EXTRACTION, ISOLATION, AND IDENTIFICATION OF COMPOUND(S) RESPONSIBLE FOR ANTI-DIARRHOEAL ACTIVITY OF CASSIA ABBREVIATA-OLIV.

BY

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A dissertation submitted in partial fulfilment of the requirement of the degree of Master of Science in Chemistry by the University of Zambia.

THE UNIVERSITY OF ZAMBIA

1994

DECLARATION

I, Martin Sehrvarzioh Tembo, declare that this dissertation is my own work and that it has not been previously submitted for degree purpose to this or any other University.

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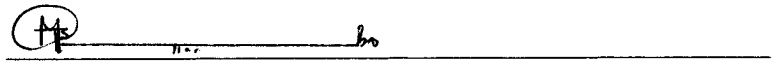
SIGNATURE: 

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APPROVAL

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DEDICATION

To Daddy, Mummy and Marsella (my sister) and to those whose lives and efforts have been dedicated to the fight against diarrhoea that people may enjoy good health.

"I pray that you may enjoy good health."

3rd John:2

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ABSTRACT

An aqueous decoction of the stem bark of the plant **Cassia abbreviata-Oliv.**, a native Zambian plant, is traditionally used as an anti-diarrhoeal remedy by many contemporary practitioners of traditional medicine in Zambia.

The present work was undertaken based on the findings of preliminary investigations on **Cassia abbreviata-Oliv.** stem bark by Dr. S. Prakash.[13(i),13(ii)]

An ethanolic extract was prepared from the dried powdered stem bark of **Cassia abbreviata-Oliv.** by Soxhlet extraction and its anti-diarrhoeal activity was evaluated in-vitro on rabbit ileum by the method of Perry et al [67]. The ethanolic extract was found to be a parasympathetic acetylcholine receptor, sympathetic ganglia and autonomic ganglia blocking agent as it antagonized the spasms induced by acetylcholine, histamine and nicotine, respectively. It was also found to have a direct inhibitory effect on the motility of the rabbit ileum because it antagonized magnesium chloride and barium chloride induced spasms. The ethanolic extract was found to demonstrate potent anti-bacterial activity against **Staphylococcus aureus, Shigella dysentrie, Proteus spp.** and **Bacillus spp.** and its minimum inhibitory concentration (MIC) was also determined. The ethanolic extract was found to contain alkaloids, tannins and nitrogen.

The ethanolic extract was separated on the basis of differences in polarity, pH, and solubility of its components into 13 fractions and anti-diarrhoeal activities of each of these fractions were evaluated in-vitro.

The most potent anti-diarrhoeal fraction, extract 13, was found to be a neuromuscular and ganglion cell blocking agent as it antagonized the spasms induced by nicotine. It was also found to have a direct inhibitory effect on the motility of the rabbit ileum because it antagonized barium chloride induced spasms as well. However, it did not antagonize acetylcholine and histamine induced spasms. Extract 13 also exhibited potent anti-bacterial activity, in-vitro, against **Shigella dysentrie** and **Proteus spp.** and its MIC was also determined.

Extract 13 was subjected to bio-assay directed separation and purification by a combination of analytical and preparative chromatographic techniques [73,74, 75,76]. At each stage of separation/purification the anti-diarrhoeal activity was monitored and only those fractions/eluates which demonstrated appreciable anti-diarrhoeal activity were chosen for further purification.

Repetition of the process of chromatographic purification linked to evaluation of anti-diarrhoeal activity yielded a pure compound which was analysed by 500 MHz ^{1}H NMR spectrometry. The ^{1}H NMR data obtained was studied and chemical structure of the compound was proposed to be either 3-(3′-hydroxy-4′-methoxphenyl)-2-propenoic acid or 3-(4′-hydroxy-3′-methoxyphenyl)-2-propenoic acid.

