*GIJMBA*
Kipsigis	*BISINDA*	Holoholo	*KIAKAIA*
Kikuyu	*MUGAYANGONDU*	Luba-Hemba	*KIAKALA*
Luo	*ACHOGO*	Kanioka	*KINAMWABI*
Kamba	*EI AW AI*	Lur	*OBARWITCH*
Marakwet	*P1SING*	Lendu	*DITI, BROSSA*
Luyha	*LUNYILI, ILKISUCHI*		

5Q~NTINTJATION: VERNACULAR NAMES OF *CLEMATISBRACHL4TA*

Tanzania

			Zimbabwe	
Pogoro	*MPENGO*		Shona	*MURIDZA*
Haya ,	*MINKAMBA*		Ndebele	*UMDLADLATHI*

Uganda

		Nigeria	
Toro	*AKANYANKAMBA*	Yoniba	*ADAPOPO*
Nyankore	*OMUNKAMBA*	Hausa	*YAMANZA*
Acholi	*OMWOMBYER*		
Luganda	*LUMAAMA*	**Ivory Coast**	
Luango	*OKORUBIJYA*	Malinke	*SANZA, KONKONDEGURASIA*

Ethiopia

		Sierra Leone	
Amhara	*HASO*	Yalunke	*YATABIA-NA*
Tigre	*KEMIDA*	**Senegal**	
		Wolof	*NDIANAU, NDANUA*

Clematis brachiata Thunb. belongs to the Ranunculaceae family, which belongs to the Ranunculales order. The Ranunculaceae family is made up of 59 genera and about 1900 species.

The leaves of most members of this family are alternate with sheathing bases and are usually divided (Agnew and Agnew, 1994; Beentje, 1994). Three *Clematis* species are found in Kenya: C. *brachiata*, C. *simensis,* and C. *sigensis.* The first two closely resemble and are used interchangeably in East Africa. *Clematis sigensis* is only found in Simba Hills in the coastal region of Kenya. Unlike the first two, it is a short shrub.

Clematis brachiata Thunb is a shrubby climber and is commonly found on forest edges, on hedges, secondary bushland and wooded grassland. It climbs on trees, bushes and rocks. A photogragh of *Clematis brachiata* growing on a fence in Kiambu district, Kenya is presented in Figure 1. Figure 2 is a close up picture of C. *brachiata* growing on the fence. In wooded grassland it is found at the base of granite hills. It is not found in semi-deserts and deserts and in areas of very high rainfall. It is widely distributed all over Africa.

It is the African equivalent of the European plant, *Clematis vitalba,* and both share the common name, Old man's beard.   This is because in the flowering season they cover supporting trees and bushes with hanging masses of white to cream colored flowers.

*

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In the winter the persistent long feathery styles of the fruit are a common site, giving the impression of white beard.

A picture of the leaves of C. *brachiata* is presented in Figure 3. The leaves are made up of five to seven leaflets. The leaflets are ovate and are about 4 X 5 cm. They are opposite, pinnate, serrate and are covered with hair on the lower surface. The leafstalks and rachis of the compound leaves act as tendrils and twine around supporting trees giving additional support to the twining stem. The round stems are hairy. Figure 4 is a picture of the flowers of *C. brachiata.* The flowers are regular and lack petals and instead have four petal-like sepals. The petiole and rachis twin around the support (Beentje, 1994). The flowers are small (22-50mm across) and have a sweet smell. The flowers form a many-flowered long-stalked terminal panicle. The petals are recurved. The stamens are yellow, long, stiff and spirally arranged. The fruit is a group of achenes with persistent elongated feathery styles, 2.5-5cm long.

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Figure ɪꜱ A picture of *Clematis brachiata* growing *on* a fence (taken in Kiambu District, Kenya, June 2000).

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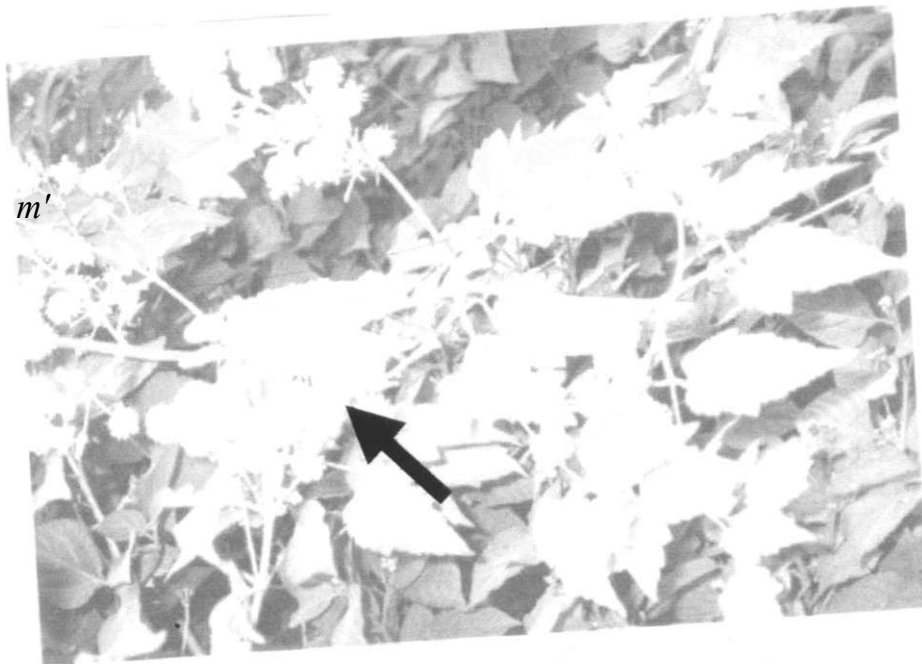
Figure 2: A close-up picture of *Clematis brachiata* growing on the fence
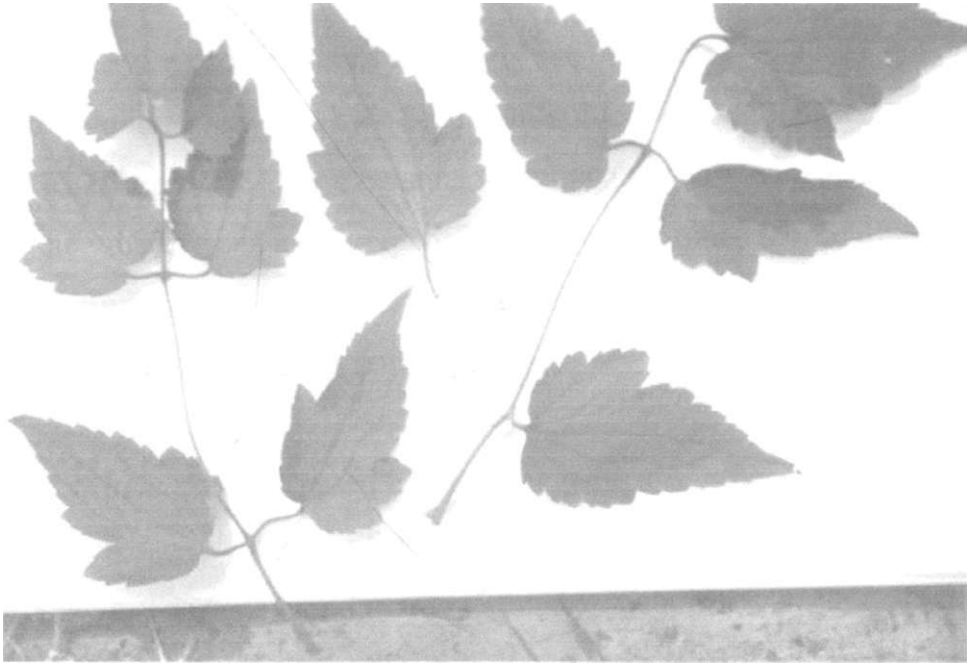
Figure 3: The leaves of *Clematis brachiata*

v*

Figure 4: The flowers of *Clematis brachiata*

1.3 MEDICINAL USES OF *CLEMATIS BRACHIATA* THUNB.

In Kenya a decoction of the roots is used to manage malaria and other febrile illnesses. The root decoction induces severe diarrhea (Kokwaro, 1993). In Tanzania, Rwanda and West Africa the leaf juice/macerate is used to manage malaria and other febrile illnesses (Neuwinger, 1994). This indicates that the plant may have antimalarial and antipyretic properties.

C. *brachiata* is also used in the management of other infectious diseases. An infusion of the leaf is used externally and orally in Kenya, Tanzania and Rwanda to manage gonorrhea, syphilis, and sore throat (Chabbra *et al.*, 1991;Kokwaro, 1993). In Rwanda, the whole plant is taken orally for the management of leprosy while in West Africa the juice of the fresh leaf is applied externally to treat leprosy and other skin diseases (Nwude and Ebong, 1980). In Zimbabwe, an infusion of the roots is mixed with the roots of *Julbernardia gloiflora* and is taken orally to treat a condition known as "green eye" in infants (Neuwinger, 1994). This suggests the plant has antimicrobial activity.

All over Africa, the leaves of C. *brachiata* are widely used to manage various skin conditions. In Kenya the leaf decoction is rubbed onto the skm for the management of skin disease though the exact nature of the skin disease is not specified. In Ulanga region of Tanzania, a root or leaf decoction is used to treat skin rash (Neuwinger, 1994).

*

The plant is widely used to manage of headaches and rheumatism. Its use as an analgesic is closely associated with its use to manage colds, blocked nose and head colds. In Kenya the whole plant is boiled and the steam is inhaled under a blanket to treat headaches and colds (Kokwaro, 1993).

In Kenya and Tanzania the fresh leaf is chewed and the juice swallowed for the management of headaches. The Luo of Kenya sniff the roots to manage headaches and colds. The flowers are sniffed to clear a stuffy nose. Elephants in Kenya have been observed in inhale the fresh roots possibly to clear blocked trunks and clear head colds (Kokwaro, 1993). In South Africa and Zimbwabwe, the stem or root powder are sniffed to clear headaches (Neuwinger, 1994). In Rwanda the leaf macerate is used to manage rheumatism. This suggests that the plant has analgesic and antiflammatory properties. The Kikuyu apply the rushed leaf or old stems to the teeth to manage toothache and sore throats (Gachathi, 1989).

C. brachiata is used to manage abdominal disorders. However, in literature the exact nature of the abdominal disorder is not specified. In Kenya, Tanzania and Burundi an infusion of the fresh leaf is used to manage abdominal disorders and especially those associated with pregnancy (Chhabra *et al*, 1984,1991; Kokwaro, 1993). In Burundi the leaf decoction is used to treat lower abdominal pain and diarrhea (Neuwinger, 1994).

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The Manja of Central African Republic use the roots alone or in combination with *Parquetina nigrescens* as an arrow poison to hunt small game. The roots are boiled and the extract is concentrated to a syrup. The rest of the roots are roasted and ground to a powder. The syrup and powder are mixed to form a paste, which is smeared on the arrowheads. The arrowhead is smoked before use (Neuwinger, 1994).

In Tanzania, the root of *Clematis brachiata* is not used for medicinal purposes because it is reputed to be highly toxic (Neuwinger, 1994). The arrow poisons used in South America are neuromuscular junction- blockers and cause death by skeletal muscle paralysis. Arrow poisons used in Africa contain cardenolides and cause death by cardiotoxicity (Neuwinger, 1994). The use of *C. brachiata* as an arrow poison strongly suggests that it contains cardenolides and may have skeletal muscle relaxant properties.

In Ethiopia the whole plant is thrown into dirty water to purify the water and the leaves are used as pesticides (Dawit, 1987). According to a traditional herbal practitioner (Olum, 1997) the Luo inhale the fresh roots to manage mental illness.

In Rwanda and Burundi, the leaf juice is used to treat urinary disorders and the hot water extract is used as an abortificient. In Tanzania, the leaf ijs taken orally for the management of epilepsy and is applied externally to rjianage snakebites.
v   ^

1.4 AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to determine and isolate the principle phytochemical components of *Clematis brachiata* and to investigate some of the claimed therapeutic uses of the plant for the management malaria, pain, infections and abdominal disorders.

CHAPTER TWO

PHYTOCHEMICAL INVESTIGATIONS

2.1 Previous Phytochemical Work Done On *Clematis brachiata* Thunb.

The leaves of *Clematis brachiata* from Tanzania contained steroids, triterpenoids, tannins, flavonoids, coumarins, carotennoids, polyoses and reducing substances. They lacked alkaloids, anthracene derivatives, anthocyanins and emodins (Chhabra *et al.*, 1984). These findings differ from an earlier study, where the whole plant macerate lacked alkaloids, saponins and tannins. The leaf from Rwanda tested positive for saponins and catechol tannins. It tested negative for alkaloids and quinones (Neuwinger, 1994). Available literature shows that no compounds have been isolated from C. *brachiata* to date.

Some compounds that have been isolated from other *Clematis* species are presented in Table 2.

TABLE 2: CLASSES OF COMPOUNDS ISOLATED FROM
VARIOUS *CLEMATIS* SPECIES

	PLANT NAME	COMPOUNDS
Flavonoids	*C. stans* (Japan)	Quercetin and rutin (Kizu *et al.,* 1995)
	C. pupurea var. *hybrida*	Clemaine (Sayed *et al.,* 1995)
Alkaloids	*C. recta* (Czech)	Magnoflorine and corytuberine (Slavik *et al*, 1995)
Lignans	*C. stans* (Japan)	Clemastanin A and B (Kizu *et al*1995)
	C. chinensis (China)	Syrigaresinol (Shao *et al.,* 1996a)
Triterpene saponins	*C. chine nsis* (China)	Oleaonolic and hederagenin based glycosides (Shao *et al* 1995; 1996b; Xu *et al.,* 1996)
Simple phenolics	*C. intricate*	Caffeic acid (Song *et al.,* 1996)
Coumarins	*C. intricata*	Scopoletin (Song *et al.,* 1996)
	C. chin ens is	Clematichinenol (Shao *et al.,* 1996a)
Sterols	*C. intricate*	Beta sitosterol (Song *et al.,* 1996)
Unsaturated lactones	*C. chinensis*	Clemochinoside A and B and Digiporlactone (Shao *et al.,* 1996b)
Essential oils	*C. hexapelata*	(Jiang *etal,* 1990)
Alk&nes	*C. intricate*	Triacontan-l-ol (Song *et al.,* 1996)

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2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

<u>REAGENTS</u>

All reagents used were of Analar grade except where the grade is otherwise indicated.

Laboratory grade 25 % ammonia solution (Alpha Chemicals Ltd., Kenya)

Glacial acetic acid (Riedel, Sleeze, Germany)

Anisaldehyde (Aldrich Chemical Co. Ltd., England)

Crystallizable benzene (Kobian Ltd., Kenya)

Laboratory grade bismuth subnitrate (May and Baker, Dagenham, England)

n-Butanol (BDH Chemicals, Poole, England)

3, 5-dinitrobenzoic acid (BDH Chemicals, Poole, England)

Absolute ethanol (Riedel de Haens AG, Seelze, Germany).

Ferric chloride hexahydrate (Seelze, Hannover AG, Germany)

Concentrated hydrochloric acid (Riedel de Haens AG, Seelze, Germany).

Laboratory grade resublimed Iodine (May and Baker Ltd., England)

Laboratory grade lead (II) acetate (May and Baker, Dangenham, England) *

Picric acid (BDH'Chemicals, Poole, England)

Potassium hydroxide^pellets (May and Baker, Dangenham, England)
  c

Potassium iodide (Sigma, St. Lious, USA)

Laboratory grade anhydrous sodium carbonate (May and Baker, Dangenham, England)

Laboratory grade sodium hydroxide (Spectra Chemicals Ltd., Nairobi, Kenya)

Concentrated sulfuric acid (Riedel de Haens AG, Seelze, Germany)

Vanillin (BDH Chemicals, Poole, England)

Magnesium filings (BDH Chemicals, Poole, England)

Mercuric chloride (May and Baker Ltd., Dangenham, England)

SOLVENTS

Acetone* (Alpha Chemicals Ltd., Nairobi, Kenya, General Reagent))

HPLC grade acetonitrile (BDH Poole, England)

Chloroform* (Kobian Ltd., Nairobi, Kenya, General Reagent)

Ethyl acetate* (Kobian Ltd., Nairobi, General Reagent)

Methanol* (Kobian Ltd., Nairobi, Kenya, General Reagent)

Petroleum ether (60-80°C)* (Kobian Ltd., Nairobi, Kenya, General Reagent)

Diethylamine (BDH Poole, England)

Dimethyl suphoxide (E. Merck, Darmstardt, Germany)

Tween 80 (BDH Poole, England)

* Solvent was distilled before use.

CHROMATOGRAPHIC MATERIALS

Pre-coated TLC plates Silica gel 6OF254 (Merck, Darmstadt, Germany)

Pre-coated TLC plates Reverse Phase Cig, (Merck, Darmstadt, Germany)

Silica gel for column chromatography (0.040 - 0.65 jim diameter) (E. Merck, Darmstadt, Germany).

REAGENTS FOR SPECTROSCOPY

Potassium bromide (E. Merck, Darmstadt, Germany)

Spectroscopic grade deuterated acetone (Sigma, Dorset, England)

Spectroscopic grade trimethyl silane (Sigma, Dorset, England)

EQUIPMENT

Analytical Balance (Sortorius, Analis, Namur, Belgium)

Centrifuge machine (International Equipment Co., Bedfordshire, England)

Clevenger's apparatus

Chromatographic tanks

Column for column chromatography

Dry Box (Perkin Elmer, Uberlingen, Germany)

Fraction Collector (Super Frac, Pharmacia Biotech, Belgium)

Gas tight box for incubation/ "Caison de Wolfe" (Bellco, Vineland, New Jersey, USA)

Kymograph (CAT. No. 1020, Scientific and Research Instrument Ltd., England)

Melting point apparatus (Btichi Melting Point Apparatus B-540, B.U.C.H.I., Switzerland).

Mill (Molly, Molinetto Machinatutto, Italy)

Micro/Macro pippetor (Socorex ISBA S. A., Switzerland)

pH meter (Sentron, pH system 1001, Roden, Netherlands)

Refrigerator

Rotary vacuum evaporator (Heidolph WB 2000, Belgium)

Solvent distiller

Soxhlet extractor

UV irradiating chamber (Model Sp-600, Too Kagaku, Japan)

Vacuum pump (AEG, Pfeiffer Balzers, Germany)

Voss Mai don shaker (Voss Instruments Ltd., Essex, England)

Water distiller

SPECTROPHOTOMETERS

Fourier Transform-Infra Red spectrometer (FT-IR) (Paragon 1000, Perkin Elmer, Beaconsfield, England)

Ultra Violet/Visible (UV/VIS) Scanning Spectrophotometer Model 8750 (Phillips, Cambridge, England)

Nuclear Magnetic Resonance Spectrometer (Varian Mercury 200, California, U.S.A.)

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2.2.2 METHODS

2.2.2.1 Collection, Drying and Identification of the Plant Materials

The plant was collected in Muguga Location, Kiambu District at a forest edge during the months of August and September, 1997. The freshly collected roots had an acrid smell, which disappeared on storage.

The leaves, stem and root parts were dried separately in an oven at 40 to 45°C for seven days and then ground to fine powder. The powders were stored in plastic bags at room temperature.

The pressed plant specimens were identified at the National Museums of Kenya and voucher specimens were deposited at the Department of Pharmacognosy and Pharmacology, University of Nairobi.

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2.2.2.2 Preliminary Screening for Various Phytochemical Groups

Spot tests for alkaloids were carried out according to the method described by Fansworth and Euler, 1962. The frothing test for saponins was carried out according to the method described by Wall *et al.,* 1954, 1952. The test for flavonoids was carried out according the method described by Geissman, 1955. The tests for bound and free anthraquinones and phenolics were carried out according the method described by Harborne, 1973. Chloroform solutions of the extracts were subjected to a test for carotenoids (Evans, 1996).

The plant extracts were subjected to modified Stas Otto extraction of glycosides and the glycosides subjected to Kedde's test for unsaturated lactone ring/cardenolide and Keller-Killian's test for a 2-deoxy sugar (Sim, 1967).

Thin Layer Chromatography (TLC) evaluation for saponins, flavonoids, coumarins and alkaloids were carried out according to the methods described by Wagner and Bladt, 1996. The presence of a steroidal nucleus was detected by TLC as described by Sim, 1967 and Waldi, 1965.

To test for essential oils, 200 g of fresh leaves and stems and 100 g of fresh root were separately subjected to hydrodistillation in a Clevenger-like apparatus for 12 hours.

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2.2.2.3 Isolation of Crystals from the Stem

About 500 g of stem powder was successively subjected to Soxhlet extraction with distilled petroleum ether and methanol for 48 hours each in that order. The extracts were reduced to dryness under vacuum at - 15 °C.

A large portion of the methanol extract dissolved in about 500 ml of water and was partitioned successively with petroleum ether; chloroform; and ethyl acetate. The three fractions were reduced to dryness under vacuum at - 15 °C.

The ethyl acetate fraction had the most activity against brine shrimp and hence was selected for isolation of bioactive compounds. To select an appropriate system for column chromatography, a variety of solvent systems were tried on TLC. Ethyl acetate was selected as the eluent because on it gave the largest number of spots with good separation.

The ethyl acetate fraction (2.74 g) was eluted on silica gel (120 g, column length 57 cm, internal diameter 2.8 cm) with ethyl acetate. The fractions were collected using a fraction collector set at 4 to 5 ml per test tube.

The fractions were monitored on pre-coated silica gel TLC plates and developed with ethyl acetate. Similar fractions were pooled together. The pooled fractions were reduced to minimal volume and left to crystallize at room temperature.

One of the pooled fractions yielded orange-red colored crystals. The crystals were cleaned by rinsing with ethyl acetate and then with ethyl acetate - methanol (1:1). The crystals were purified further by recrystalizing from a concentrated methanol solution. These crystals were given the code name OCB-3.

The aqueous portion obtained above was reduced to about 100 ml under vacuum at -15 °C and transferred to a flask. Acetone was added carefully along the wall till a white precipitate was observed. The flask was then covered with aluminum foil to prevent vaporization of acetone and placed in a fridge at 4 °C. An off-white precipitate formed along the walls after seven days. They were scrapped off and transferred to another container and rinsed repeatedly with methanol till white. The precipitate was given the code name FAO-FRS.

A flow diagram summarizing the isolation process of crystals from the stem is presented in Figure 5.