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LIST OF ABBREVIATIONS

1.	ADP	Adenosine-5′-disphosphate
2.	ATP	Adenosine-5′-triphosphate
3.	CHF	Chloroform
4.	cm	Centimetre
5.	DMSO	Dimethyl sulphoxide
6.	2DTLC	Two Dimensional Thin Layer Chromatography
7.	EtOAc	Ethylacetate
8.	g	Gramme
9.	^{1}H NMR	Proton Nuclear Magnetic Resonance
10.	HPLC	High Performance Liquid Chromatography
11.	I.R.	Infra Red
12.	I.U/l	International Units per Litre
13.	J value	Coupling Constant
14.	kg	Kilogramme
15.	l	Litre
16.	Me	Methyl (CH_3-)
17.	MHz	MegaHertz
18.	MIC	Minimum Inhibitory Concentration
19.	mg	Milligramme
20.	ml	Millilitre
21.	mm	Millimetre
22.	nm	Nano metre

23.	N.M.R.	Nuclear Magnetic Resonance
24.	pH	$-\text{Log}[H^-]$
25.	PMR	Proton Magnetic Resonance
26.	ppm	Parts per Million
27.	Rf	Relative Migration Rate Factor $= \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}}$
28.	T.L.C	Thin Layer Chromatography
29.	T.S.A	Trypticase Soy Agar
30.	T.S.B	Trypticase Soy Broth
31.	uv	Ultra Violet
32.	μg	Microgramme
33.	μl	Microlitre

CHAPTER ONE

INTRODUCTION

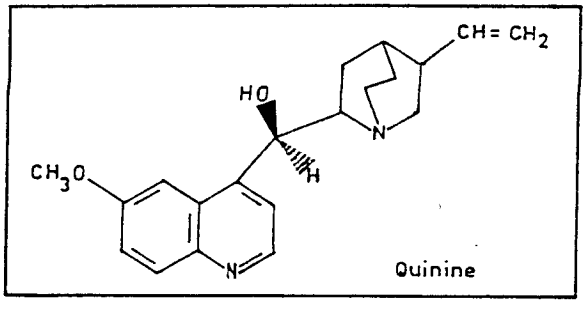
INTRODUCTION

1.1 The History of the Use of Medicinal Plants by Man

Mankind has a venerable history of the use of higher plants and preparations taken from them for treatment of various diseases.

One of the earliest records of the use of herbal medicine is that of chaulomoogra oil from a species of **Hydnocarpus Gaertn** [1]. Its use was recorded in pharmacopoeia of the Emperor Shen Nung of China between 2730 and 3000 BC. The seeds of opium poppy, **Papaver somniferum L.**, were excavated from some ancient Egyptian tombs which indicated their use as far back as 1500 BC, which could have been probably used for the same medical purpose as they are used today [1]. Suffice it to say that some 3000 years BC, man was well aware of the medicinal properties of some plants growing around him [2]. By trial and error, man discovered that certain plant materials were effective against a number of diseases [3]. It was Hippocrates, a Greek philosopher, who first regarded medicine as a science and he is now referred to as the father of medicine [1,3].

One of the greatest herbal remedies, **Cinchona bark**, obtained from South American Indians, who had long used it as a remedy for chills and fevers, was introduced into Europe in the 17th Century by a Jesuit missionary who had accompanied the Spanish conquistadors on their exploration of Central and South America. It soon became the preferred medicine for fevers, chills and malaria in Europe. Two centuries later, in 1820, its active principle, quinine, was isolated [3].



In the 18th Century, Withering introduced the use of an extract prepared from leaves of the Fox-glove plant, in England, for treatment of dropsy, a heart condition characterized by excessive accumulation of liquid in the lower limb of the patient. He used this extract on the personal recommendation of a country folk who had been using the elixir for several years [3]. This represents a good example of an enquiring medical practitioner following up and developing a lead from folk culture. The active principle, digitalis, is still used today for threatened heart failure and it is even now obtained from Fox-glove, **Digitalis purpurea L,** leaves.