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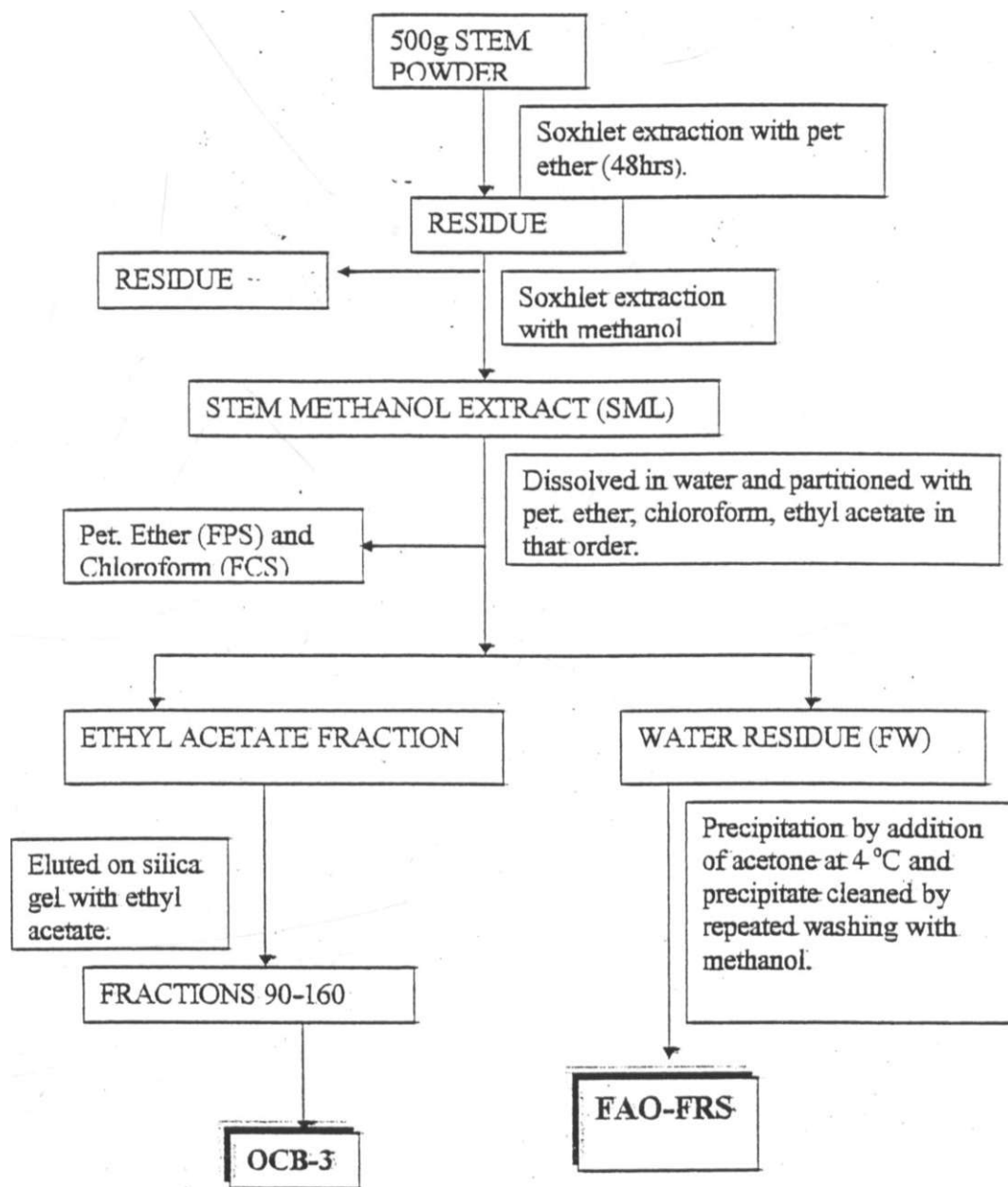
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Figure 5: A flow diagram showing: the isolation of OCB-3 and FAO-FRS from the stem *of Clematis brachiata*

2.2.2.4 Isolation of crystals from the roots

Two hundred and fifty grams of dried root powder was subjected to cold maceration with 80 % methanol for seven days to give an orange colored extract. The extract was reduced to dryness under vacuum at - 15 °C to give 10.1 g of dry extract. It was dissolved in about 200 ml of water and acidified with 10 % hydrochloric acid till pink to litmus. The acidified solution was partitioned with chloroform to remove all acidic and neutral components.

Dilute ammonia solution was added to the solution till it turned blue to litmus and it was partitioned with chloroform to extract basic components. The solution was acidified again and 100 ml of Mayer's reagent were added to precipitate quaternary alkaloids. The solution kept at 4 °C. After seven days a cream colored precipitate formed and it was filtered off. The filtrate formed white crystals at 45 °C. The crystals were cleaned by first rinsing with ethanol, then 70 % v/v ethanol and finally 50 % v/v ethanol. The rinses were pooled together and let to recrystallize again. The crystals were given the code name FAO-WR.

 A flow diagram summarizing the isolation process of crystals from the stem is presented in Figure 6.

```
                    ┌─────────────────┐
                    │ 250g-DRIED      │
                    │ ROOT POWDER     │
                    └─────────────────┘
                             │
                             │         ┌──────────────────────────┐
                             │         │ Cold maceration with 80% │
                             │         │ methanol for seven days. │
                             │         └──────────────────────────┘
                             ▼
                    ┌─────────────────┐
                    │ 10.1g of DRIED  │
                    │ ROOT EXTRACT    │
                    └─────────────────┘
                             │
 ┌──────────────────────┐    │    ┌──────────────────────────────────────┐
 │ CHLOROFORM EXTRACT   │    │    │ Extract dissolved in 200ml water,      │
 │ (contains acidic and │◄───┤    │ acidified with 10% hydrochloric acid   │
 │ neutral components.) │    │    │ and partitioned with chloroform.       │
 └──────────────────────┘    │    └──────────────────────────────────────┘
                             ▼
                    ┌─────────────────┐
                    │ AQUEOUS RESIDUE │
                    └─────────────────┘
                             │
 ┌──────────────────────┐    │    ┌──────────────────────────────────────┐
 │ CHLOROFORM EXTRACT   │    │    │ Basified with dilute ammonia solution  │
 │ (contains basic and  │◄───┤    │ and partitioned with chloroform.       │
 │ neutral components.) │    │    └──────────────────────────────────────┘
 └──────────────────────┘    │
                             ▼
                    ┌─────────────────┐
                    │ AQUEOUS RESIDUE │
                    └─────────────────┘
                             │
 ┌──────────────────────┐    │    ┌──────────────────────────────────────┐
 │ Cream colored        │    │    │ Acidified again and 100ml of Mayer's   │
 │ precipitate of       │◄───┤    │ reagent added and left at 4°C for      │
 │ quaternary alkaloids │    │    │ seven days                             │
 └──────────────────────┘    │    └──────────────────────────────────────┘
                             ▼
                    ┌─────────────────┐
                    │ FILTRATE        │
                    └─────────────────┘
                             │
                             │    ┌──────────────────────────────────────┐
                             │    │ Crystal formation at 45 °C and         │
                             │    │ crystals cleaned by repeated washing   │
                             │    │ with ethanol, then 70% ethanol and     │
                             │    │ finally 50% ethanol-water.             │
                             │    └──────────────────────────────────────┘
                             ▼
                    ┌─────────────────┐
                    │ FAO-WR          │
                    └─────────────────┘
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Figure 6: A flow diagram showing the isolation of FAO-WR from the roots *of Clematis brachiata*

2.2.2.5 Evaluation of the Isolates for Purity by Thin Layer Chromatography.

The purity of FAO-FRS and FAO-WR were studied using the chromatographic conditions listed in Table 3.

TABLE 3: THIN LAYER CHROMATOGRAPHIC CONDITIONS USED TO ASSESS THE PURITY OF FAO-WR AND FAO-FRS

Code	STATIONARY PHASE	MOBILE PHASE (IN VOLUME PARTS)
A	Silica gel 60 F254	Chloroform-glacial acetic acid-water-methanol (60:32:8:12)
B	Silica gel 60 F254	Chloroform-methanol-water (70:30:4)
C	Silica gel 60 F254	Chloroform-glacial acetic acid-water-methanol (15:24.5:3:2)
D	Silica gel 60 F254	Methanol-glacial acetic acid-water (4:3:2)
E	Reverse Phase, Ci8	Methanol-water (9:1)
F	Reverse Phase, Cm.	Methanol-water (5:5)
G	Reverse Phase, Cig "	Methanol-water (1:9)

As OCB-3 was relatively apolar compared to FAO-FRS and FAO-WR, it was monitored on silica gel using ethyl acetate - methanol - water (100: 13.5: 10).

2.2.2.6 High performance Liquid Chromatography Separation of FAO-FRS

The analysis was carried out on a Beckman System Gold apparatus (Programmable Solvent Module 126) composed of pump, automatic injector, diode array UV detector Module 168 and an integrator GOLD V180. High performance Liquid Chromatography (HPLC) was carried out on an Ultraspore ODS column (250 x 4.6 mm i. d.) particle size 5 jim.

Two different solvent systems were used. The first solvent system was a linear gradient of 85 % v/v acetonitrile in water to 10 % v/v acetonitrile in water in 22minutes. The flow rate was 1.0 ml/min.

The second solvent system was a linear gradient of 99 % v/v acetonitrile in water to 40 % v/v acetonitrile in water in one minute, then to 10 % v/v in 20 minutes. The flow rate was 1.0 ml/min.

2.2.2.7 Spectroscopic Evaluation of the Isolates

OCB-3 was dissolved in acetone-ck and Proton NMR was carried out at 200 MHZ and ^{13}C NMR was carried at 50 MHZ on Varian Mercury 200 instrument at the Department of Chemistry, Chiromo Campus, Faculty of Science, University of Nairobi. Homonuclear COSY and DEPT spectra were obtained.

The Infra Red (IR) spectrum of OCB-3 was obtained in potassium bromide disks using a Perkin Elmer Fourier Transform Infra Red Spectrometer (Paragon 1000). The IR spectra of FAO-FRS and FAO-WR were not obtained as they were not pure.

The UV spectra of FAO-FRS, FAO-WR and OCB-3 were obtained using a UV/VIS scanning spectrophotometer. FAO-FRS and FAO-WR were dissolved in water: methanol (2:1) and OCB-3 was dissolved in methanol.

2.2.2.8 Melting Point Determination

OCB-3 was dried at 50 °C for two and a half hours before the melting point was determined. A capillary tube (internally diameter of 1.15 ±_0.05 mm, external diameter of 1.55 + 0.05mm) was packed with 3 to 8 mg of the crystalline material. The melting point was determined using Buchi Melting Point Apparatus.

2. 3 RESULTS AND DISCUSSION

2.3.1 RESULTS

2.3.1.1 Yields of the extracts obtained from *Clematis brachiata*

The yields of the various soxhlet extracts from the stem are presented in Table 4.

2.3.1.2 Phytochemical Groups Detected in the Roots, Stem and Leaves of *Clematis brachiata*

Preliminary investigations by TLC and spot tests revealed the presence of saponins, coumarins, anthraquinones, flavonoids, triterpenoids, steroidal saponins, cardenolides and alkaloids in the root, stem and leaf. The stem and leaves have tannins while the root lacked tannins. No essential oils and cyanogenic glycosides were detected.

The relative amounts of various phytochemical groups were deduced from the relative intensity and sizes of the spots on TLC. The relative amounts of various phytochemical groups detected in the various plant parts are presented in Table 5.

The results of spot tests done on the fractions obtained from the stem by partitioning with petroleum ether, chloroform and ethyl acetate are presented on Table 6.

*

TABLE 4: THE YIELDS OF VARIOUS EXTRACTS OBTAINED
FROM THE STEM

EXTRACT	Percentage Yield of the dried stem % ± SE (n = 4)
SOXHLET PETROLEUM ETHER EXTRACT (SPS)	0.780 ±0.05
SOXHLET METHANOL EXTRACT (SMS)	14.90 ±0.21
* Petroleum ether fraction (FPS)	0.69 ±0.03
* Chloroform fraction (FCS)	0.27 ±0.03
* Ethyl acetate fraction (FES)	0.64 ±0.13
* Aqueous potion (FWS)	13.21 ±0.29

TABLE 5: RESULTS OF SPOT TESTS DONE ON THE
SOXHLET METHANOL EXTRACT OF THE STEM, LEAF AND
ROOT OF *CLEMATIS BRACHIATA*

Phytochemical group	LEAVES	ROOTS	STEM
Alkaloids	+	+++	+
Anthraquinones	-	+++	-
Anthrones / anthranols	+	+	+
Cardenolides	+	+	+
2-Deoxy sugar	-	-	-
Carotenoids	+	+	+
Coumarins	+	+	+
Cyanogenic glycosides	-	-	-
Essential oils	-	-	-
Flavonoids	+++	+	+
Steroids	+	+	+
Triterpenoid	+++	+	+
Saponins			
Tannins	+	-	+

KEY

+++   Present in relatively large amounts
++   Present in moderate amounts
+   Present in small amounts
      Not detected

y

TABLE 6: PHYTOCHEMICAL GROUPS DETECTED IN THE
PETROLEUM ETHER. CHLOROFORM. ETHYL ACETATE
FRACTIONS AND WATER PORTION OF THE STEM
EXTRACT

Phytochemical Group	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous portion
Alkaloids	-	+	+	
Anthraquinones	+	+	+++	
Carotenoids	++			
Coumarins	-	+	+++	+
Flavonoids			+	+++
Steroids	++	+		
Cardenolides				+
Saponins			+	+++
Tannins			+	+++

KEY

+++ Present in relatively large amounts
++   Present in moderate amounts
+   Present in small amounts
      Not detected

TLC evaluation for various phytochemical groups was carried out. The Rf values of the anthraquinones, coumarins, saponins and flavonoids detected in various extracts are presented in Table 7.

2.3.1.3 TLC evaluation of the crystals for purity

FAO-WR and FAO-FRS did not move from the solvent front when developed on silica gel with the solvent systems listed in Table 3. When developed on Reverse Phase C-18 with solvent systems E, F and G (Table 3), FAO-WR gave a single pale blue fluorescence in long UV at Rf 0.74, 0.51 and 0.44 respectively. This spot turned yellow when sprayed with concentrated sulphuric acid.

FAO-FRS gave a pale blue fluorescent spot at Rf 0.74 and 0.76 when developed on reverse phase with solvent system E. On developing with system G, it gave two blue fluorescent spots at Rf 0.82 and 0.54. The spot at Rf 0.82 was due to a methanol soluble impurity. These spots turned yellow when sprayed with concentrated sulphuric acid.

OCB-3 gave a single red fluorescent spot at Rf 0.8 when developed on silica gel with ethyl acetate-methanol-water (100: 13.5: 10). On exposure to ammonia the spot turned yellow-green.

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TABLE 7: THE R_fx 100 VALUES OF PHYTOCHEMICAL GROUPS DETECTED IN VARIOUS EXTRACTS OF [1]*CLEMA T1S BRA CHI A TA*

CODE	**ANTHRAQU1NONE**	**COUMAR1NS**	**FLAVONOIDS**	**SAPONINS**
Mobile Phase 'li	A $_t$	A	B	C
MR	*4* (20; 56.4; 80; 9527-50;65-92)	1 spot(88)	2 spots (80; 100)	streaking
SMS	3 spots (20; 56.4; 80)	2 spots (38; 88)	2 spots (80; 98)	streaking
SML	3 spots (20; 56.4; 80)	3 spots (38; 88; 100)	3 spots (80; 86; 98)	streaking
FPS	1 spot(22)	Not detected	Not detected	Not detected
FCS	2 spots (22; 46)	1 spot(100)	Not detected	Not detected
FES	3 spots (22; 50; 80)	5 (38; 54; 74; 83; 88)	2 spots (80; 98)	streaking
AW	Not detected	1 spot (38)	3 spots (80; 86; 98)	Slight streaking

Key continued on the next page.

CHROMATOGRAPHIC CONDITIONS

A: Ethyl acetate-methanol-water (100: 13.5: 10). Detected using 8 % w/v ethanolic potassium hydroxide spray reagent.

B: Ethyl acetate-formic acid- water-methanol (10: 2:2:1), detected by spraying with alcoholic 1 %w/v ferric chloride reagent.   ,

C: Chlorofo/m - glacial acetic acid - methanol - water (64: 32: 12:8). Detected using Anisaldehyde- sulfuric acid spray reagent.

EXTRACT CODES

SMS - soxhlet methanol extract of the stem

SML - soxhlet methanol extract of the leaf

MR - cold methanol extract of the root

FPS - petroleum ether fraction of the stem

FCS - chloroform fraction of the stem

FES - ethyl acetate fraction of the stem

AW - Aqueous residue of the stem

2.3.1.4 Results of HPLC evaluation of FAO-FRS

With the first solvent system (a linear gradient of 85 % v/v acetonitrile in water to 10 % v/v acetonitrile in water in 22minutes), only two broad unresolved peaks were seen at retention times 7.4 and 9.90 min (see Appendix B).

The second solvent system (a linear gradient of 99 % v/v acetonitrile in water to 40 % v/v acetonitrile in water in one minute, then to 10 % v/v in 20 minutes), only two peaks were seen (see Appendix B). The first peak had a retention time of 5.7 minutes (peak area 10.3 %). The second peak was a broad hump from retention time 8.0 to 14.0 minutes. The hump had five points of inflection with the following retention times: 9.7 min (21.6 %); 10.4 min (41.2 %); 11 min (6.7 %); 11.2 min (23.6 %); and 12.8 min (2.7 %).

The UV maxima of all the peaks were between 210 and 215 nm.

2.3.2 Discussion

2.3.2.1 Phytochemical investigations done on *Clematis brachiata*

The presence of anthraquinones in the root of C. *brachiata* explains the fact that a decoction of the root when used to treat malaria/fever causes severe diarrhea. Purgative activity is associated with 1,8-dihydroxyl substitution (Figure 7). Whenever quinones occur in various plants, they tend to concentrate in the roots, heartwood and bark (Evans, 1996).
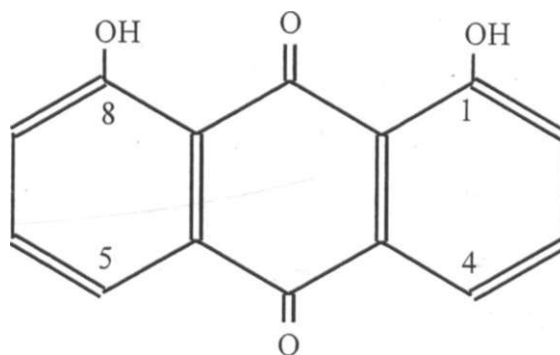
Figure 7:
The structure of 1, 8 dihydroxy anthraquinone

The absence of essential oils was very surprising as literature indicates that different plant parts are steamed and inhaled for the management of headaches and colds.   In this work, the method used to test for essential oils was extraction with Clevenger-like apparatus and it lacks sensitivity. Essential oils could have been present in trace amounts. Trace amounts of essential oils can be detected using the trap method which was not used in this study.

The presence of cardenolides explains the use of the plant by the Manja as an arrow poison. Unlike the arrow poisons used in South America, the arrow poisons used in Africa have cardenolides (Neuwinger, 1994).

The presence of sterols, triterpenoids, coumarins and tannins in the leaves concurs with a previous study done by Chabbra *et al*., 1991a. However, unlike his study, the leaves tested positive for alkaloids. The alkaloids are most abundant in the root and were also detected in the stem. The alkaloids could not be detected easily in methanol extracts. They were detected in the chloroform extracts during the isolation of FAO-WR from the root. The whole plant macerate of the plants collected in Tanzania tested negative for alkaloids, saponins and tannins (Neuwinger, 1994).

The saponins detected by TLC gave a lot of streaking. This was expected because saponins are glysosides and are therefore hydrophilic. This makes separation on normal phase silica gel very difficult.

The presence of tannins in the leaf and stem supports the traditional use of the plant for the management of skin disease and skin bites as tannins have demulcent and stryptic properties (Evans, 1996). Carotenoids are associated with the Ranunculaceae family and they give cross sections of the roots and stem a yellow color. Hence the family is called the Buttercup family. Cyanogenic glycosides are common in many *Clematis* species but these were not detected in *Clematis brachiata* (Evans, 1996).

Partitioning the stem methanol extract of concentrated some phytochemical groups in certain fractions based on their polarity. Non-polar groups like sterols and carotenoids were concentrated in the petroleum ether fraction. Highly polar groups like saponins, cardenolides and tannins concentrated in the aqueous fraction. Coumarins, flavonoids and anthraquinones, which have intermediate polarity, concentrated in the chloroform and ethyl acetate fractions.

2.3.2.2 The partial characterisation of isolates from *Clematis brachiata*

FAO-FRS

It was white in color is freely soluble in water and methanol-water (2:1). The yield was 6.4 g (1.39 % of the dried stem powder). The UV spectrum (methanol-water (2:1), 7.24 mg/ml) was transparent above 200 nm showing that it lacks aromatic groups (Appendix A).

HPLC evaluation of FAO-FRS on a Hypersil C-18 column revealed that it is a complex mixture of aliphatic compounds. The resolution of peaks on HPLC was poor.

FAO-WR

It was obtained as a white crystalline material. The yield was 170 mg (0.068 % of the dried root powder). Its melting point was greater than 400 °C. It tested positive for an unsaturated lactone. The UV spectrum (methanol-water (2:1)) was transparent above 200 nm showing that it lacks aromatic groups (Appendix C).

Though TLC evaluation of FAO-WR showed single spots in a variety of solvent systems, this was not sufficient to prove it is a pure compound.

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OCB-3

The yield of OCB-3 was 13.2 mg of crystalline material (0.029 % of the dried stem powder). It was orange-red in colour with an uncorrected melting point of 173-174.9 °C. It was soluble in acetone and 50 % methanol-ethyl acetate and was insoluble in chloroform and water.

The UV, IR, ^{13} Carbon, Proton, COSY and DEPT NMR spectra are appended (see Appendices E to L). UV max (methanol) nm: 217, 256 and 351. IR (KBr) cm^{-1}: 3420.8,2933.3, 1654.5, 1608.3, 1508.6, 1448.5, 1362.1, 1272.4, 1202.4, 1170.5; 1117.2; 1088.5; 996.1; 958.6; 814.8.

The Aglycone moiety

When developed on silica gel with ethyl-acetate-methanol-water (100:13.5:10) OCB-3 gave a single red-brown fluorescent spot at Rf 0.8 in long UV light and turned yellow-green in viscible light when exposed to ammonia. This color reaction suggested the presence of a flavone or a flavanol. The presence of a flavone/flavanol was supported further by the UV absorption spectrum. Most flavones and flavanols give two major absorption bands in UV.

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The bands are called Band I and Band II and occur at 320-385 nm and 250-285 nm respectively (Markham, 1989). Band 1 represents B-ring absorption and Band II represents A-ring absorption (see Figure 8). The peak at 350nm showed that OCB-3 may be a flavone or a 3-hydroxyl substituted flavonol. Flavonols with free 3-hydroxy groups absorb at higher wavelengths (Marby *et al*., 1969).

The aromatic nature of the molecule is indicated by IR absorption in the following regions: 1600-1585 cm^{-1}; 1500-1400 cm^{-1}; and 900-675 cm^{-1}. The peaks at 996.1, 958.6 and 814.8 cm^{-1} are due to out of plane bending of ring C-H bonds. The bands at 1608, 1508.6, 1448.5 cm^{-1} could be due to skeletal vibrations involving C=C stretching within the ring. As expected these skeletal bands appear as doublets. Aromatic C-H stretch vibrations occur between 3100 to 3000 cm^{-1}. This was not observed in the IR spectrum of OCB-3 and was probably obscured by the broad peak due to O-H stretch vibration.

The peak at 2933.3 cm^{-1}, which is due to an aliphatic C-H stretch vibration, suggests the molecule has an aliphatic substituent.

The strong broad peak at 3420 cm^{-1} could be due to free hydroxyl groups of an alcohol or a phenol. The presence of aliphatic and phenolic hydroxyl groups is supported further by the C-0 stretch vibrations occurring between 1260 to 1000 cm^{-1}.

The strong bands at 1362.1 and 1202.4 cm"^{1} could be the C-0 stretch and O-H bend of a phenol. These bands arise from an interaction between the O-H bend and the C-0 stretch. The band at 1117.2 and 1088.5 cm"^{1} could be due to the C-0 stretch vibrations of a secondary alcohol.

The strong peaks at 1654.5 and 1608.3 cm'^{1} could be due to a C=0 stretch vibration. As the carbonyl vibration occurs at a low wavenumber, it is conjugated to an aromatic system.

Fifteen aromatic carbons were observed between 5 95 to 8 170 in the ^{13} Carbon NMR spectrum. From DEPT, there were five proton substituted aromatic carbons. This was supported by proton NMR which showed five aromatic protons between 5 6.2 to 5 7.8. The chemical shift at 8 178.6 confirmed the presence of a carbonyl group. Carbon-13 NMR spectroscopy is useful for distinguishing between various flavonoid classes (Grayer, 1989). In the case of OCB-3, the carbonyl shift occurred between 8 172-179 and the C-3 shift at 8 135.164, and this confirmed that OCB-3 is a flavanol and not a flavone.

COSY revealed two separate aromatic systems. In the first aromatic system, a proton at 8 6.26 (doublet) was coupled to a proton at 8 6.47 (doublet) (J = 2.0 HZ). The low coupling constant indicated meta coupling.

<1

The first aromatic system was due to ring-A (see Figure 8). In 5, 7 dihydroxy substituted flavonols, H-6 and H-8 in ring-A generally resonate in the ranges 5 6.0-6.8 and 8 6.3-6.9 respectively and appear as two meta-coupled doublets (Markham, 1989). The hydroxy 1 signals were not observed as OCB-3 was partially trimethylsilylated.
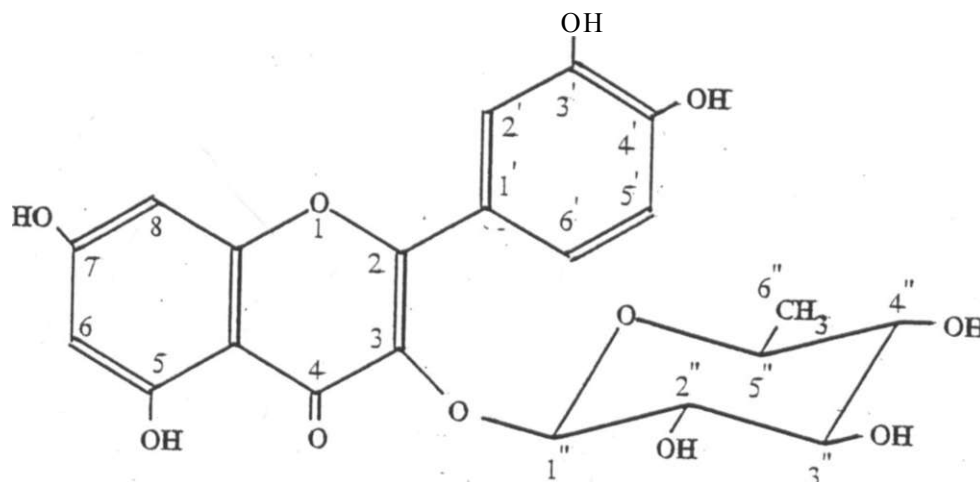
Figure 8: The structure of OCB-3: quercetrin (quercetin 3-0-beta-rhamnoside).

The second aromatic system had three protons at 8 6.98 (doublet), 8 7.38 (double doublet) and 8 7.49 (doublet).

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COSY revealed the proton at 5 7.38 is strongly coupled to the proton at 5 6.98 (J = 8.4 Hz, ortho coupling). The proton at 5 7.49 is coupled to 8 7.38 (J = 2.2 HZ, meta coupling). The second aromatic system is due to ring-B.

In flavones/flavanols, dihydroxyl substitution at C-3' and C-4' produces a three proton signal. The C-5' proton appears as a doublet and is centered between 5 6.7-7.1 (J ~ 8.4). The H-2' and H-6' proton signals occur at between 5 6.7 to 7.1 (Markham, 1989).

No proton signal associated with the C-ring was observed indicating that the hydroxyl group at C-3 is conjugated to a sugar substituent.

In the spectrum of partially trimethylsilylated flavone/flavonols the 5-hydroxyl is not trimethylsilylated and its signal appears downfield at about 5 12.7 (Marby *et al*, 1969). The singlet at 5 12.72 (intramolecular chelated phenolic OH) is due to the free hydroxyl group at C-5.

The substitution pattern on the A and B rings, OCB-3 suggested that the aglycone was quercetin. The carbon-13 chemical shifts of OCB-3 corresponded well to the chemical shifts of quercetin given in literature (see Table 8). Variations of 0 to 3 ppm in the chemical shifts of quercetin obtained in literature are acceptable due to differences in equipment, solvents and operating conditions.

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There were no significant differences between the shifts associated with A-and C- rings.

The most significance differences between the chemical shifts of quercetin and OCB-3 were two downfield shifts at C-2 (10.4 ppm) and field shift C-4 (2.8 ppm). These differences were expected as 3-O-glycosylation causes downfield shifts of about 10 ppm and 3 ppm on the C-2 and C-4 respectively (Markham, 1989). An upfield shift of 2 ppm on C-3 was expected but was not observed.

The chemical shifts in acetone are generally downfield compared to the chemical shifts of quercetin obtained in dimethyl sulphoxide (DMSO- c^) because the carbonyl function in acetone has a deshielding effect.

The proton NMR chemical shifts and coupling constants of OCB-3 fell within the literature values for 3-O-glycosylated 5,7, 3", 4' oxygenated flavones (Marby *el ai,* 1969) (see Table 9). The H-6 and H-8 signals of OCB-3 proved that the aglycone was not glycosylated at C-7 as the H-6 and H-8 signals of 7-O-glycosylated flavones/flavonols are shifted downfield (Marby *et al.,* 1969).

TABLE 8: THE CARBON NMR SHIFTS OF OUERCETIN. 3-0-GLYCOSYLATED DERIVATIVES OF OUERCETIN AND OCB-3

	Quercetin	A	B	C	**OCB-3**
	DMSO- d6	DMSO- d_6	DMSO- d_6	CD_3OD	CD_3OCD_3
C-2	146.9	156.5	156.3	159.2	**157.3**
C-3	135.5	133.5	133.6	133.0	**135.2**
C-4	175.8	177.5	177.4	179.9	**178.6**
C-5	160.7	161.0	161.1	163.3	**162.2**
C-6	98.20	98.60	98.70	100.4	**98.80**
C-7	163.9	164.0	164.6	166.4	**164.5**
C-8	93.3	93.5	93.5	95.4	**93.9**
C-9	156.2	156.5	156.1	158.8	**157.7**
C-10	103.1	104.5	103.7	105.8	**105.0**
c - r	122.1	121.5	120.8	123.2	**122.1**
C-2'	115.3	114.5	115.6	118.2	**115.4**
C-3'	145.0	144.5	144.9	146.2	**145.2**
C-4'	147.6	149.0	148.6	150.4	**148.5**
C-5'	115.6	116.5	115.3	116.5	**116.0**
C-6'	120.0	121.5	122.0	123.4	**122.0**

A-quercetin   3-0-[6"[1]-alpha-L-rhamnosyl-6"-beta-D-glucosyl]-beta-glucoside (Sharaf *etal.,* 2000).

B-quercetin 3-O-alpha- L-arabinopvranoside (Fraisse*etal.,* 2000).

C-quercetin 3-0-(6"-caffeoyl)-beta-D-galactopvranoside (Datta*etal.,* 2000).

OCB-3 quercetin 3-O-beta-rhamnoside.

TABLE 9: THE PROTON NMR SPECTRUM OF 3-0-
GLYCOSYLATED DERIVATIVES OF OUERCETIN

	Literature values * DMSO-d$_6$	A DMSO-d$_6$	B DMSO-d$_6$	C CD$_3$OD	OCB-3 Acetone- d6
H-6	6.0-6.2 (d, J-2.5)	6.20 (J = 2)	6.18 (J = 2.2)	6.16 (J = 2.2)	**6.26** (J = 2)
H-8	6.3-6.5 (d, J ~ 2.5)	6.40 (J = 2)	6.38 (J = 2.2)	6.34 (J = 2.2)	**6.48** (J = 2)
H-2*	7.2-7.5 (d, J ~ 2.5)	7.50	7.50 (J = 2.0)	7.81 (J =2)	**7.49** (J = 2.2)
H-5'	6.7-7.1 (d, J ~ 8.5)	6.80 (J = 9)	6.83 (J = 8.5)	6.79 (J = 8.8)	**6.98** (J = 8.4)
H-6'	7.3-7.7 (dd, J~ 8.5,2.5)	7.50	7.65 (J = 8.5)	7.58 (J = 8.8)	**7.38** (J = 8.4)

* The chemical shifts of 3-0-glycosylated 5,7, 3', 4' tetrahydroxyflavones (Marby *etal*1969).

A-quercetin 3-0-[6'''-alpha-L-rhamnosyl-6''-beta-D-glucosyl]-beta-glucoside (Sharaf *etal.,* 2000).

B-quercetin 3-O-alpha- L-arabinopvranoside (Fraisse *etal.,* 2000).

C-quercetin 3-0-(6''-caffeoyl)-beta-D-galactopyTanoside (Datta*etal.,* 2000).

OCB-3 quercetin 3-O-beta-rhamnoside.

The Sugar Substituent

In flavones and flavonols the carbons shifts of the hydroxylated carbons of sugars occur between δ 60-83. The anomeric carbon of O-glycosides resonate at about 100 ppm while the anomeric carbons of C-glycosides resonate at about 74 ppm (Markham, 1989).

The presence of four methine carbons at δ 70-72 in ^{13}C NMR spectrum provided evidence for the presence of a sugar moiety. The anomeric carbon shift at δ 102.1 showed that OCB-3 is an 0-glycoside.

Proton NMR showed a three-proton doublet at δ 0.918 suggesting the presence of a methyl group on the sugar moiety. The presence of a methyl group was confirmed by DEPT which showed a methyl group at δ 17.20. This indicated that the sugar moiety was rhamnose which is characterized by a C-6" methyl group that resonates at about δ 0.93 in proton NMR.

The chemical shift of the H-1" (anomeric) proton varies according to the site of attachment of the sugar moiety to the flavone/flavanol. The anomeric proton shifts of 7-O-rhamnosides and 3-0-rhamnosides are 5.1-5.3 ppm and 5.0-5.1 ppm respectively (Markham, 1989). In OCB-3, the anomeric proton signal appeared at 5.5 ppm which was higher than the literature values for both 7-0- and 3-0- rhamnosides.

c

Thus it was not useful for inferring the position of attachment of the sugar moiety to the aglycone. Running the spectrum in acetone may have contributed the low shift of the anomeric proton. Variations of between 0-0.3ppm are observed when the spectrum is run in various solvents (Markham, 1989). The methyl proton signal is useful for distinguishing between 7-0- and 3-0-rhamnosides. In 3-0-rhamnosides the methy 1 signal appears as a sharp doublet at about 0.85 ppm (Marby *et al.,* 1969). In 7-0 rhamnosides, the methyl signal appears as a complex signal resonating at about 1.2 ppm since in this position the sugar has freer rotation. In OCB-3, the methyl signal occured as a doublet 0.91 ppm providing evidence that it is a 3-0-rhamnoside.

Additional evidence for 3-O-glycosylation is provided by the UV spectrum of OCB-3. Band I occurs between 354 to 387 nm in flavonols with free 3-OH groups. In the case of OCB-3, the UV shift occurred at 351 nm indicating substitution at the 3-OH group.

The sugar ^{13}Carbon^{rt} shifts of OCB-3 corresponded closely with the shifts of 3-O-rhamnoside given in literature (see Table 10).

TABLE 10: THE CARBON-13 CHEMICAL SHIFTS OF
RHAMNOSE IN FLAVONE/FLAVONOL 3-O-RHAMNOSIDES
AND OCB-3

	C-1"	C-2"	C-3"	C-4"	C-5"	C-6"
3-O-rhamnosides*	101.9	70.4°	70.6^C	71.5	70.1^c	17.3 ~
DMSO-d$_6$						
X	103.7	72	72	73.2	71.9	17.7
Methanols						
Y	103.5	73.8	72.2	72.0	71.9	17.9
Methanol-^						
OCB-3	102.1	70.7	71.3	72.3	70.7	17.1
Acetone-cU						

^{c} The assignments may be reversed.

KEY

* The chemical shifts of rhamnose in flavanol-3-O-rhamnosides (Markham 1989).

X mvricetin 4' methyl ether 3-O-rhamnoside (Braca *etal.,* 2001).

Y myricetin 3', 5' dimethyl ether-3-O-rhamnoside (Braca*etal.,* 2001).

OCB-3 quercetin-3-O-beta-rhamnoside.

COSY reveals the couplings within the sugar moiety. The proton assignments of the sugar moiety are only tentative and can only be confirmed by INEPT NMR. H-1" (doublet) is coupled to H-2^M (J = 1.2 Hz). H-2" which is a doublet doublet is also coupled to H-3" (J = 3.4 Hz). H-3" (doublet doublet) is coupled to H-4" (J = 5.8 Hz).

The proton shifts of H-4" and H-5" overlap at 5 3.4 which appears as a double quartet. H-5" is coupled to the methyl group at C-6" (J = 2.8 Hz) and to H-4" (J = 8.5 Hz).

As the coupling constant of the anomeric proton is 1.2 Hz the sugar is in the beta configuration (axial-axial or axial-equitorial coupling). This is unusual as flavonoid rhamnosides usually occur as alpha-L-rhamnosides.

Taking into consideration all the spectroscopic data, OCB-3 is 3-0-beta-L- rhamnosyl, 3', 4', 5, 7 tetrahydroxyl flavone. The common name of this compound is quercitrin or quercetin 3-0-beta-rhamnoside (Figure 8). The UV maxima (methanol) of quercitrin occur at 256 nm and 350 nm (Marby *et al*1969). This corresponds well to the UV maxima (methanol) of OCB-3 (256 and 351 nm).

The proton and carbon-13 NMR assignments of OCB-3 are as follows: [1]H-NMR (200 MHZ, acetone de): 5 6.26 (1H, d, J 2 Hz, H-6), 6.47 (1H, d, J 2 Hz, H-8), 7.49 (1H, d, J 2.2 Hz, H-2'), 6.98 (1H, d, J 8.4 Hz , H-5'), 7.38 (1H, dd, J 8.4 and 2.2, H- 6'), 12.72 (1 OH, C-5), 5.50 (1H ,d, J 1.2 Hz, H−1"), 4.21 (1H, dd, J 1.2 Hz and 3.4 Hz, H−2"), 3.73 (1H, dd, J=3.4Hz and 5.8 Hz, H-3"), 3.40 (2H, double quartet, J 2.8 Hz and 5.8 Hz, H-4" and H-5"), 0.92 (3H, d, J 2.8 Hz, H-6").; [13]C NMR (50 MHZ, acetone d*): 5 157.3 (C-2), 135.2 (C-3), 178.6 (C-4), 162.2 (C-5), 98.8 (C-6), 164.5 (C-7), 93.9 (C-8), 157.7 (C-9), 105.0 (C-10), 122.1 (C-l'), 115.4 (C-2'), 145.2 (C-3'), 148.5 (C-4'), 116.1 (C-5[1]), 122.0 (C-6'), 102.0 (C-1[M]), 70.7 (C-2[M]), 71.3 (C-3"), 72.3 (C-4"), 70.8 (C-5"), 17.10 (C-6").

CHAPTER THREE

PHARMACOLOGICAL INVESTIGATIONS

3.1 INTRODUCTION AND LITERATURE SURVEY

3.1.1 <u>Studied pharmacological effects of various *Clematis* species</u>

There are about fifty documented *Clematis* species of medicinal importance. About 20 species are found in China where they are widely used as analgesics and to manage: rheumatism; febrile illness; sinusitis, colds; sexually transmitted diseases; dermatological; and abdominal disorders. Almost all *Clematis* species have vesicant effects on the skin. Various *Clematis* species are claimed to have diuretic, antitumor, purgative, abortificient, and antihelminthic activities.

Many *Clematis* species have been studied for their antimicrobial effects. Many species lack antimicrobial activity. The few that have demonstrated *in vitro* antimicrobial activity include C. *gouriana* (Bashar, 1991) and C. *insodentata* A. Rich (Omer *et al.,* 1998). Extracts from C. *vitalba* obtained from North America demonstrated very high activity against *Mycobacterium tuberculosis* (Mc Cutcheon *et ai,* 1997). In Guatemala the extracts of the roots of C. *dioca* showed strong activity against *Niesseria gonorrheae* (Caceres *et ai,* 1995).

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Capuchin monkeys in Costa Rica have been observed to apply parts of *Clematis dioca* topically due to its insect repellent properties. The indigenous peoples of the Americas similarly use the plant topically for its insect repellant effects (Baker, 1996). Methanol extracts of *Clematis florida* have been demonstrated to have larvicidal activity against *Thecodiplosis*, which is a pest of the *Pinus* species (Kim *et al.,* 1996).

Extracts of C. *chinensis* have a hypotensive effect in rats (Chiu *et al.,* 1995).

Extracts of *Clematis flamna,* used traditionally in Turkey for the management of various inflammatory conditions, inhibited interleukin and tumor necrosis factor synthesis thus providing evidence for antiinflammatory activity (Yesilada *et al*., 1997).

3.1.2 Previous pharmacological tests done on *Clematis brachiata* Thunb.

The leaf macerate and leaf juice from Rwanda showed antimalarial activity (Hakizamungu and van Puyvelde, 1988).

An ethanol-water (80 %) extract of the leaf (Rwanda) inhibited lymphocyte (mononuclear leukocytes) proliferation thus providing evidence for potent anti-inflammatory activity (Lasure, *et al*., 1995).

Ethanol extracts of the dried root from Rwanda showed uncertain activity against coxsackie, measles and polio viruses. The extract was inactive against herpes and semlicki-forest viruses (Vlietinck *et al.* 1995).

A methanol extract of the leaf (1.0 mg/ml) from Rwanda showed 100 % activity against *Trichmonas vaginalis* (Hakizamungu *et al.,* 1992).

The methanol extract of the dried root (10 mg/ml) from Tanzania was inactive against *Shigella boydii* and *Niesseria gonorrheae* but showed weak activity against *Staphylococcus aureus* (Chabbra and Usio, 1991).

3.1.3. Malaria and other infectious diseases

3.1.3.1 Background on Malaria and other infectious diseases

Diseases caused by protozoa include malaria, trypanosomiasis and leishmaniasis. Malaria is the most serious parasitic infection in man. It has a global incidence of 200 million cases per year and an annual mortality of 2.7 million people per year (Palmer *et al.*, 1998). The global incidence of malaria is increasing due to drug resistance, human migration, climatic changes and poverty (Woube, 1997; Lindsay *et al.*, 1998). In the recent decades there has been an emergence of parasites resistant to drugs. Chloroquine resistance is widespread in nearly all malaria zones. Widespread resistance to the first line antimalarial drug, Suphadoxine-Pyrimethamine (SP), is expected to develop soon. Resistance to antibacterials and antifungals has rendered many low cost drugs ineffective thus straining the health budgets of poor developing countries. Most organisms are resistant to multiple drugs in the essential drug list (WHO, 1997). Drug resistance is a global problem and hence the pressure to develop new antimicrobials.

Plants have provided lead molecules for the management of malaria (Kirby, 1997). The first effective antimalarial, quinine, was isolated from Chincona-and still remains useful for the management of severe and complicated malaria.

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Recently artemisinin isolated from *Artemisia* species has proved to be useful for the management of drug resistant severe and complicated malaria (Bouree, 1997).

The *in vivo* tests for antimalarial activity include: the Roehl test; Davey's test; four-day suppressive Peter's test; the twelve-day Rane test; and the thirty day Rane test (Chinchilla *et al*, 1998). The disadvantage of *in vivo* methods is that they make use of non-human malaria parasites such as *Plasmodium berghei* and the results may not apply to humans. Few compounds can be screened at a time.

The earliest *in vitro* tests for antimalarial activity include: macro-test (Rieckmann, 1978); micro test; and the visual micro test (Rieckmann, 1982). The first two methods depend on the microscopic detection of parasites obtained from infected humans or monkeys. The third method depends on the visual detection of pigment formed when the ling forms mature to schizonts. The first semi automated *in vitro* test for antimalarial activity was described by McCormick and Canfield (1972) and is based on the suppression of incorporation of 6-^{14}C-Orotic acid into the deoxyribonucleic acid of P. *knowlisi* obtained from Rhesus monkeys. Desjardins *et al.,* 1979 developed a method based on the inhibition of uptake of radioactive hypoxanthine by *P. falciparum* grown in an in vitro culture. This method was modified by Le Bras *et al*1983 and Li *et al.,* 1983. Rieckmann's method is reliable and accurate but depends on parasite growth in primate hosts. The isoptic method is rapid and has better precision.

A good correlation was established between the morphologic and isotopic methods (Desjardins *et al.,* 1979).

The method described by Valecha *et al.,* 1994 is a modification of the Rieckmann's test where the parasites are cultured *in vitro* and parasitemia is determined using a thin blood film as opposed to a thick blood film.

3.1.3.2 Compounds isolated from the Ranunuculaceae family with antimicrobial activity.

Four different kinds of isoquinoline alkaloids have been isolated from the Ranunculaceae family: protoberberine group; bisbenzylisoquinoline type; aporphine; and quinolizidine - based alkaloids.

Berberine, palmatine, and jatorrhizine are examples of protoberberine alkaloids that have *in vitro* antimalarial activity. Their *in vitro* activity is comparable to that of quinine but they lack *in vivo* activity against *Plasmodium berghei.* In a clinical trial, a combination of pyrimethamine and berberine showed very good efficacy against multidrug resistant *P. falciparum* (Sheng *et al.,* 1997). Protoberberine alkaloids also have antibacterial activity. Berberine has activity against *Mycobacterium* (Newall *et al.,* 1996).

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Magnoflorine and corytuberine are aporphine alkaloids isolated from C. *recta* (Slavik and Slavikova, 1995). Aporphine alkaloids isolated from *Thalictrum faberi* (Ranunculaceae) have potent *in vitro* anti malarial activity (Lin *et al.,* 1999).

Bisbenzylisoquinolines are quaternary ammonium compounds. They are made up of two isoquinoline units joined together by two or more ether bridges. Examples include tiliacorine, tiliacorinine and nor-tiliaconrinine. Pheanthrine and cocsuline inhibit *Leishmania* species, *Trypanosoma cruzi* and *Mycobacterium smegmatis* (Neuwinger, 1994). Bis benzylisoquinoline alkaloids demonstrated *in vitro* antimalarial activity against multidrug resistant *P. falciparum* (Frappier *et al*., 1996; Angerhofer *et al.,* 1999).

Over 25 triterpene saponins isolated from *Cimicifuga* species had *in vitro* antimalarial activity. Activity was linked to triepoxy substitution on the triterpene moiety (Takahara *et al*., 1998).

Ranunculin, anemomin and protoanemonin are unsaturated lactones and are widely distributed in the Rannunculaceae family. Ranunculin is a glycoside with an acrid smell and it is rapidly hydrolyzed to protoanemonin which is highly volatile . Protoanemonin is rapidly dimerized to anemonin. Ranunculin, protoanemonin and anemonin have bactericidal, fungicidal and antihelmintic activity (Neuwinger, 1994).

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Diterpenoids isolated from *Delphinium denudatum* demonstrated *in vitro* antifungal activity (Atta-ur-Rahman *et al.,* 1997).

Quercetin has been isolated from *Clematis stans* from Japan (Kizu et al., 1995). It has strong *in vivo* antimalarial activity against *Plasmodium berghei.* Its glycoside lacks antimalarial activity (Castros *et al*., 1996). It has cytotoxic (Jagadeewaran *et al*., 2000) and moderate antiviral activity (Amaral *et al*., 1999).

3.1.4. Pain

3.1.4.1 Background on pain management

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. The term pain is avoided when describing animal models of nociception. Nociception is defined as activity in nociceptors and nociceptive pathways and other neurophysiological processes induced by a noxious stimulus.

Many people suffer from severe and intractable pain. The most important classes of drugs used in the management of pain are opioids and non-steroidal anti-inflammatory agents (NSAJAs). The latter are only useful for the management of mild to moderate pain. Only two thirds of patients who suffer from chronic pain experience good or moderate pain relief when treated with opiates.

The remaining third do not respond due to either primary insensitivity to opiates or development of tolerance. Patients with neuropathic pain show primary insensitivity to opiates.

The use of opiates is also associated with respiratory depression, nausea and vomiting (Mc Quay and Moor, 1997). NSAIAs may cause gastric ulceration or damage to the liver and kidneys. Local anesthetics are used to manage localized pain and neuropathic pain (Oskarsson *et al.,* 1997).

Analgesics and local anesthetics were developed from compounds isolated from higher plants. Cocaine was the first local anesthetic and was isolated from *Erythroxylon coca* in 1860 and has been in clinical use an ophthalmic anesthetic since 1884. Morphine was isolated from *Papaver somniferum* by a German pharmacist in 1803. The plant has been in use about 6000 years. Opioids are the most potent analgesics in clinical use. The willow bark was used in folk medicine for the relief of mild pain and fever for many years. The active ingredient is salicin, which on hydrolysis yields salicylic acid from which NSAIAs were developed.

3.1.4.2 The analgesic and local anesthetic effects of compounds isolated from the Ranunculaceae family

Aconitum alkaloids are used in Japanese and Chinese herbal medicine as analgesics, antirheumatics and in the management of various neurological conditions. Diterpene alkaloids are highly oxygenated complex alkaloids and all known types have been isolated from the *Aconitum* and *Delphinium* genera.

Diterpene alkaloids have analgesic, anti-inflammatory and local anaesthetic activity; class I antiarrhythmic activity and anticonvulsant activity (Ameri *et al.,* 1998). They exert their anesthetic effect by suppressing the inactivation of voltage dependent sodium channels. They bind to site two of the alpha sub unit of the sodium channel.

Seven saponins based on hederagenin and oleanolic acids were isolated from C. *chinensis* and demonstrated antinociceptive activity in laboratory animals (Xu *et al*., 1996). Oleanolic and ursolic acid based triterpenes are selective inhibitors of cycloxygenase 2 activity (Suh *etal.,* 1999).

Anemonin and protoaneonin have shown *in vivo* analgesic, sedative and antipyretic effects *in vivo* (Newall *et al.,* 1996).

Various flavonoids and coumarins isolated from the Ranunuculaceae family have analgesic and anti-inflammatory activity. They act by inhibiting cycloxygenase, lipoxgenase and phosholipase A_2 (Lindahl and Tagesson, 1997).

Some quaternary bisbenzylisoquinolines have analgesic and anti inflammatory effects (Neuwinger, 1994).

3.1.5. <u>Abdominal disorders</u>

3.1.5.1 Background on abdominal disorders

The most common abdominal disorders are: dyspepsia; intestinal spasms such as colic; peptic and duodenal ulcers; and diarrhea secondary to infection. The abdominal disorders associated with pregnancy are nausea, vomiting and reflux oesophagitis. In the third world, diarrhea is often a result of protozoal infection in the gastrointestinal tract. Protozoa that cause diarrhea include *Giardia lamblia, Entamoeaba histolytica* and *Crpytosporidium parvum. Giardia lamblia* infects 200 million people each year and causes 10 000 deaths yearly. *Entamoeba histolytica* infects 42 million people yearly.

Clematis brachiata is widely used for the management of abdominal disorders though the exact nature of abdominal disorders is not specified in literature. It may have antimicrobial, spasmolytic, anti ulcer or anti-emetic activity.

The plant kingdom has produced many spasmolytics such as papaverine, tropane alkaloids, essential oils (peppermint, dill and anise). The spasmolytics in use have adequate efficacy. On the other hand, the management of nausea and vomiting due to travel illness, pregnancy and cancer chemotherapy is inadequate. Antiemetics in use lack adequate efficacy.

3.1.5.2   Spasmolytic and anti ulcer activities of compounds isolated form the Ranunculaceace family

The flavonoids, apigenin, kaempferol and quercetin, isolated from *C.stans* and C. *koreana* (Kizu *et al.*, 1995), have spasmolytic activity (Mata *et al*1997).   They inhibit acid production in isolated parietal cells by inhibiting the proton pump. They stimulate the production of Prostaglandin E_2 and inhibit the growth of *Helicobacter pylori* in a dose dependent manner (Beil *et al.,* 1995).

Berberine, the prototype of protoberberine alkaloids, is used in modern therapeutics for the management of diarrhea. The pharmacological activities that make it useful for the management of diarrhea and other abdominal disorders are: antiprotozoal; cholagogue; spasmolytic; anticholinergic; antihistamine; sedative; immunostimulant; alpha 2 adrenoceptor agonist; and antimicrobial effects (Brunton, 1996),

The roots of *Clematis brachiata* are said to cause diarrhea suggesting the presence of anthraquinones. Rhein, an anthraquinone, causes dose dependent bacteriostatic activity against *H. pylori* (Chung *et al.,* 1999) and increases intestinal epithelial cell proliferation by increasing Prostaglandin E_2 levels (Nishikawa *et al.,* 1997).

3.2 *MATERIALS AND METHODS*

3.2.1 MATERIALS

DRUGS USED IN PHARMACOLOGICAL TESTS

Chloroquine diphosphate courtesy of Lab and Allied, Nairobi

Clotrimazole courtesy of Universal Pharmacy, Nairobi

Ciprofloxacin courtesy of D. A. R. U., Nairobi

Indigo carmine stain C. 1.73015 (BDH stains. BDH chemicals, England)

Sennokot® tablets (Reckitt and Colman, Nairobi, Kenya)

Procaine hydrochloride (Aldrich Chemical Co. Ltd., Gillingham Dorset, England, Analar)

Morphine hydrochloride (Mac Farlan Smith Ltd., Edinburgh, U.K.)

Normal saline (Infusion Kenya Ltd., Nairobi)

Veet-0® (Reckett Collman, Nairobi)

SALTS FOR PHYSIOLOGICAL SOLUTIONS

Calcium chloride (Associated Chemical Enterprises, Reuven Estates, South Africa)

Sodium chloride (Associated Chemical Enterprises, Reuven Estates, South Africa).

Potassium chloride (Associated Chemical Enterprises, Reuven Estates, South Africa)

Magnesium chloride (S. D. Fine Chemicals, Nairobi, Kenya)

Sodium bicarbonate (Kanha Laboratory, Nairobi, Kenya)

Sodium dihydrogen ortho phosphate (BDH Chemicals, Poole, England)

Glucose (Aldrich Chemical Co., Gillinghan-Dorset, UK)

MATERIALS USED TO TEST FOR ANTIMALARIAL ACTIVITY

Buffer tablets - pH 7.2 (Merck, Darmsadt, Germany, ACROS Organics, New Jersey, USA)

Citrate monohydrate (ACROS Organics, New Jersey, USA)

Giemsa stain (Belani Fine Chemicals, India)

D-Glucose (Sigma Co., St. Louis, USA)

Glycerol (ACROS Organics, New Jersey, USA, Analar)

D (+) Mannitol (ACROS Organics, New Jersey, USA)

D-Sorbitol (BDH Ltd., Poole, England, Analar)

Sodium bicarbonate (ACROS, Organics, New Jersey, USA, Analar)

Sodium chloride ((ACROS, Organics, New Jersey, USA, Analar))

Tri-sodium citrate (Associated Chemicals, South Africa)

Citric acid

HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid) (ICN Biomedicals Inc., Ohio, USA)

RPMI 1640 powder medium, without glutamate, without sodium bicarbonate (Life Technologies, Grand Island, N.Y., U. S. A.)

Gas mixture (5% oxygen, 3% carbon dioxide, and 92% nitrogen, East African Oxygen Nairobi)

MATERIALS USED TO TEST FOR ANTIMICROBIAL
ACTIVITY

Thayer Martin Medium without Inhibitors (Becton Dickinson and
Co., Cockeysville, MD, USA)
Mueller Hinton agar (Oxoid, Unipath Ltd., Hampshire, England)
Sabouraud Dextrose Agar for Fungi (Topley House, Bury,, UK)

MICRO ORGANISMS

The bacteria and fungi were provided courtesy of D.A.R.U.
Plasmodium falciparum was provided courtesy of K.E. M.R.I,
parasitology laboratory.

Aspergillus niger

Candida albicans

Escherichia coli

Niesseria gonorrheae

Plasmodium falciparum

Staphylococcus aureus

LABORATORY ANIMALS

The animals were obtained from the National Public Health
Laboratories, Nairobi, Kenya.

Guinea pigs
New Zealand rabbits
Swiss albino mice

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BRINE SHRIMP BIO ASSAY MATERIALS

Brine shrimp eggs (Interpret Ltd., Dorking, England)

Marine salt obtained from Nairobi Pet Shop, Nairobi, Kenya

Baker's yeast (Excel Chemical Ltd., Nairobi)

EQUIPMENT

Analgesymeter (Ugo Basile Comerio- va, Model V220, Italy)

Anaerobic jar (Oxoid, Hampshire, England)

Antibiotic zone reader (I. E. C., England)

Centrifuge machine(I.E.C. Centra-CLD, I.E.C., Bedfordshire, England)

Culture flasks (Becton Dickinson, France)

Gas tight box for incubation/ "Caison de Wolfe" (Bellco, Vineland, New Jersey, USA)

Incubator (Type B4057, Bergen, Norway)

Kymograph (CAT. No. 1020, Scientific and Research Instrument Ltd., England)

Laminar flow hood (Analis ah 120, Heto Holten, Denmark)

Liquid nitrogen cryopreservation container (Minnesota Valley Engineering Inc. Ltd., New Prague, U. S. A.)

Marsh II automated cell harvester (Microbiological Associates, Bethesada, USA)

Membrane filters (0.22 p.l, Becton Dickinson and Co., Cockeysville, MD, USA)

Micro/Macro pippetor (Socorex ISBA S. A., Switzerland)

Microscope (Nikkon, Japan)

Organ bath

PH meter (pH system 1001, Senton, Roden, Netherlands)

Portable autoclave (Dixon's Surgical Instruments Ltd., England)

Refrigerator

Rotary vacuum evaporator (Heidolph WB 2000, Belgium)

Liquid scintillation spectrometer (Searle Model Delta 300)

Solvent distiller

Top loading balance (Analis, Belgium)

Vacuum pump

Voss Maldon shaker (Voss Instruments Ltd., Essex, England)

Water distiller

3.2 METHODS

3.2.2.1 Preparation of extracts

Two hundred and fifty grams of the dried leaf, root and stem powder were separately subjected to extraction by cold maceration with one liter of 80 v/v % methanol - water for seven days. The solvent was changed daily. The extract was filtered and reduced to dryness under vacuum at - 15 °C. The extracts were used for pharmacological investigations. The yields of the cold methanol extracts of the stem, leaf and roots were 20.0 % w/w, 17.0 % w/w and 40.4 % w/w of the dried plant part respectively.

3.2.2.2 Brine Shrimp Lethality Bioassav

Hatching the shrimps

A two-chambered plastic container was filled with artificial seawater (33 g of marine salt, 6 mg of baker's yeast in one liter of water). The chambers were separated by a plastic divider with several holes of diameter 2 mm. One chamber was darkened and the other chamber was illuminated. About 50 mg of brine shrimp eggs were sprinkled in the darkened side. After 48 hours the phototrophic larvae migrated to the illuminated side and were harvested using a pipette.

Preparation of test solutions

A stock solution was prepared by dissolving 50 mg of the extract in 5 ml of brine solution to attain a concentration of 10 000 jil/ml. In cases where solubility was poor, the extract was first dissolved in 0.1 ml of dimethyl sulfoxide or Tween 80 and then made up to 5 ml using the brine shrimp solution.

Procedure

Using artifical seawater, serial dilutions of the extracts were prepared and solutions with the following concentrations were obtained: 1000 jig/ml; 100 |ig/ml; and 10 ng/ml. Five milliliters of each dilution of the extracts were transferred to five different test tubes. Each test tube was inoculated with 10 larvae. A set 50 larvae in five different test tubes were grown without exposure to the extracts/isolates and these served as the negative control.

After 24 hours, all the live larvae at concentration were counted and percentage death was calculated.

3.2.2.3 Test for Antimalarial Activity

3.2.2.3.1 Growth and maintenance of the parasite culture

<u>Preparation of culture medium</u>

All glassware used was sterilized by dry heat at 140 °C for 30 minutes. Pipette tips were sterilized by autoclaving at 121 °C for 15 minutes at a pressure of 2 bars.

RPMI 1640/HEPES solution was prepared by dissolving 5.94 g of HEPES acid and 10.4 g RPMI medium 1640 in distilled water to a final volume of 960 ml. The RPMI 1640 solution was sterilized using a 22 jim membrane filter. The medium was stored at 4 °C for a maximum of four weeks.

Sterile 5 % w/v Sodium bicarbonate solution was prepared by dissolving 5 g of Sodium bicarbonate in up to 100 ml of freshly distilled water. It was sterilized by using a 22 jim membrane filter. It was stored for a maximum of two weeks at 4 °C.

Heat treated frozen-pooled human serum, obtained from Kenya Medical Research Institute, Nairobi, Kenya, was thawed at 37 °C.

Complete culture medium (CMS) was prepared by mixing freshly prepared 1.89 ml sterile 5 w/v % sodium bicarbonate solution; 43.1 ml sterile RPMI 1640/HEPES solution; and 5 ml of thawed serum.

O positive blood was collected from a donor who had not been on medication for at least six months. The blood was drawn into a syringe containing sterile citrate anticoagulant (2.2g Tri-sodium citrate; 0.8 g citric acid; and 2.45 g of dextrose in up to 100 ml of freshly distilled water).

Three milliliters of the anticoagulant were added to a 15 ml centrifuge tube under aseptic conditions. The blood was mixed well with the anticoagulant and stored at 4 °C and discarded three months after collection. At least once a week the blood was mixed by swirling.

The blood was washed four times with Wash Medium (48 ml of sterile 1640/Hepes stock solution and 2 ml 5 % w/v sodium bicarbonate) to remove any leukocytes that may interfere with the analysis.

The haematocrit was adjusted to 50 % by adding one packed cell volume of wash medium to the packed cells. The cells were resuspended, flushed with culture gas and stored at 4 °C for a maximum of one week.

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Initiation and maintenance of the Culture

A cyropreserved a multidrug resistant strain of *Plasmodium falciparum*, Vl/S, was obtained form KEMRI Parasitology Laboratories, Nairobi, Kenya. Vl/S strain was obtained in 1969 from an American soldier and is resistant to chloroquine, quinine and pyrimethamine (Martin *et al.*, 1973; Canfield, 1971). A multidrug resistant strain was chosen for study due to the high prevalence of chloroquine resistant strains worldwide.

The frozen parasites were rapidly thawed to room temperature **and** the isotonicity was adjusted as described by Watkins *et al.,* 1987

The parasitised red blood cells were washed with CMS three times and the supernatant was discarded. For every 0.25 ml packed cell volume of parasitised red blood cells, 0.7 ml 50 % haematocrit red blood cells and 9.05 ml CMS were added to give to final haematocrit of 6 %. The parasites being microaerophilic were flushed with oxygen, carbon dioxide and nitrogen and incubated at 37.2 °C. The medium was changed daily to remove any toxic metabolic wastes and to renew nutrients.

The parasitemia was determined after every three days by Giemsa stain thin film technique. When the parasitemia exceeded 2%, it was diluted by adding'appropriate volumes of C M S and washed red blood cells.

x

The growth rate per 48 hours was calculated using the following formula:

Growth Rate = (Pf /Pi) * 2/ n

Where Pf is the Final parasitemia;

Pi is the Initial parasitemisa; and

n is the number of days in the culture (Chulay *et al.,* 1984).

When the culture had achieved a growth rate of more than 3 % in 24 hours, it was considered adapted to *in vitro* culture and ready for *in vitro* drug sensitivity tests.

Preparation of Stock Test Solutions

About 10 mg of FAO-FRS, FAO-WR and chloroquine diphosphate were separately dissolved in 10 ml of distilled water to produce stock solutions of concentrations 1000 jig/ml.

The alcoholic plant extracts, which were poorly soluble in water, were initially solubilized by dissolving in 1 mg of extract in 50 fil of 70 % v/v ethanol and distilled water added to obtain stock solutions of concentration 1000 jig/ml.

The final concentration of ethanol in the stock test solutions was less than 0.1 % and at this concentration it had no measurable effect on parasite growth. The stock drug solutions were sterilized by membrane filtration under aseptic conditions.

. v^x

3.2.2.3.2 Testing for schizontocidal activity by optical microscopy

The method used was a slight modification of that described by (Valecha *et al.*, 1994). Fifty microliters of CMS were added to all the wells in a 96 well microtitration plate made up of 8 rows (A to H) and 12 columns (1 to 12). Fifty microliters of the stock drug solution were to three different three wells in the first row. The first nine wells accommodated 3 different test extracts/isolates. Fifty microliters of CMS were added to each of the last three wells on the first row. After mixing well, 50 [i\ removed from each of the wells in the first row and transferred to the adjacent well on the second row. The contents of the second row were mixed well and 50 [x\ were removed from each well on the second row and transferred to the adjacent well on the third row. This process was repeated till all the wells had been loaded with drug. Hence a two-fold dilution of the stock solution in triplicate was achieved down each column.

A 50 |il suspension of *P. falciparum* Vl/S strain infected red blood cells of parasitemia 0.4 % and haemotocrit 1.5 % were added to each well.

The plate was covered and incubated at 37.2 °C in a humidified anaerobic chamber for three days. The parasitemia in each well was determined by Giemsa stain thin smear technique.

The percentage inhibition at each concentration was determined using the following formula:

% Inhibition = $[1 - (Po/P_c)] \times 100$

P**d**: Mean parasitemia of the wells with the drug

P_c: Mean parasitemia of the wells without the drug

3.2.2.3.3 Test for antiplasmodial activity by inhibition of uptake of radioactive hypoxanthine

The method was adapted from that described by Jensen *et al.,* 1982. Fifty microliters of CMS were added to each well to a 96 well microtitration plate made up of 8 rows (A to H) and 12 columns (1 to 12). The hematocrit of the culture was reduced to 1.5 % by diluting with CMS.

One hundred microliters of parasitised red blood cells were added to each of the first eight wells on the Row A and these served as the negative controls. One hundred microliters of 1.5 % hematocrit non parasitized red blood cells were added to each of the last four wells on the first row. This provided a control for background radiation. To row B, 50 fil of the stock solution of each drug was added to two adjacent wells. Six different drugs were accommodated on each row. After mixing the drug solutions well with culture medium, 50 jal were transferred from th^well in Row B to the adjacent well in row C.

This process was repeated down the column till a serial two - fold dilution was achieved across each row in each column.

One hundred microliters of parasitized red blood cells were added to each of the wells containing drug solution. The plates were flushed with gas mixture and transferred to a humidified gas tight box. The box was flushed with gas mixture for about twenty minutes and transferred to an incubator at 37.2 °C. After 48 hours when the parasite was at the stage of mature trophozoite, 50 (il of radiolabeled hypoxanthine solution (0.5 mCi per 25 jal) were added to each well. The plates were incubated for an additional 18 hours after which they were harvested.

Harvesting The Parasites And Scintillation Counting

The particulate contents of each well were deposited separately on small disks of filter paper (Whatmann No. 939). The disks were washed with copious amounts of distilled water and then dried at 60 °C for three minutes. Each disk was placed in a glass scintillation vial containing 10 ml of a toluene based scintifluor. Counting was done using a liquid scintillation spectrometer.

3.2.2.4 Test for antifungal and antibacterial activity

Preparations solutions of the extracts/isolates

The extracts that were poorly water-soluble were initially solubilized by dissolving in 50 ^l of dimethyl sulphoxide or Tween 80. The solutions were then made up to 5 ml using water. Clotrimazole and ciprofloxacin were used as the positive controls and were prepared by dissolving in water to obtain final concentrations of 100 ^ig/ml each.

 Test for antimicrobial activity using the well diffusion method

All glassware was sterilized by dry heat at 200 °C for one hour. Ten milliliters of water was sterilized autoclaving at 121 °C, 2 bar pressure for 15 minutes. All work was carried out using aseptic techniques. All bacteria except *Neisseria gonorrheae* were grown on Mueller Hinton medium which was prepared by dissolving 38 g of agar powder in one liter of distilled water and sterilized by autoclaving. *N. gonorrheae* was grown on Thayer Martin Medium without Inhibitors. All fungi were grown on Sabouraud Dextrose medium which was prepared by dissolving 62 g of Sabouraud Dextrose agar in one liter of water and sterilized by autoclaving at 121 °C, 2 bar pressure for 15 minutes.

The extracts were tested for activity against *Candida albicans, Aspergillus niger, Staphylococcus aureus, Bacillus subtilis Escherichia coli* and *Neisseria gonorrhea.* Working cultures were grown using the slope technique. A microbial suspension of each organism was prepared by gently suspending the organism in the slope culture in about 10 ml of sterile water. Three milliliters of the microbial suspension was added to one liter of sterile molten medium at a temperature of about 45 °C. Twenty milliliters of seeded molten medium was added to different ten petri dishes and the medium was left to set for about one hour. A borer was used to drill six wells in each petri dish.

Fifty microliters of test solution was added to each well. Six replicates were prepared for each extract. Sterile water was used as the negative control and ciproflocaxin and cotrimoxazole were used as the positive controls. Each petri dish accommodated six different drugs. All bacteria were incubated at 37.4 °C for 24 hours and the fungi were cultured at 24 °C for three days. *N. gonorrhae* was grown in an anaerobic jar. After incubation, the zones of inhibition were measured using a venire caliper.

3.2.2.5 Test for local anesthetic activity

Twelve adult guinea pigs of either sex, weighing between 300 and 400 g were obtained from the National Public Health Laboratories, Nairobi, Kenya. They were fed on animal pellets, vegetables and water *ad libitum* and bred in an open space under a twelve-hour dark/light cycle.

Twenty four hours before the experiment, the hair on the lower back was clipped with a scissors and a depilatory cream, Veet-o   was used to shave the back clean. Four sites near the midline were chosen.

The cold methanol extracts of the stem and leaf were dissolved in water and filtered before use. The concentrations of the extracts were: stem extract (260.0 mg/ml); leaf extract (12.60 mg/ml; and FAO-FRS (21 mg/ml). Sterile water was used as the negative control. Into each site, 0.4 ml of drug/extract were injected intradermally and formed wheals which were outlined using a felt pen. The doses were determined empirically.

The wheal was pricked lightly six times with a pin and the number of no responses by the animal was recorded. This was done every five minutes for the first half-hour and then at 10 minute intervals for the remaining half-hour. Each drug was tested on six different animals.

$$v^x$$

<1

3.2.2.6 Tests for antinociceptive activity

3.2.2.6.1 The hot plate method

The test for antinociception (hot plate method) was done as described by Woolfe and Mac Donald (1944). Swiss albino mice of either sex, maintained under standard laboratory conditions (room temperature, 12-hour light/dark cycle, food and water *ad libitum)* were obtained from the National Public Health Laboratory. A hot plate was set at a constant temperature of between 55 to 60 °C. The mice weighing 18 to 31 g were screened by putting them on the hot plate. Those that failed to respond by standing on the hind legs and licking the front paws within 30 minutes were omitted form the test. The time taken to respond was recorded and this was the pre test response.

The mice were divided into groups of eight each. The mice in the control group were injected with 0.25 ml of normal saline intraperitoneally. The mice in the second group received 10 mg/kg of body weight of morphine. Morphine was used as the positive control because peripherally acting analgesics like NSAJAs give inactive in this test for antinociception. Two groups received the stem and leaf extracts (300 mg/kg of body weight). The last group received 100 mg/kg of body weight FAO-FRS. All the drugs were administered intraperitoneally. The doses were determined empirically.

Half an hour later, the response time of each mouse on the hot plate was determined.
x

3.2.2.6.2 The tail pressure method

The method used was a slight modification of that described by Randell and Selitto, 1957. Swiss albino mice weighing 18 to 31 g were divided into six groups of six to eight animals each. The pre-test response was obtained by placing the tail (2 - 3 cm from the tip) of each mouse under the Teflon tip of an Analgesymeter. The pressure on the tail was increased gradually till the animal began to struggle. To avoid bruising the animal the pressure was restricted to a maximum of 25 units. The pressure was read on a scale of 0 to 25 units.

The control group received 0.25 ml of normal saline. The mice in the second group received 10 mg/kg of body weight of morphine. The mice in test groups received 100 mg/kg of body weight of the plant extracts and FAO-FRS. All the drugs were given intraperitoneally.

An hour later the post drug response was obtained.

3.2.2.7 The effects on the gastrointestinal system

3.2.2.7.1 Effect on the isolated rabbit ileum

Male New Zealand rabbits were obtained from the National Public Health Laboratory and bred under standard laboratory conditions (room temperature, 12 - hour light/dark cycle, food and water *ad libitum)*. A rabbit was killed by a blow at the back of its head. The neck was cut and the animal was let to bleed.

The abdomen was cut open and a 4 to 5 cm piece of the ileum was cut out and mounted in a 25 ml double walled organ bath containing Tyrode's solution of the following composition: Sodium chloride - 160 g; Potassium chloride - 4 g; magnesium chloride - 2 g; calcium chloride - 4 g; sodium dihydrogen orthophosphate - 1.0 g; sodium bicarbonate - 20 g; and glucose - 20 g per liter.

The tissue was aerated with 95 % oxygen and 5 % carbon dioxide and thermostatically maintained at 37 °C. It was allowed to stabilize for 20 minutes so that the required tone would develop. The movements of the intestine were recorded on a kymograph.

Drug solution was added to the organ bath and it was allowed to act till the maximum response was obtained. The organ bath was drained and the tissue rinsed twice. It was let to rest for 2 to 3 minutes and rinsed again before adding 'the next drug.

The effect of the extract on at six different tissues was observed. The dose response effects of FAO-FRS and the stem extract were studied by recording the effect of the drug six times at four different concentrations.

3.2.2.7.2 Effects on intestinal movement

Swiss albino mice weighting 19 to 30 g were obtained from the National Public Health Laboratory. They were starved for 24 hours and then divided into groups of eight to ten.

The control group received 0.2 ml of 1.0 % w/v indigo carmine solution by gavage. The second group received 0.2 ml Senokott (10 mg/ml) and 0.2 ml indigo carmine dye. Each of the other groups receive 500 mg/kg of body weight of the plant extract orally and 0.2 ml of indigo carmine solution. The last group received 100 mg/kg of body weight FAO-FRS and 0.2 ml indigo carmine solution. The doses were determined empirically.

One hour later the mice were killed by a blow on the head and the entire alimentary canal was dissected out and carefully stretched out. Using a ruler the distance the dye had migrated was measured and expressed as a ratio .of the length of the entire alimentary canal.

N
%

3.2.2.8 Data analysis

<u>Brine shrimp lethality assay</u>

The percentage deaths were corrected by subtracting the corresponding deaths in the control group. The LD_{50} at 95 % confidence limit was determined using the Finney Probit Computer Program for analysis of quantal data (kindly supplied by Prof. J. C. Mc Laughlin, University of Purdue, U. S. A.).

<u>*In vitro* test for antimalarial activity by inhibition of uptake of radioactive hypoxanthine</u>

The IC_{50} was determined using the Q-PRO program (kindly provided by Wellcome Trust Laboratories, Kenya).

<u>Test for Local Anesthetic activity - Guinea Pig Wheal Method</u>

For each set of data, the median and mode was obtained. The Kruskal Wallis non- parametric test was used to compare the groups for differences. The group medians were tested for differences using Mood's multiple sample median test. Where a significant difference in the various drug groups was established, a non-parametric comparison of the control group to other groups was done using a Dunnett-like method..

Test for antinociceptive activity

Data from the tail pressure and hot plate method was analyzed using the paired Student-t test.

Effects on the isolated rabbit ileum

The mean ratio of amplitude of contractions before drug and amplitude after drug where determined and plotted against the appropriate bath concentration of the extract.

Effect on intestinal movement

Data was analyzed by using single factor Analysis of Variance (ANOVA).

Results and discussion

3,3'1 Results

3-3-1.1 Brine shrimp lethality assay

^{ihe} LD$_{50}$ of various extracts against brine shrimp larvae are presented on Table \ \

I^BLE 11: THE LD^o OF VARIOUS EXTRACTS AND ISOLATES *Qℒ-QlEMATlS BRACHIATA* AGAINST BRINE SHRIMP LARVAE

EXTRACT Og/mi)	LD$_{50}$ (jag/ml)	upper confidence limit (jig/ml)	lower confidence limit (Hg/ml)	g-statistic
SML	66.5	122.02	37.48	0.0610(3 df)
s p T	571.72	2101.4	249.63	0.1103 (3df)
SMS	365.60	1127.72	99.70	0.4825 (2 df)
FPS '	116.55	235.54	116.54	0.0650 (3 df)
FCS~	59.79	90.18	38.80	0.0646 (2 df)
FES	23.08'	35.14	15.07	0.0541 (3 df)

CONTINUATION: LD$_{50}$ OF EXTRACTS OF C. *BRACHIATA*				
FW	108.36	178.20	67.67	0.0524 (3 df)
FAO-FRS	>1000	-	-	-
FAO-WR	>1000	-	-	-

KEY

SML - soxhlet methanol extract of the leaf

SPS - soxhlet petroleum ether extract of the stem

SMS - soxhlet methanol extract of the stem

FPS - petroleum ether fraction of the stem

FCS - chloroform fraction of the stem

FES - ethyl acetate fraction of the stem

FW - Aqueous residue of the stem

FAO-FRS - Crystals obtained from the stem

FAO-WR - Crystals obtained from the root

3.3.1.2 Test for antimalarial activity

The Minimum Inhibitory Concentration of Extracts of *Clematis brachiata*

The minimum inhibitory concentrations (MIC) of the methanol extracts of the root stem and leaves were: 65.25 jj.g/ml; 173.25 jig/ml; and 248.25 jig/ml. The MICs were determined using the method based on optical microscopy.

The MIC of chloroquine was less than 5.06 ng/ml. None of the isolates, FAO-FRS and FAO-WR, inhibited parasite growth at concentrations of 45.55 jig/ml and 49.55jig/ml respectively.

Inhibition of Uptake of Radioactive Hypoxanthine

The calculated percentage inhibition of parasite growth by the cold methanol extracts of the root and stem is presented on Table 12.

TABLE 12: PERCENTAGE INHIBITION OF UPTAKE OF RADIOACTIVE HYPOXANTHINE BY *PLASMODIUM FALCIPARUM (V*I/S STRAIN)

STEM EXTRACT Well concentration (jig/ml)	Percentage Inhibition	ROOT EXTRACT Well concentration (jug/ml)	Percentage Inhibition
168.5	9.9	172.86	94.2
84.25	2.7	86.43	81.10
42.13	8.9	43.21	55.60
21.06	15.4	21.61	100
10.53	8.4	10.80	15.80
5.27	11.4	5.40	100
2.63	18.4	2.70	1.6

3. 3. 1.3 Test for antimicrobial activity

None of the extracts or isolates inhibited bacterial or fungal growth even at very high concentrations (10 mg/ml). The positive controls, ciproflaxcin (zone of inhibition 6 cm + 1.2, n = 3) and clotrimazole (zone of inhibition 7 cm + 2.60, n = 3) inhibited bacterial and fungal growth respectively.

3.3.1.4 Test for local anesthetic activity

Both Mood's multisample for differences between group medians and Kuskal's Wallis non-parametric tests show that there were significant differences between all five groups up to 50^{th} minute (95 % confidence limit). In the 60^{th} minute there was no difference between the test groups and control groups. This shows that for all test groups the anesthetic effect had worn off at the end of one hour.

Dunnett-like non-parametric multiple comparison of the test group with the control group showed that FRS (8.4 mg i.d.) and procaine hydrochloride (1.25 mg i.d.) had significant anesthetic activity till the 25 and 50^{m} minutes respectively. The methanol extracts of the stem (104 mg i.d.) and leaves (5 mg i.d.) had significant anesthetic activity till the 30^{th} and 40^{th} minutes-respectively. All tests were done at 95% confidence limits. &

3.3.1.5 Tests for antinociception

The Hot Plate Method

The time taken for the mice to respond to heat stimulus is presented in
Table 13.

TABLE 13: HOT PLATE METHOD - MEAN TIMES TAKEN FOR
MICE TO RESPOND TO THE HEAT STIMULUS

	Pre-test Response (minutes) X ± SD (n)	Post-test Response (minutes) X ± SD (n)
Normal Saline 0.4ml ip	9.78 + 2.46 (9)	11.89 + 3.53 (9)
Leaf extract (300mg/kg ip)	8.44 + 2.32 (8)	12.38 + 4.77 (8)
Stem extract (300mg/kg ip)	11.75 + 3.38 (8)	18.25 + 4.4.(8)
FAO-FRS extract (lOOmg/kg ip)	10.81+3.81 (8)	15.00 + 3.18(8)
Morphine (lOmg/kg ip)	13.09 + 5.07 (9)	18.15 + 4.66 (9)

In all groups, except the control group, there was a significant difference
between the pre-drug response and the post-drug response (P=0.05 (1)).

Tail Pressure Method

The pre-drug and post-drug responses of the mice to the pressure stimulus are presented in Table 14.

TABLE 14: TAIL PRESSURE METHOD - RESPONSES OF THE MICE TO THE PRESSURE STIMULUS

	Pre-test Response (Pressure) $X + SD$ (n)	Post-test Response (Pressure) $X \pm SD$ (n)
Leaf extract (lOOmg/kg i.p.)	8.00 + 3.53(8)	11.89 + 3.53 (8)
Stem extract (lOOmg/kg i.p.)	10.14 + 1.80 (7)	14.83 ± 5.41 (7)
FAO-FRS extract (lOOmg/kg i.p.)	8.00 ± 2.92 (9)	13.06 ± 5.98 (9)
Morphine (lOmg/kg i.p.)	9.25 + 2.32 (6)	13.58 + 3.50 (6)

In all groups there was a significant difference between the pre-drug response and the post-drug response (P=0.05 (1)).

3.3.1.6 Effects on the gastrointestinal system

Effect on the Isolated Rabbit Ileum

The cold methanol extracts of the root, stem and leaf consistently caused relaxation of the isolated rabbit ileum (see Figure 10). At a bath concentration of 0.4 mg/ml the effect of the root extract was brief and the tissue recovered rapidly taking only about 5 seconds to recover to the pre-existing tone. The effects of the stem and leaf extracts on the other hand were more prolonged. The tissue took about 27 seconds to recover from the effects of the of stem extract at a bath concentration of 0.915 mg/ml. The leaf extract had the most prolonged effect. At a bath concentration of 0.34 mg/ml, tissue recovery did not begin even after 30 seconds of exposure to the extract. However at lower bath concentrations recovery was much faster. The effects of the extracts were dose dependent. The log dose- response curve of the effects of the stem extract on the ileum is presented in Figure 12.

FAO-FRS had mixed effects on the isolated rabbit ileum. At low concentrations is caused relaxation of the ileum. As the concentration increased, it caused initial brief relaxation followed by increased intestinal activity.

At higher concentrations it increased intestinal activity. The kymograph tracings of the effects of FAO-FRS on the isolated ileum are presented in Figure 11. The log dose- response curve of the contractile effect of the FAO-FRS on the ileum is presented in Figure 13.
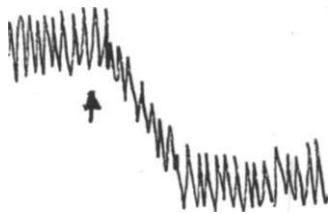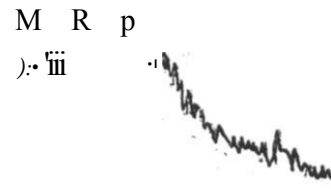
a. Effects of the Adrenaline

(Bath concentration 0.04 (ig/ml)
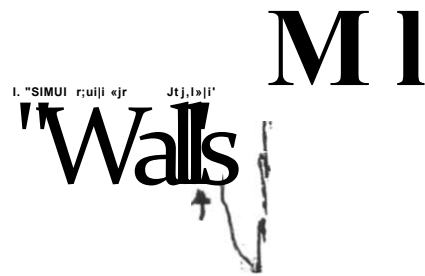
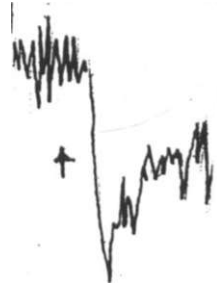b. Effects of the Root extract

(Bath concentration 4 mg/ml)

c. Effects of the Stem extract

(Bath concentration 0.92 mg/ml)

d. Effects of the Leaf extract

(Bath concentration 0.34 mg/ml)

Figure 10: The Kymograph tracings of the effects of extracts of *Clematis brachiata* **on the isolated rabbit ileum.**

iiBiil

a. Effects of the Acetylcholine

(Bath concentration 0.25 ng/ml)

I

b. Effects of the FAO-FRS

(Bath concentration 0.03 mg/ml)

c. Effects of the FAO-FRS

(Bath concentration 0.18 mg/ml)

Figure 11: The Kymograph tracings of the effects of FAO-FRS on the isolated rabbit ileum.

0   0.2   0.4   0.6   0.8   1   1.2   1.4   1.6

LOG $_{10}$ BATH CONCENTRATION

Figure 12: The log dose response curve of the relaxant effect of the methanol extract of the stem on the isolated rabbit ileum
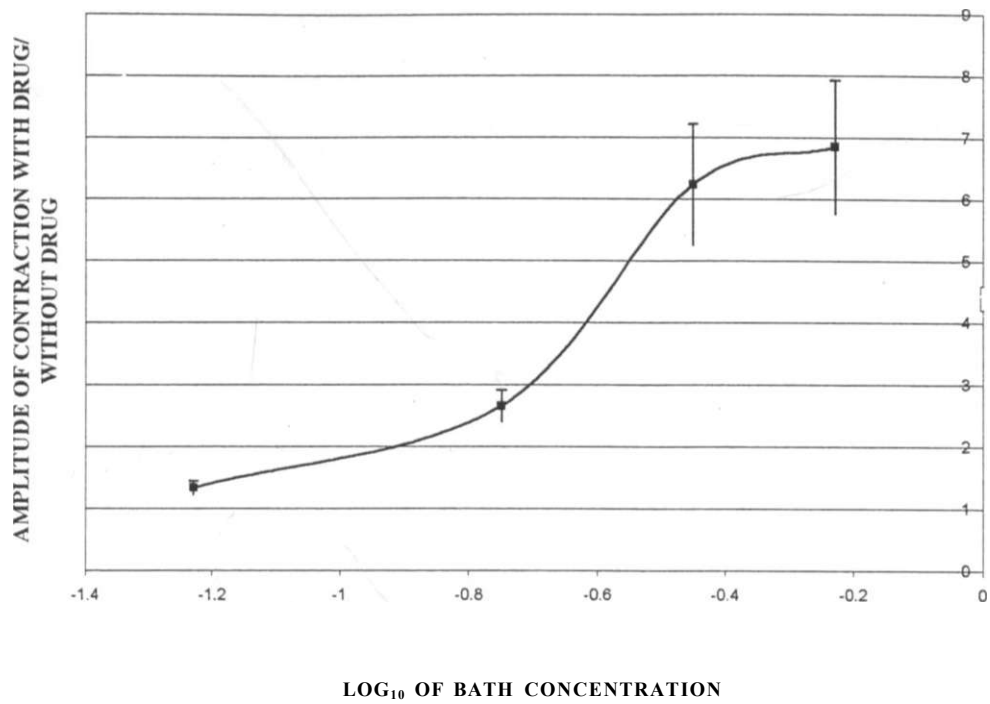
Figure 13: The log dose response curve of the contractile effect of the FAO-FRS on the isolated rabbit ileum

Effect on gastrointestinal movements

The mean ratio of the distance moved by the dye to the length of the alimentary canal when groups of mice received extracts of *Clematis brachaita* is presented in Table 15. There was no significant difference between the test groups and the control groups.

TABLE 15: EFFECT ON OF EXTRACTS OF *CLEMATIS BRACHIATA* ON GASTROINTESTINAL MOVEMENTS OF MICE

Extract/Isolate (500mg/Kg orally)	Mean of Ratio of Distance Moved By the Dye to the Length of the Alimentary canaL $X \pm S^2$ (N)
Control	0.7384 + 0.0293 (10)
FAO-FRS	0.8173 ±0.0005 (7)
STEM EXTRACT	0.8217 ±0.0013 (6)
LEAF EXTRACT	0.8187 ±0.0013 (6)
ROOT EXTRACT	0.8245 + 0.0019 (6)

3.4 Discussion

Brine Shrimp Lethality Assay

The brine shrimp lethality assay is used to detect compounds with cytotoxic and insecticidal activity (Meyer *et al*., 1982; Mc Lauhglin, 1988; and Mc Lauhglin *et al.,* 1991). Out of all the extracts, the ethyl acetate fraction of stem (FES) had greatest activity against brine shrimps (LD_{50}=23.08 ng/ml). Spot tests showed that it has the highest amounts of coumarins, flavonoids and anthraquinones. It tested positive for alkaloids. These groups have cytotoxic activity (Schneller, 1997; Johnson *et al*, 1997; Sanders *et al*, 1998; Weher *et al*, 1998).

The chloroform fraction of the stem had the second highest activity against brine shrimps. Its composition was similar to that of FES. The fractions obtained by partitioning the soxhlet methanol extract of the stem (SMS) had greater activity than the unpartitioned extract. Partitioning improves the activity of an extract by concentrating active compounds in certain fractions.

The activity of the soxhlet methanol extract of the leaves (LD_{50} = 66.50 fig/ml) was about seven times that of the stem. The leaves have good activity against brine shrimps. The crude extracts have relatively high lethality and this supports the observation in Tanzania that the plant is generally toxic (Neuwinger, 1994).

The petroleum ether fractions/extracts and the aqueous portion of the stem had the least activity against the brine shrimps. The activity lies in extracts of intermediate polarity.

The isolates, FAO-WR and FAO-FRS, showed no activity against the shrimps (LD_{50} > 1000 jig/ml). This shows that they lack cytotoxic or insecticidal activity.

Antimalarial Activity

The leaf and stem showed very low antimalarial activity against the multiple drug resistant VI/S strain of *Plasmodium falciparum* (MIC > 168 |ig/ml). The IC_{50} of the cold methanol extract of the root extract against the drug resistant strain is about 39.24 fag/ml. Based on a scale used by Weenen *et al.* (1990), the root extract has good antimalarial activity. However, it was less effective than chloroquine whose IC_{50} is less than 5.00 jig/ml.

The anthraquinones (Sittie *et al.,* 1999), flavonoids (Castros *et al.,* 1996) or isoquinoline-based alkaloids (Lin *et al.,* 1999; Angerhofer *et al.,* 1999; Frappier *et al.,* 1996) or triterpene saponins (Takahara *et al.,* 1998) could be responsible for the antimalarial activity associated with the roots. Compared to all the other plant parts the roots have the highest relative amounts of anthraquinones and alkaloids.

Despite the fact the plant extract has lower activity than chloroquine it could still yield compounds with better activity than chloroquine. Crude plant extracts often have less activity than pure isolates. In the case of development of artemisinin, the crude extract of *Artemia annua* showed less activity than artemisinin.

These results support the ethnobotanical use of the roots in Kenya for the management of malaria. However caution must be taken when interpreting the antimalarial activity of plant extracts. Seasonal and regional variations, traditional methods of preparation and the effects of storage need to be taken into consideration. Traditional healers may prescribe a herb for its anti-pyretic effects rather than for direct antimalarial effects (Weenen *et al.,* 1990). The therapeutic efficacy of C. *brachaita* may lie in both direct antimalarial effects and antipyretic activity.

FAO-FRS and FAO-WR were not thoroughly screened for antimalarial activity. Higher doses should have been used to determine their actual MIC values. It was only observed that their MIC is greater than 45(ig/ml.

In Rwanda and Tanzania the leaves are used for the management of malaria and the leave extract of Rwandese plants have *in vitro* antimalarial activity (Hazimungu and van Pulyede, 1988). However, the leaves obtained from Kiambu district, Kenya showed very low antimalarial activity. This discrepancy can be explained by the different environmental characteristics (climate and soil quality) leading to different phytochemical characteristics and hence differences in the pharmacological profiles (Capasso *et al.,* 2000).

The experimental design did not allow for comparison between the isotopic method and the morphologic method. A study done by Desjardins *et al.* (1979) shows that there is a good correlation between the morphologic and isotopic methods. Morphologic method is reliable and accurate. The isoptic method is rapid and has better precision.

The method based on optical microscopy is cheaper and safer compared to the method based on radioactive uptake of hypoxanthine. It is useful in laboratories that do not have expensive equipment. However, it is very tedious and time consuming.

Its reproducibility and reliability is highly dependent on good slide preparation technique and the method is strenuous to the eyes of the observer. It is mostly used for preliminary screening for antimalarial activity and determination of minimum inhibitory concentrations. Despite the high costs involved, the isoptic method is more popular method as it is semi-automated.

Antimicrobial activity

None of the extracts and crystals tested showed any antibacterial or antifungal activity. The positive controls, ciproflaxcin (zone of inhibition 6cm + 1.2, n=3) and clotrimazole (zone of inhibition 7cm + 2.60, n=3) inhibited bacterial and fungal growth respectively.

Lack of antimicrobial activity was unexpected as the plant is used in the management of respiratory disorders, glue eye, skin disorders and gonorrhea. Low rates of diffusion of extract into the medium or a possible interaction between components of the medium and plant extract could have led to the masking of antimicrobial activity of the extract. The antimicrobial activity of the plant is probably dependent on *in vivo* metabolic activation.

The plant is used traditionally to manage syphillis and leprosy. However, there were no facilities to test for activity against *Mycobacterium* and *Trepenomes*.

4.

Local anesthetic activity

In all test groups, the medians and modes decreased progressively
with time showing that the local anesthetic effect is time dependent.
The onset of effect was immediate. The isolate FAO-FRS had the
shortest duration of effect (25 minutes), followed by the cold
methanol extract of the stem (30 minutes). The duration of action of
the cold methanol extract of the leaves was 40 minutes. Procaine
hydrochloride, a short acting local anesthetic, had the longest duration
of action (50 minutes).

The crude extracts of C. *brachiata* have a longer duration of action
than the isolate, FAO-FRS. There could be other components in the
extracts that modulate the anesthetic activity of FAO-FRS.

The anesthetic activities of the extracts of the leaf and stem support
the use of old stems and leafs by the Kikuyu of Kenya for the
management of toothache and sore throats. Local anesthetics are used
clinically to alleviate the pain associated with sore throats and
toothache.

The local anesthetics in clinical use are a heterogenous group of
compounds. They are made up of a lipophilic group linked to a
hydrophilic group by either an ester bond or an amide bond (Miller,
1998).

The short acting local anesthetics are the ester type while those with a longer duration of action have an amide bond.

This suggests that FAO-FRS could be made up a lipophilic group linked to a hydrophillic group by an ester group.

Many species in the Ranunculaceae family are used as local anesthetics. Diterpene alkaloids isolated from *Aconitum* and *Delphinium* species have a local anesthetic effect and inhibit neuronal sodium currents by binding to the inner surface of the sodium channels (Ameri, 1998).

Antinociceptive activity

From the hot plate and tail pressure methods, the cold methanol extracts of leaf, stem and root demonstrated significant analgesic activity (95% confidence limit). This supports the use of the leaf for the management of headaches. The plant could provide lead molecules of the development of analgesic compounds of high efficacy without the dependency associated with narcotic analgesics.

FAO-FRS (150mg/ kg i.p.) had analgesic effects. Local anesthetics at high doses have systemic analgesic activity due to their depressant effects at the level of the central nervous system (Abram and Yakshi, 1994).

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The hot plate method tests for central analgesic activity. Peripherally acting analgesics like (Non Steriodal Anti inflammatory Agents) NSAIAs will not prolong reaction time on the hot plate even in high doses. The antinociceptive effects of FAO-FRS and the various plant extracts are thus mediated centrally.

The local anesthetic effect of FAO-FRS partially explains the observed analgesic effect of the cold methanol extracts of the leaf and stem. Other components may come into play as the extracts were effective at relatively low concentrations.

In China all documented *Clematis* species are widely used as analgesics. Several hederaginin and oleanolic acid based triterpene saponins isolated from *C. chinensis* demonstrated antinociceptive activity (Xu *et al.,* 1996). They act by selectively inhibiting cycloxygenase II (Suh *et al.,* 1999).

Compounds isolated from the Ranunculaceae family with analgesic activity include ranunculin (Neuwinger, 1994); and diterpene alkaloids (Ameri, 1998); flavonoids and coumarins (Lindahl and Tagesson, 1997); and bis benzlyisoquinolines (Newall, 1996). Most of these classes of compounds are to be present in C. *brachiata* and could be responsible for the analgesic effects of extracts.

$v^{\overset{*}{v}}$

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The leaves are steamed and fresh roots are inhaled for the management of headaches and colds in Kenya, Tanzania and Zimbabwe (Kokwaro, 1993; Neuwinger, 1994). This suggests that the plant has essential oils with an analgesic effect. However, during phytochemical screening no essential oils were detected in the plant. A more sensitive method, like the trap method, should have been used to test for essential oils which could have been present in trace amounts.

During collection, the roots produced an acrid smell that disappeared after a few days. This strongly indicates the presence of Ranunculin, a highly volatile unsaturated lactone which is almost invariably found in all species belonging to the *Ranunculaceae* family (Neuwinger, 1994). Ranunculin has an acrid smell. Ranunculin is a glycoside rapidly hydrolyzes to protoanemonin, which dimerizes to anemonin. Protoanemonin and anemonin are volatile and have an acrid smell. The three compounds have strong analgesic and antipyretic activity and could be the volatile components inhaled for the management of headaches and colds.

Effects on the GIT

Effects on the isolated rabbit ileum

Since FAO-FRS demonstrated local anesthetic effect, it was expected to have a spasmolytic effect on the isolated rabbit ileum. The initial spasmolytic effect may have been due to the membrane stabilizing effects of FAO-FRS.

The spasmogenic effect may be due to direct effects of FAO-FRS on receptors found on the GIT. The spasmogenic effect of FAO-FRS is dose dependent and the log-dose-effect curve shows the expected sigmoidal shape.

The cold methanol extracts of the root, stem and leaves all caused relaxation of the isolated rabbit ileum. Relaxation may have been due to the membrane stabilizing effect of extracts. Local anesthetics are known to cause relaxation of the GIT. Flavonoids such as apigenin and quercetin (Mata *et al.,* 1997) and protoberberine Brunton, 1996) alkaloids have a spasmolytic effect on the ileum. The effect of the stem extract is dose dependent and the log-dose-effect curve shows the expected sigmoidal shape. Though, the root and leave extracts showed spasmolytic effects their dose - response effects were not studied.

The spasmolytic effects of the leave and stem extracts may in part explain the traditional use of an infusion of the leaves for the management of abdominal disorders especially those associated with pregnancy.

In the case of the root, an increase in the contractions of the isolated ileum was expected as it is known to induce diarrhea. However, it caused a very brief relaxation of the ileum. The presence of anthraquinones in the roots explains the purgative effect of the root extract when used traditionally for the management of diarrhea.

Effect on Intestinal Movement

Since the leaf and stem extracts demonstrated spasmolytic effects they were expected to increase intestinal transit time in the mouse model. However, there was no significant difference between the test groups and the control group. This may be explained by the fact that the extracts may contain components that may increase gastro intestinal motility *in vivo.*

CHAPTER FOUR

CONCLUSION

The following phytochemical groups of compounds were detected in *Clematis brachiata* collected from Kiambu District, Kenya: alkaloids, anthraquinones, sterols, carotenoids, triterpenoid saponins, coumarins, flavonoids, tannins and cardenolides. No cyanogenic glycosides and essential oils were detected. The roots had the highest relative amount of anthraquinones and alkaloids but had no tannins and carotenoids. The presence of anthraquinones explains use of the roots as a purgative.

A flavanol, quercitrin, was isolated from the stem. It is orange-red in color and has an uncorrected melting point of 173-174.9 °C. It is poorly soluble in water but dissolves well in acetone and ethyl acetate-methanol (1:1).

FAO-FRS was isolated from the stem mixture. It is off white in color and is highly water-soluble. HPLC evaluation showed that it is a complex mixture of aliphatic compounds. It has local anesthetic and antinociceptive effects. On the isolated rabbit ileum, it causes relaxation at low doses and contraction at high doses. Its contractile effect is dose dependent. It has no antimicrobial effects. It had no significant activity against brine shrimps.

FAO-WR was isolated from the roots. The yield was 170 mg (0.068% of the dried root powder). It tested positive for an alpha/beta unsaturated lactone. It was transparent in UV. It lacks antibacterial, antifungal and antiplasmodial activity. It also lacks activity against brine shrimps.

The cold methanol extract of the roots had good antimalarial activity (IC5o= 39.90 jig/ml) against a chloroquine resistant strain of *Plasmodium falciparum* (VI/S). This provides scientific rationale for the use of the roots of *Clematis brachiata* for the management of malaria in Kenya. The leaf and stem extracts had low antimalarial activity.

C. *brachiata* have good activity against shrimps and hence they could provide compounds with insecticidal and cytotoxic activity if appropriate tests are carried out.

FAO-FRS and the cold methanol extracts of the leaves and stem demonstrated local anesthetic and antinociceptive activities thus providing evidence of their efficacy for the management of headaches, sore throats and toothache.

Though the plant is used for the management of skin disorders, chest infections and sexually transmitted diseases, the plant extracts showed no significant *in vitro* antifungal and antibacterial activity.

The stem, leaves and root extracts caused relaxation of the isolated rabbit ileum. The spasmolytic effects of the leaves and stem extracts provide scientific rationale for their use in the management of other abdominal disorders. The extracts had no significant effect on intestinal transit time in the mouse model.

The phytochemical and pharmacological investigations carried out were preliminary and there is still the option of future work.

RECOMMENDATIONS

Not all claimed therapeutic effects of the plant were investigated. Since it is claimed that the plant has abortificient, anticonvulsant, antimycobacterial, neuroleptic and antivenin properties it would be useful to study the plant further to provide scientific rationale for these claimed activities.

Though *in vitro* and studies done on animal models provide scientific rationale for the use of the plant as an analgesic, local anesthetic, spasmolytic and for the management of malaria, its efficacy and safety need to be proved by animal toxicity tests and conventional clinical trials.

BIBLIOGRAPHY

1. Abram S. E. and Yakash T. (1994). Systemic lidocaine blocks nerve injury-induced hyperalgesia and nociceptor-derived spinal sensitization in the rat. *Anesthesiology* 80: 385-391. (Discussion 325A).

2. Agnew A. D. Q. and Agnew S. (1994)
Uplands Kenya Wild Flowers. A flora of the ferns Herbaceous Flowering plants of upland Kenya; East African Natural History Society, Nairobi. Pg. 44.

3. Amaral A.C. P.; Kuster R. M.; Goncalves J. L. S.; and Wiggs M. D., 1999. Antiviral investigation on the flavonoids of *Chamlaesyce thymifolia. Fitoterapia.* 70 (3): 293-295.

4. Ameri A. (1998). The effect of Aconitum alkaloids on the central nervous system. *Prog. Neurobiol.* 56 (2): 211-35.

5. Angerhofer C. K.; Guinaudeau H.; Wongpanich V.; Pezzuto J.M.; and Cordell G. A. (1999). Antiplasmodial and cytotoxic activity of natural bisbenzylisoquinoline alkaloids. *J. Nat. Prod.* Jan; 62: 59-66.

6. Atta-ur-Rahman; Nasreen A.; Akhtan F.; Shekhani M. S.; Clardy J.; Parvez M.' and Choudhary M. I. (1997). Antifungal diterpenoids from *Delphium derudatum. Journal of Natural Products.* 60 (5): 472-4. y

7. Baker M. (1996). Fur rubbing: use of medicinal plants by capuchin monkeys (*Cebus capucinus*). *American Journal of Primatology.* 38 (3): 263-270.

8. Basilar M. A. (1991). Antifungal activity of *Clematis gouriana* against chickpea root pathogens. *Bangladesh Journal of Plant Pathology 1* (1-2): 39-40.

9. BeentjeH. (1994). Kenya Trees and Shrubs and Lianas. National Museums of Kenya, Nairobi. Pg. 59

10. Beil W.; Birkholz C.; and Sewing K. F. (1995). Effects of flavonoids on parietal cell acid secretion, gastric mucosal prostaglandin production and *Helicobacter pylori* growth. *Arzneimittelforschung* (Germany). June; 45 (6): 697-700.

11. Bouree P. (1997). Malaria: what treatment today? (Editorial) Original Title: *Paludisme: quel traitement aujourd'hui*? (French) *PresseMed.* 26(4): 156-7.

12. Braca A., Bilia A. R., Mendez J. and Morelli I. (2001). Myrecetin glycosides from *Licania densiflora. Fitoterapia.* 72: 182-185.

13. Brunton C. C. (1996). The pharmacological basis of the use of berberine in the management of diarrhea. In: Hardman J. G.; Limbird L. E.; Molinoff P. B.; Ruddon R. W.; and Gilman A. G. (Editors). New York: Mc Graw-Hill. *The pharmacological basis of therapeutics*: Pg 917-936.

14. Caceres A.; Menendez H.; Mendez E.; Cohobon E.; Samayoa B. E.; Jauregui E.; Peralta E.; and Carrillo G. (1995). Antigonorrheal activity of plants used in Guatemala for the treatment of sexually transmitted diseases. *Journal of Ethnopharmacology.* 48 (2): 85-8.

15. Canfield C. J.; Whiting E. G.; Hall W. H.; and Mac Donald B. (1971). Treatment of acute falciparum malaria form Vietnam with trimethoprim and sulfalene. *Am. J. Trop. Hyg.* 20: 524-526.

16. Castros O.; Chinchilla M.; and Guerrero O. (1996). Chemical and biological evaluation of the effect of plant extracts against *Plasmodium berghei.* Original Title: *Evaluacion quimica y biologica del effecto de extracts de plantas contra Plasmodium berghei.* (Spanish). *Rev Biol Trop.* Aug; 44 (2A): 361-7.

17. Capasso R.; Izzo A. A.; Pinto L.; Bifulco T.; Vitobello C.; and Mascolo N. (2000). Phytotherapy and quality of herbal medicines. *Fitoterapia.* 71:'S58-S65.

*

18. Chhabra S. C.; Mahunnah R. L. A.; and Mshiu E. N. (1984). Plants used traditional medicine in Eastern Tanzania. V. Angiosperms *(Passifloraceae* to *Sapindaceae).* *Journal ofEthnopharmacology.* 33 (1/2): 143-157.

19. Chhabra S. C. and Usio F. C. (1991). Antibacterial activity of some Tanzanian plant used in traditional medicine. *Fitoterapia.* 626: 499-503.

20. Chhabra S. C.; Usio F. C.; and Mshiu E. N. (1984). Phytochemical screening of Tanzanian medicinal plants. I. *Journal of Ethnopharmacology.* 11 (2): 157-179.

21. Chinchilla M.; Guerrero O. M.; Abarco G.; Barrios M.; and Castro O. (1998). An *in vivo* model to study the antimalaric capacity of plant extracts. *Rev. Biol. Trop.* 46 (1): 35-39.

22. Chiu K. W.; Wong S. W.; and Sham S. K. (1995). Observations on blood pressure responses to injections of medicinal plant extracts in rats. *Amer. J. Chinese Med.* 23 (1): 91-99.

23. Chulay J. D.; Watkins W. M.; and Sixsmith D. G. (1984). *Am. J. Trop Med Hyg.* 33: 325-330.

24. Chung J. G.; Tsou M. F.; Wang H. H.; Lo H. H.; Hsieh S. E.; Yen Y. S.; Wu L. T.; Chang S. H.; Ho C. C.; and Hung C. F. (1998). Rhein affects arylamine N-acetyltransferase activity in *Helicobacter pylori* from peptic ulcer patients. *Journal of Applied Toxicology.* 18 (2): 117-23.

25. Datta B. K.; Datta S. K.; and Sarker S. D., 2000. Quercetin 3-0-(6"-caffeoyl)-beta-D-galactopyranoside from *Polygonum viscosum. Fitoterapia.* 71 (4): 459-460.

26. Dawit Abebe (1987). In: *Medicinal and poisonous plants of the Tropics. Proceedings of Symposium 5-35 of the 14[th] International Botanical Congress, Berlin.* 24[th] July/August, page 79.

27. Desjardins R. E.; Canfield R. E.; Hayness C. J.; and Chulay J. D. (1979). *Antimicrob. Agents Chemother.* 16: 710-718.

28. Evans C. E. (1996). Phenolics and phenolic glycosides.
In: *Trease and Evan's Pharmacognosy.*, pp218-254. 14[th] Edition. Published by W B Saunders Co. Ltd. London, Toronto, Sydney, and Tokyo.

29. Farnsworth N. R. and Euler K. L. (1962). An alkaloid screening procedure utilizing Thin-Layer-Chromatography. *Lloydia.* 25: 186.

30. Frappier F.; Jossang A.; Soudon J.; Calvo F.; Rasoanaivo P.; Ratsimamanga-Urveg S.; Saez J.; Schrevel J.; and Grellier P. (1996). Bis benzylisoquinolines as modulators of chloroquine resistance in *Plasmodium falciparum* and multidrug resistance in tumor cells. *Antimicrob. Agents Chemother.* 40 (6): 1476-1481.

31. Fraisse D.; Heitz A.; Carnat A.; Carnat A. P.; and Lamaison J. L., 2000. Quercetin 3 arabinopyronoside, a major flavonoid compound form *Alchemilla xanthochlora. Fitoterapia.l\ (4): 463-464.

32. Gachathi F. N. (1989). *Kikuyu Botanical Dictionary.* GTZ, Nairobi. Pg. 64.

33. Geissman T. A. (1955). Anthocyanins, Chalcones, Aurones, Flavones and related water-soluble plant pigments. In: "*Modern methods of plant analysis. Vol. IIF* Paech K. and Tracey M. V. (Editors). Springer-Verlag-Berlin-Gottingen. Page 405-498.

34. Grayer R. J. (1989). Flavanoids. In: *Methods in Plant Biochemistry. Volume One. Plant Phenolics.* Ed. J. B. Harborne. Academic Press, London.

35. Hakizamungu-E.; van Puyelde L.; and Wery M. (1992). Screening of Rwandese medicinal plants for *anti-trichomonas* activity. *Journal of Ethnopharmacology.* 36 (2): 143-146.

36. Hakizamungu E. and van Puyvelde L. (1988). *L' Usage deplants* medicinales *dans le traitement du paludisme en medecine traditionelle Rwandese.   Bull De Liason ACCT: Medecine traditionelle et Pharmacopee* 2: 11-17.

37. Harborne J. B. (1973). In: *Phytochemical methods.* First Edition. Chapman and Hall, London. Pg. 33-88.

38. Jagadeeswaran R.; Thirunavukkarasu C.; Gunasekaran P.; Ramamurty N.; and Sakthisekaran D., 2000. *In vitro* studies on the selective cytotoxic effect of crocein and quercetin. *Fitoterapia.* 71 (4): 395-399.

39. Jensen J. B.; Boland M. T.; and Hayness M. (1982). *Experimental Parasitology.* 54: 416-424.

40. Jiang B.; Jia X.; Ye X.; Ding J.; Yu X.; and Wu Y. (1990). Studies and comparisons on chemical components of essential oils from *Clematis hexapleta* Pall. *Chung Kuo Chang Yao Chih.* 15 (8): 488-90, 512.

Natschke S. L.; Mauger A.; Bowers-Daines M. M.; Lange B.C.; and Lee K. H. (1997). Antitumour agents - CLXVII. Synthesis and structure-activity correlations of the cytotoxic anthraquinone 1, 4-bis-(2,3-epoxpropylamino)-9,10 anthracenedione, and related compounds. *Bioorganic and Medicinal Chemistry.* 5 (8): 1469-79.

42.[1] Joshi B. S. and Kaul P. N. (2001). Alternative medicine: herbal drugs and their critical appraisal - Part One. *Prog. Res.* 56: 1-76.

43. Kim S. I.; Ahn Y. J.; Lee S. G.; Kim J. B.; and Byun B. H. (1996). Larvicidal activities of extracts from domestic and Japanese plants against the pine needle gall midge *(Diptera: Cecidomyiidae). Korean Journal of Applied Entomology.* 35 (2): 153-158.

44. Kirby G. C. (1997). Plants as a source of antimalarial drugs. *Trop. Doc.* 27 (Suppl. 1): 7-11.

45. Kizu H.; Shimana H.; and Tomimori T. (1995). Studies on the constituents of Clematis species. VI. The constituents of *Clematis stans* Sieb. *et* Zucc. *Chem Pharm Bull.* (Tokyo) 43(12): 2187-94.

46. Kokwaro J. O. (1993). *Medicinal Plants of East Africa-,* Kenya Literature Bureau, Nairobi. Pg. 181.

47. Lasure, A.; van Pole, B; Clerck, L. S.; Bridts, C. H.; Stevens, W. J.; Rwangaro, P. C.; Pieters, 1.; and Vlietinck, A. J. (1995). Screening of Rwandese plant extracts for their influence on lymphocyte proliferation. *Phytomedicine* 1 (4); 303-307.

48. Le Bras J.; Deloron, P.; Ricour A; Andrieu B.; Savel, J.; and Couland, J. P. (1983). *Plasmodium falciparum*: Drug sensitivity *in vitro* of isolates before and after adaptation to continuous culture. *Exp. Parasitol.*, 56: 9-14.

49. Li Z. L.; Gu H. M.; Warhurst D. C.; and Peters W. (1983). *Trans Roy Soc Med Hyg.* (77): 522-523.

50. Lin L. Z.; Hu S. F.; Chu M.; Chan T. M.; Chai H.; Angerhofer C. K.; Pezzuto J. M.; and Cordell G. A. (1999). Phenolic aporphine-benzylisoquinoline alkaloids form *Thalictrum faberi*. *Phytochemistry.* 50 (5): 829-34.

51. Lindahl M. and Tagesson C. (1997). Flavonoids as phospholipase A2 inhibitors: importance of their structure for selective inhibition of group II phospholipase A2. *Inflammation.* 21 (3): 347-56.

52. Lindsay S. W. and Martens W. J. (1998). Malaria in the African Highlands: past, present and future. *Bull World Health Org.* 76 (1): 33-45.

53. Martin, D. C.; Arnold, D. F.; Clyde A.; Ibrahim, P. E.; Carson, K. E.; Rieckmann K. E.; and Willerson D., Jr. (1971). A quinoline methanol (WR 30,090) for treatment of acute malaria. *Antimicrob. Agents Chemother.* 3: 214-219.

54. Marby J. T.; Markham K. R.; and Thomas M. B.,1969. *The Systemic Identification ofFlavonoids.* Springer-Verlag New York Inc., New York, U. S. A. Chapter One. Pg. 1-45.

55. Markham K. R., 1989. Flavones, Flavonols and their glycosides. In: *Methods in Plant Biochemistry. Plant Phenolics.* Academic Press Limited. Edited by Harborne J. B. and Dey. Chapter 6. Pg. 197-235.

56. Mata R.; Rojas A.; Acevedo L.; Estrada S.; Calzada F.; Rojas I.; Beje R.; and Linares R. (1997). Smooth muscle relaxing flavonoids and terpenoids from *Conyza filaginoides. Planta Med.* 63 (1): 31-5.

57. McCormick, G. J. and Canfield C. J. (1972). *In vitro* evaluation of antimalarial drug combinations. *Proc. Helminthol. Soc. Wash.* (special issue) 39: 292-297.

58. Mc Cutcheon A. R.; Stokes R. W.; Thomson L. M.; Ellis S. M.; Hancock R. E. S.; and Towers G. H. N. (1997). Antimycobacterial screening of British Columbian medicinal plants. *Int. .J Pharmcog.* 35 (2):77-83.

59. McLaughlin J.L.; Chang C.J.; and Smith D.L. (1991). Bench - Top Bioassays for the Discovery of Bioactive Natural Products: an update. Atta-ur-Rahman (Ed.), *Studies in Natural Products Chemistry, Vol. 9,* Elsevier Science Publisher B. V., Amsterdam.

60. Mc Laughlin J. L. (1988). Brine Shrimp and Crown gall tumors: Simple bioassays for the discovery of plant Antitumor agents. *Proceedings, N1H Workshop, Bioassays for Discovery of Antitumour and Antiviral Agents from Natural Source*s, Bethesda, Oct. 18 -19, Pg. 22.

61. Mc Quay H. J. and Moor R. A. (1997). The Opioid problem. In: *Handbook of Experimental Pharmacology. Vol. 130. The Pharmacology of Pain.* Dickenson A. and Besson J. M. (Eds.). Printed by Springer-Verlag Berlin Heidelberg,

62. Meyer B. N.; Ferrigni N. R.; Putnam J. E.; Jacobsen L. B.; Nichols D. E. and McLaughlin J. L. (1982). Brine Shrimp: A convenient general bio assay for active plant constituents. *PlantaMedica.* 45, :3V- 34.

*

<1

63. Miller R. D. (1998). Local Anesthetics. *In: Basic and Clinical Pharmacology.* Seventh Edition. Edited by B. G. Katzung. Published by Mc Graw Hill. P425-433.

64. Neuwinger H.P. (1994). *African Ethnobotany. Poisons and Drugs.* Chapman and Hill. Weinheim. Pg. 754-756.

65. Newall C. A.; Anderson L. A.; and Phillipson J. D. (1996). *Herbal Medicines A guide for Health-care professionals.* The Pharmaceutical Press, Pg. 80-83; 151-152; and 209-222.

66. Nishikawa A.; Kase Y.; Hayakawa T.; Yanagisawa T.; Kanno J.; and Hayashi Y. Enhancement of cell proliferation and prostaglandin biosynthesis by 1, 8 dihydroxyanthraquinone in the rat large intestine (1997). *Carcinogenisis.* 18 (6): 1259-63.

67. Nwude N. and Ebong, O. O. (1980). Some plants used in the treatment of leprosy in Africa. *Leprosy Rev.* 51: 11-18.

68. 01um(1997). Personnal communication. Muguga location, Kiambu district, Kenya.

69. Omer M. E. A.; A1 Magoul A. Z.; and El Egani A. A. (1998). Sudanese plants used in folkloric medicine: Screening for antibacterial activity. Part IX. *Fitoterapia* LXIX, 6: 540-545.

70. Oskarsson P.; Ljunggren J. G.; and Linns P. E. (1997). Efficacy and safety of mexiletine in the treatment of painful diabetic neuropathy. The Mexilitine Study Group. *Diabetes Care.* 20(10): 1594-7.

71. Palmer C. J.; Makler M.; Klaskala W. I.; Lindo J. F.; Baum M. K.; and Ager A. L. (1998). Increased prevalence of *Plasmodium falciparum* in Honduras, Central America. *Rev Panam Salud Publica.* 4 (1): 40-2.

72. Randell L. O. and Selitto J. J. (1957).   A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther III* 409-418.

73. Rieckmann K. H. (1982). Visual *in vitro* test for determining the drug sensitivity of *Plasmodium falciparum.* *Lancet* (1): 1333-1335.

74. Rieckmann K. H. (1978). Drug sensitivity of *Plasmodium falciparum.* An *in vitro* microtechnique. *Lancet* (1): 22-23.

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75. Sanders M. M.; Liu T. K.; Wu H. Y.; Desai S. D.; Mao Y.; Rubin E. H.; La Voie E. J.; Makhey D.; and Liu L. F. (1998). Selective toxicity of topoisomerase-directed protoberberines against glioblastoma cells. *Biochemical Pharmacology.* 56 (9): 1157-66.

76. Sayed H. M.; El-Moghazy S. A.; and Kamel M. S. (1995). Chemical constituents of stems and leaves of *Clematis pururea var. hybrida* cultivated in Egypt. *Indian J. Chem.* (34B) 12: 1111-1113.

77. Schneller T.; Latz-Bruning B.; and Wink M. (1997). Biochemical activities of berbeine, palmatine and sanguinarine mediating chemical defence against microorganisms and herbivores. *Phytochemistry.* 44 (2): 257-66.

78. Shao B. P.; Wang P.; Qin G. W.; and Xu R. S. (1996a). Phenolics from *Clematis chinensis. Nat. Prod. Lett.* 8 (2): 127-132.

79. Shao B. P.; Qin G.; Xu R.; Wu H.; and Ma K (1996b). Studies on bioactive components from Chinese Medicinal plants. *Phytochemistry.* 42 (3) :821-5.

80. Shao B.P.; Qin G.; Xu R.; Wu H.; and Ma K. (1995). Triterpeniod saponins from *Clematis chinensis. Phytochemistry.* 38 (6): 1473 - 9.

81. Sharaf M.; El-Ansari M. A.; and Saleh N. A.M. (2000). Quercetin triglycoside from *Caparis spinosa*. *Fitoterapia*. 71 (1): 46-49.

82. Sheng W. D.; Jiddawi M. S.; Hong X. Q.; and Abdulla S. M. (1997). Treatment of chloroquine-resistant malaria using pyrimethamine in combination with berberine, tetracyline or cotrimoxazole. *East African Medical Journal*. 74 (5): 283-4.

83. Sim S. K. (1967). *Medicinal plant glycosides*. University of Toronto Press. Toronto, Buffalo. Pg. 26, 49.

84. Sittie A. A.; Lemmich E.; Olsen C. E.; Hviid L.; Kharazmi A.; Nkrumah F. K.; and Christensen (1999). Structure activity studies: *In vitro* antileshimanial and antimalarial activities of anthraquinones from *Morinda lucida*. (letter). *Planta Med*. 65 (3): 256-61.

85. Slavik J. and Slavikova L. (1995). Quaternary isoquinoline alkaloids and some diterpenoid alkaloids in plants of the Czech Republic. *Collect Czech Chem Commun*. 60 (6): 1034-1041.

86. Song Z.; Zhao Y.; Duan J.; and Wang X. (1995). Chemical constituents of *Clematis intricata* Bunge. *Chung Kuo Chung Yao Tsa Chih*. 20(10) :613-4, 640.

*

*

<1

87. Suh N.; Wang Y.; Honda T.; Gribble G. W.; Dmitrowsky E.; *et al. (1999). Cancer Research* (59): 236.

88. Takahara M.; Kusano A.; Shibano M.; Kusano G.; Koizumi K.; Suzuki R.; and Kim H. S. (1998). Antimalarial activity and nucleoside transport inhibitory activity of the triterpenic constituents of *Cimicifuga* spp. *Biol Pharm Bull.* 21 (8): 823-8.

89. Thompson C. A. (1997). As patients embrace herbal remedies, dearth of scientific evidence frustrates clinicians. *Am J Health Syst Pharm.* 54 (23): 2656, 2658, and 2664.

90. Valecha N.; Biswas S.; Badoni V.; Bhandarik S.; and Sati O. (1994). *Indian Journal of Pharmacology* .26: 144.

91. Vlietinck A. J.; van Hoof L.; Totte J.; Lasure A.; Vanden Berghe D.; Rwangabe P. C.; and Mvukiyumawani J. (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *Journal of Ethnopharmacology.* 46 (1): 31-47.

92. Wagner H. and S.Bladt. With Colored photographs by Rickl V. (1996). In *Plant Drug Analysis. A Thin Layer Chromatography Atlas.* With 184 colored photographs. Chapter 18. By Springer-Verlag Berlin Heilberg. Pg. 349-358.

v^x

c

93. Waldi D. (1965). Spray Reagents for Thin-Layer chromatography. In Stahl E. (Ed)"*Thin Layer Chromatography. A Laboratory Handbook".* Springer-Verlag. Berlin, Heidelburg, New York. Academic Press, New York, London. Pg. 483-502.

94. Wall M. E.; Krider M. M.; Krewson C. F.; Eddy C. R.; Wilaman J. J.; Correll D. S.; and Gentrey H. S. (1954). Steriodal sapogenins XIII. Supplementary table of data for steriodal sapogenins VII. *Agr. Research Service Cir.,* 363:17.

95. Wall M. E.; Eddy C. R.; Mc Clennan M. L.; and Klump M. E. (1952). Detection and estimation of steriodal sapogenins in plant tissue. *Anal. Chem.* 24: 1337.

96. Watkins W. M.; Howells R. E.; Brandling Bennet A. D.; and Koech D. K. (1987*)Am. J.Jrop. Med. Hyg.* 37: 445-451.

97. Weher U. S.; Steffen B. and Siegers C. P., (1998). Antitumor activities of comarin, 7-hydroxy-coumarin and its glucoronide in several human tumor cell lines. *Res. Commun. Mol. Pathol. Pharmacol.* 99 (2): 193 - 206.

98. Woube M. (1997). Geographical distribution and dramatic increases in incidences of malaria: consequences o f the resettlement scheme in Gambela, S. W. Ethiopia. *Indian Journal ofMalariology.* 34 (3): 140-63.

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99. Weenen H.; Ngunya M. H. H.; Bray D. H.; Mwasumbi L. B.; Kinabo L. S.; and Kilimali V. A. E. B. (1990). Antimalarial activity of Tanzanian medicinal Plants. *PlantaMed.* 56: 368-370.

100. World Health Organisation (WHO), (1997). World Health Organization Technical Reports Service. 867: 1-74.

101. Woolfe G. and Mac Donald A. D. (1944). The evaluation of the analgesic action of pethidine hydrochloride (demerol). *J. Pharmacol. Exp. Ther.* 80: 300-307.

102. Xu R.; Zhao W.; Xu J.; Shao B.; and Qin G. (1996). Studies on bioactive saponins from Chinese medicinal plants. *Advances in Experimental Medicine and Biology.* 404: 371-82.

103. Yesilada E.; Ustun O.; Sezik E.; Takaishi Y.; Ono Y.; and Honda G. (1997). Inhibitory effects of Turkish folk remedies on inflammatory cytokines: interleukin- 1 alpha, interleukin-1 beta and tumor necrosis factor alpha. *Journal of Ethnopharmacology.* 58(1): 59-73.

APPENDIX A

The UV Spectrum of FAO-FRS

(Concentration: 7.24 mg/ml, Solvent: methanol-water (1:2))

WAVELENTGH (nm)
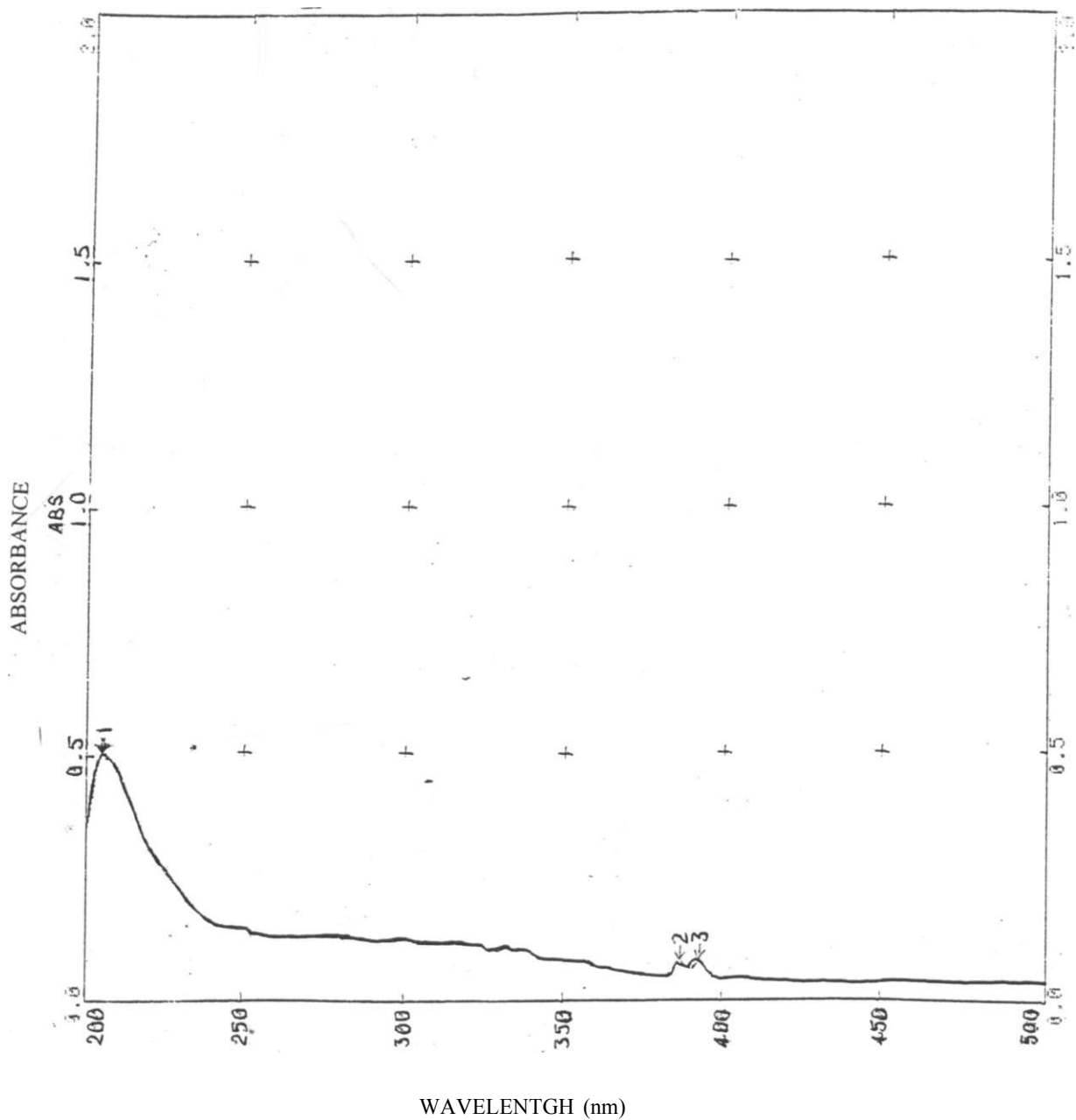
APPENDIX B

HPLC CHROMATOGRAPHS OF FAO-FRS

ACETONITRILE - WATER (99:1 TO 60:40) IN 20 MINUTES
SYSTEM 1: SOLVENT GRADIENT ELUTION

- ' TIME   (minutes)
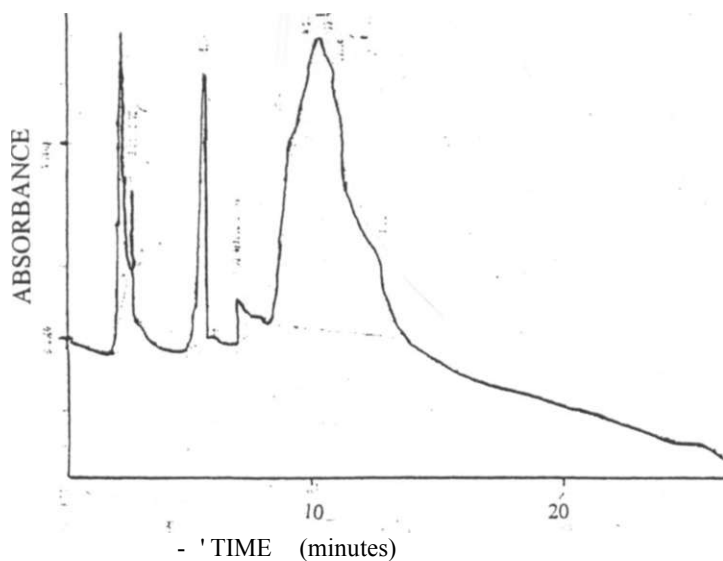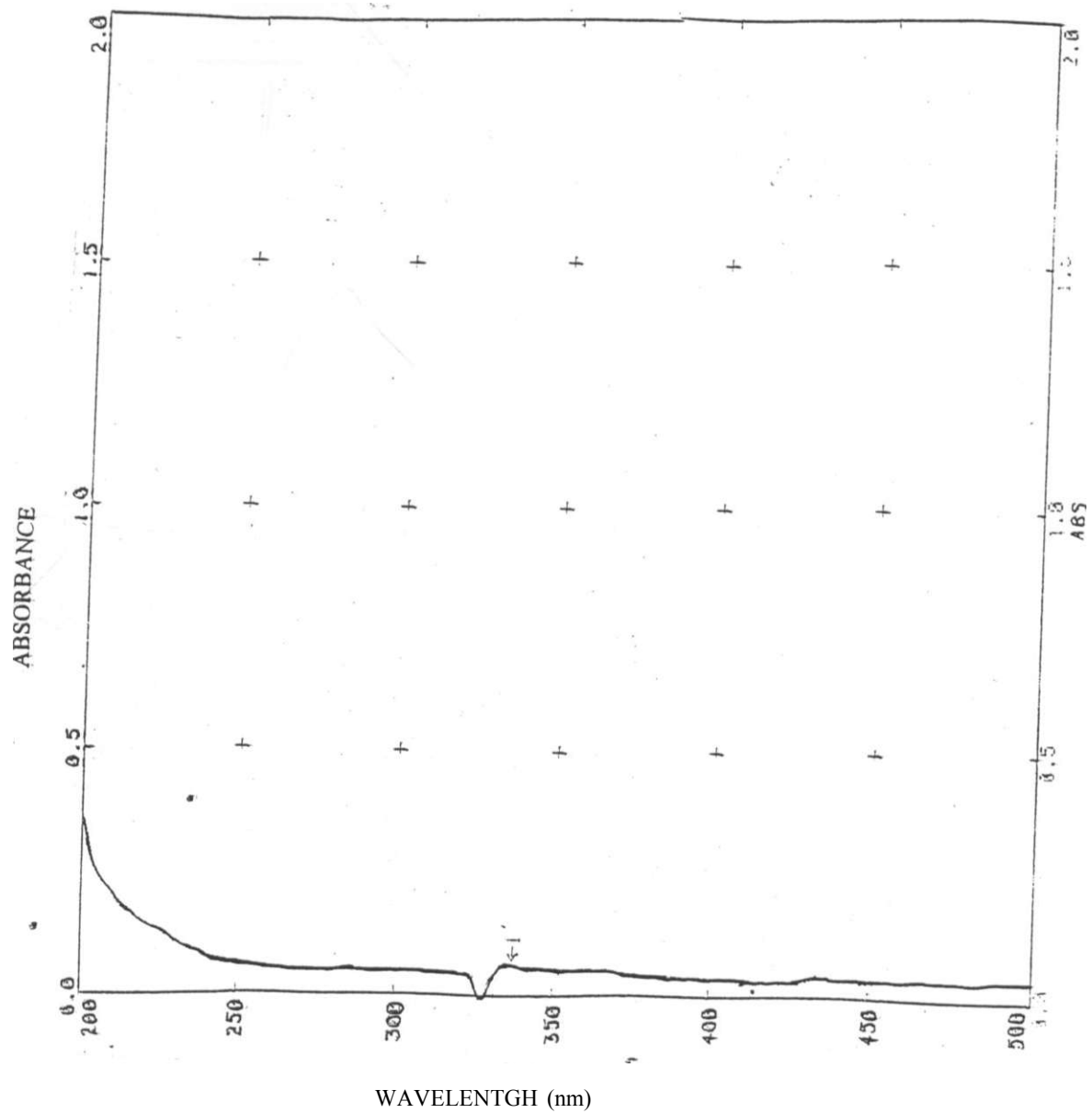
SOLVENT SYSTEM 2: GRADIENT ELUTION ACETONITRILE
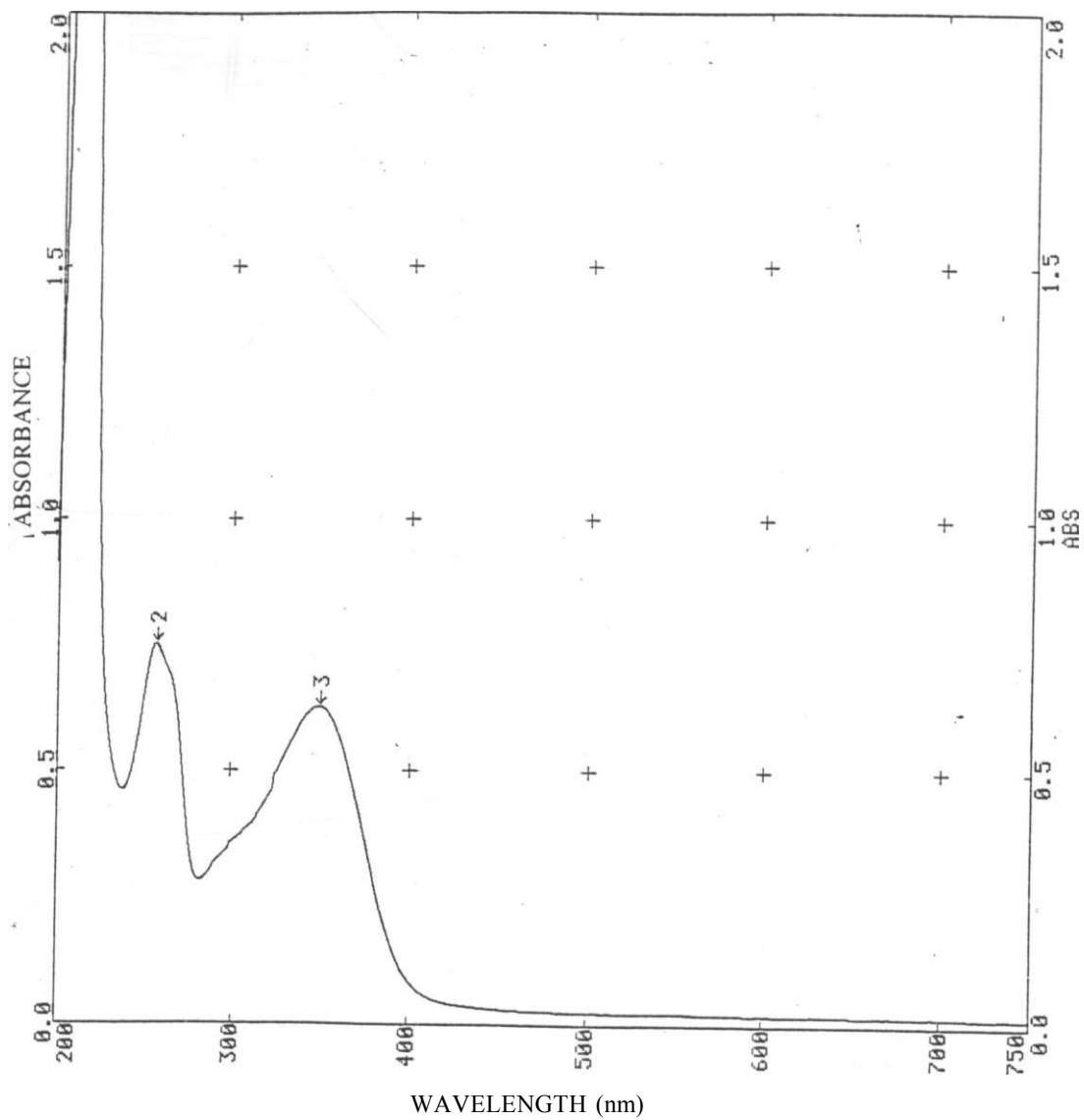- WATER (85:15 TO 10:90) IN 20 MINUTES

TIME (minutes)

V^x

158
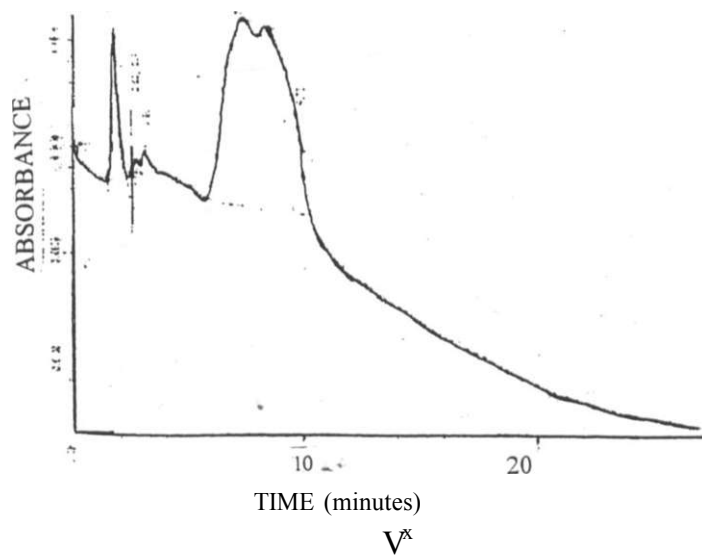
APPENDIX C

THE UV SPECTRUM OF FAO-WR

(Concentration: 3.45mg/ml, Solvent: methanol-water (1:2))

WAVELENTGH (nm)

APPENDIX D

The UV Spectrum of OCB-3 (Solvent - Methanol)

4^J

APPENDIX E

THE IR SPECTRUM OF OCB 3 (KBr)
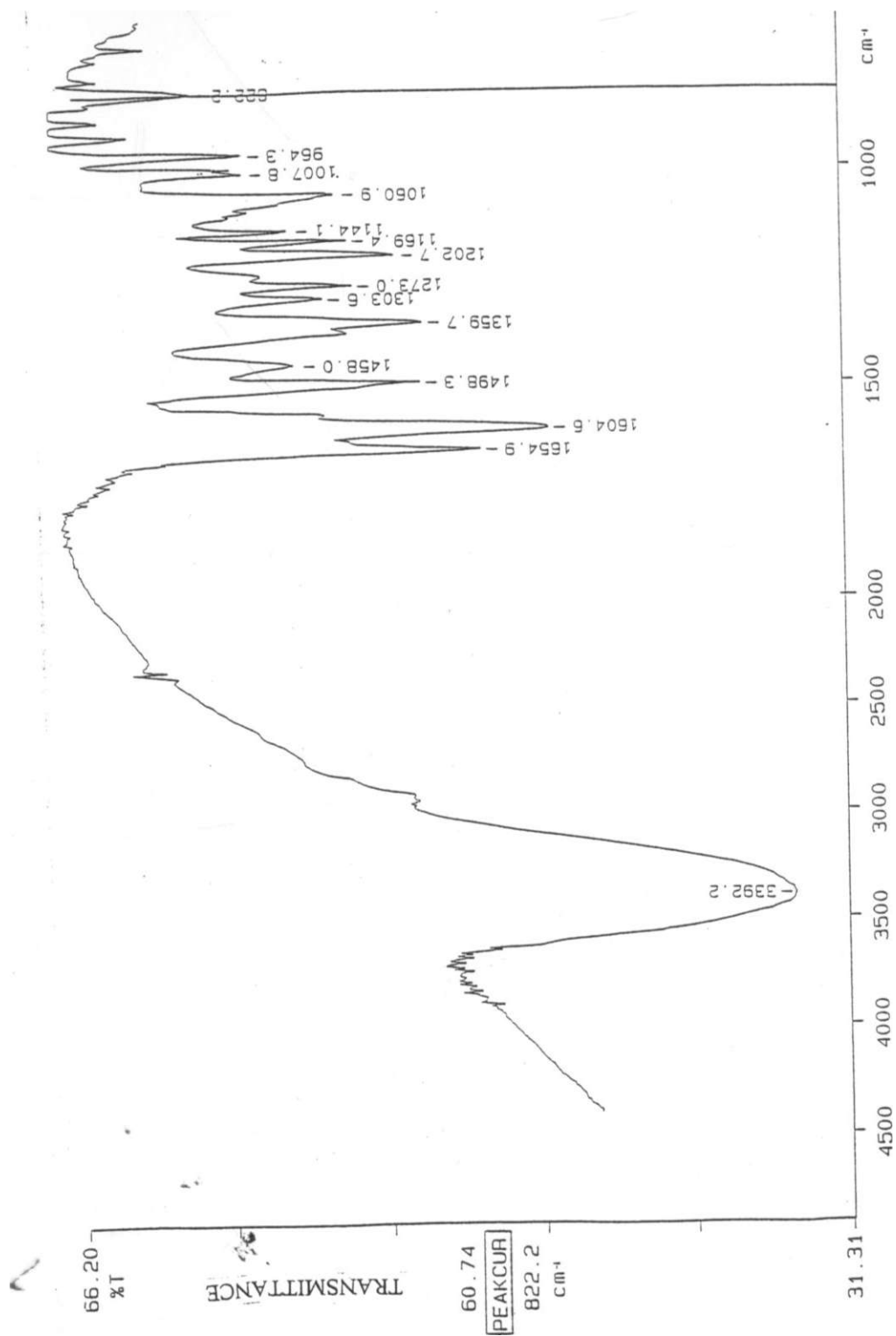
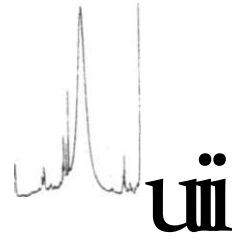VPPENDIX F: THE PROTON NMR SPECTRUM OF OCB-3

acetone -
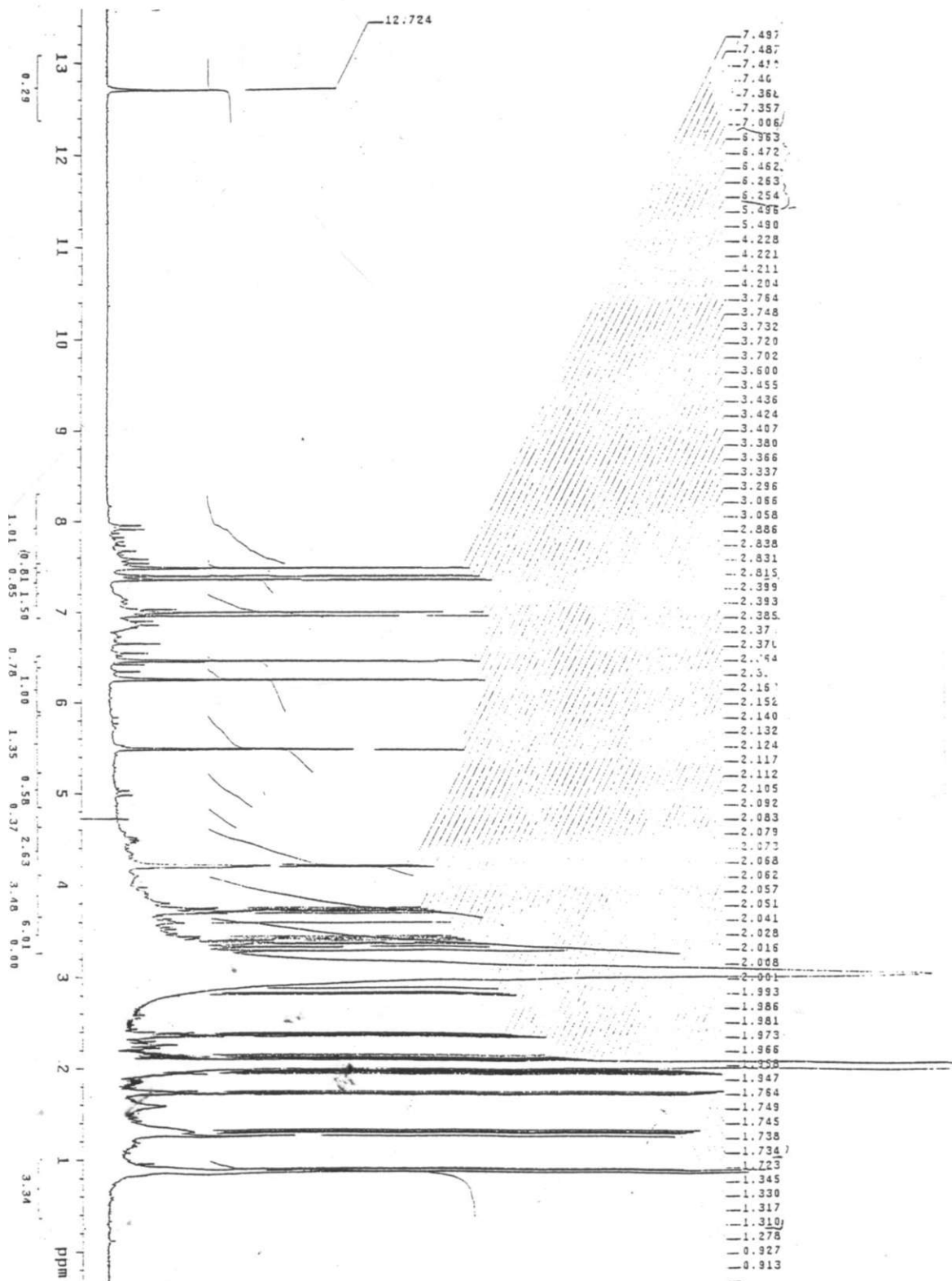
¹³CARBON NMR SPECTRUM OF OCB-3
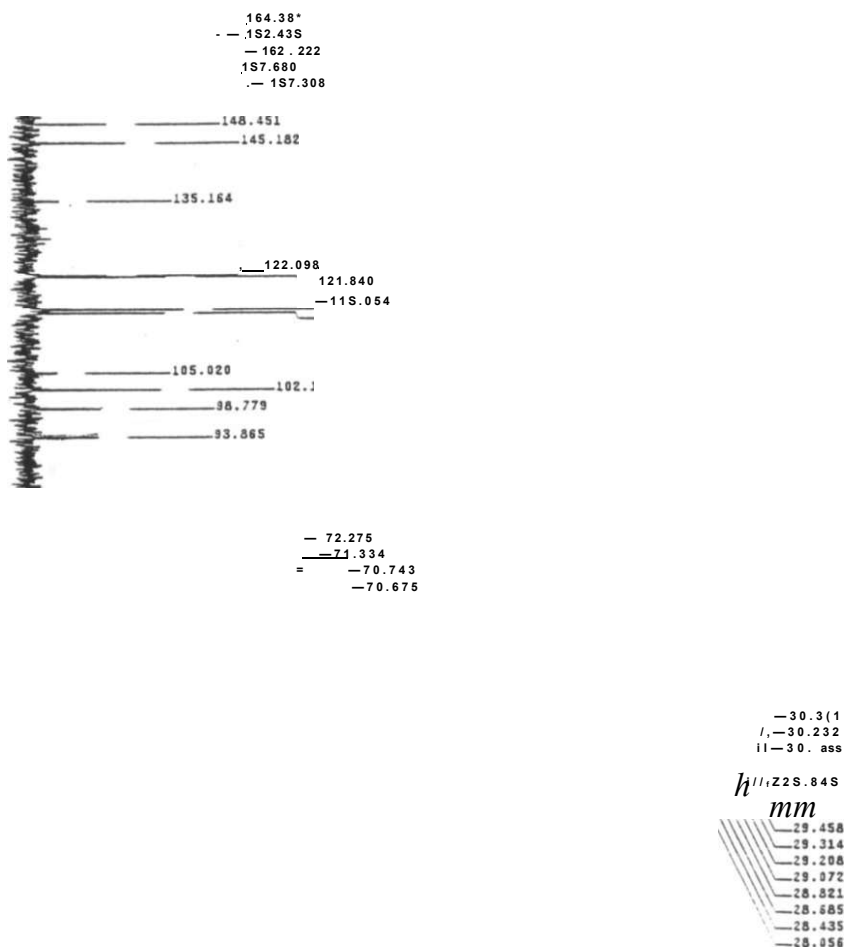
;nt: Acetone d^.

164.38*
- — 1S2.43S
— 162 . 222
1S7.680
.— 1S7.308

148.451
145.182

135.164

122.098
121.840
—11S.054

105.020
102.1
98.779
93.865

— 72.275
—71.334
=   —70.743
—70.675

—30.3(1
/,—30.232
i l—30. ass

h^{//},Z2S.84S
mm
29.458
29.314
29.208
29.072
28.821
28.685
28.435
28.056

u Uï

-o-]

2-

4 q

4 'H

6 -

$J^{3'}$

8-

10-

12-j

14- 14   12   10   8   6   −0

F1 (ppm)

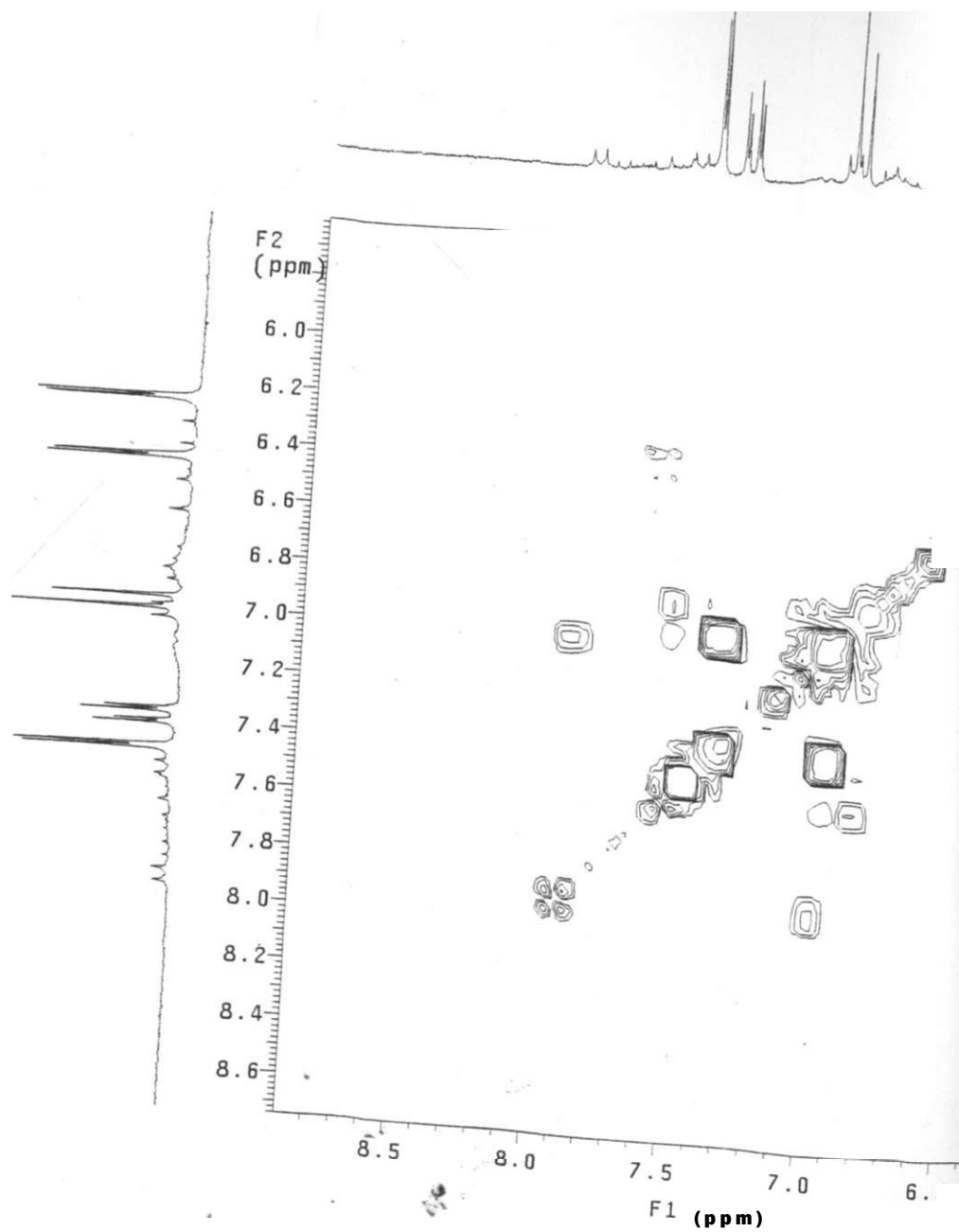# APPENDIX I

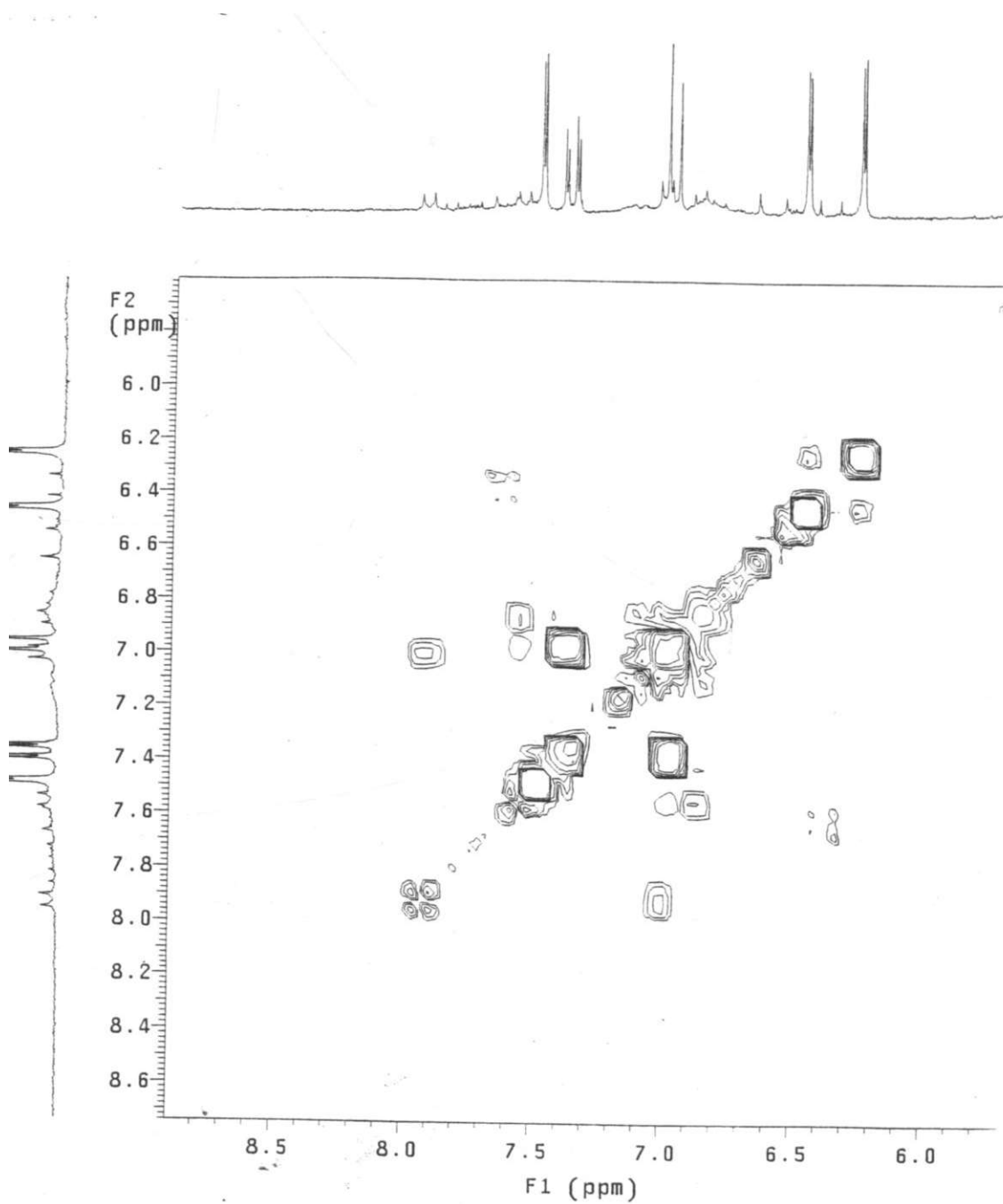## THE HOMONUCLEAR COSY SPECTRUM OF OCB-3 (So

"84.5)
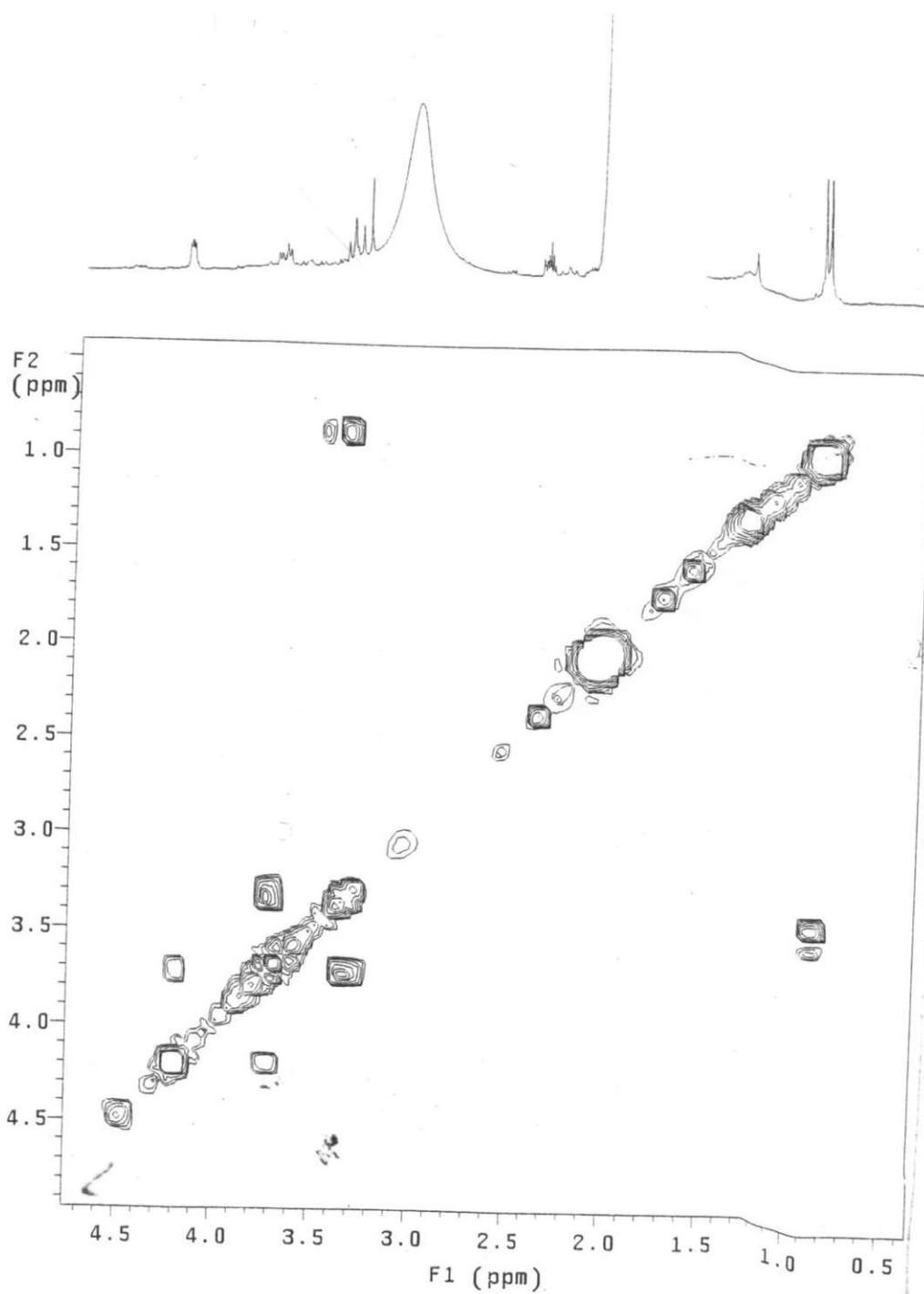
APPENDIX J

TOEHOMONUCLEAR COSY SPECTRUM
^{OF} OCB-3 (56.0

NDIXJ

iOMONUCLEAR COSY SPECTRUM OF OCB-3 (86.0 -

THE DEPT NMR SPECTRUM OF OCB-3 (80 - 8200).