The importance of higher plant materials in the development of drugs has been substantiated by numerous researches and a large number of currently used drugs owe their origin to plant sources [4,5,6]. Apart from quinine and digitalis, other currently used drugs of plant origin include morphine, artermisinine, emetine, sanguinarine, berberine, etc. [5,7]. Plant materials have also been used as a source of novel structural leads and some of these lead compounds have been developed into useful drugs by enhancing their potencies and/or improving their ADME (absorption, distribution, metabolism and excretion) characteristics by structural modifications or synthetic molecular manipulations.

In the past two decades or so, the interest in therapeutic constituents (bio-active secondary metabolites) of higher plants has reawakened worldwide and the literature in this area is now becoming substantial [8]. Improved understanding of biochemical events underlying a number of diseases at molecular level and introduction of convenient bio-assays has led to rapid progress in the field of plant based and other naturally occuring therapeutic agents.

Although therapeutic potential of most of the American, Asian and European medicinal plants has been investigated, far less scientific studies have been undertaken to evaluate the medicinal potential of varied and exotic African plant flora. Natural products chemists in East, Central, South and West Africa have investigated a moderate number of plants traditionally used as medicine in their respective countries and in few cases, significant success has also been achieved, for example, patenting of **Fagora** roots by Sofowara et al of Nigeria as a remedy for sickle cell anaemia [9].

Zambia has abundant indigenous medicinal plants which have been used by contemporary practitioners of traditional medicine for treatment of various diseases on an empirical basis. Some preliminary studies have been carried out including chemical and physiological studies on **Aristolachia Petersiana** Klotz by Siamwiza and Kasitu [10], investigation of antitubercular potential of an undisclosed Zambian medicinal plant by National Council of Scientific Research [11], antimicrobial study of some Zambian medicinal plants by Prakash et al [12], and anti-diarrhoeal study of **Cassia abbreviata-Oliv**. by Prakash et al [13(i),13(ii)]. However much scientific work remains to be done on numerous plant materials traditionally used as medicine in Zambia. Hence, there is a need to evaluate the medicinal potential of these materials with a view to rationalize or reject their folkloric usage before the

dwindling plant species becomes extinct.

1.2 Diarrhoea

Diarrhoea is an intestinal disorder characterized by abnormally frequent passing of more or less liquid faeces. It results in excessive loss of water and essential salts from the body, leading to severe dehydration [14,15]. Diarrhoea poses a serious global health problem causing significant morbidity [14] and mortality [16,17] by dehydrating the patient if uncontrolled, particularly in the paediatric age group [16]: For example in the year 1989. estimates reveal acute episodes in the range of 6.5 million in U.S.A. alone with 9,100 deaths [16].

In Zambia, both the incidence and the severity of diarrhoea are quite high particularly in children. Statistical data on diarrhoeal patients obtained from University Teaching Hospital, U.T.H., Lusaka, presented in Table 1 for a period of four years, from 1990 to 1993, reveals that on an average, 44.58% of out-patients suffering from diarrhoea were admitted into hospitals/clinics each year for treatment and the mean associated mortality was approximately 9% per year [18].

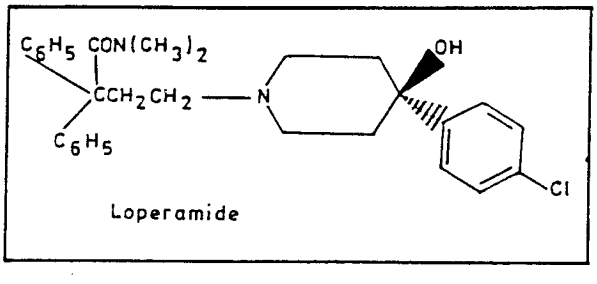
Year	% of the diarrhoea patients admitted	% of the diarrhoea patients that died
1990	54.3	8.5
1991	24.33	4.3
1992	43.20	12.1
1993	56.50	12.0

TABLE 1: Percentage of The Total Diarrhoea Out-patients Admitted and Associated Mortality During 1990-1993

Aetiological origins of diarrhoea are varied - ranging from a variety of infections such as bacterial [19], protozoal, parasitic and viral [20] to thyrotoxicosis, medullar cancer of thyroid with amyloid stoma, carcinoid tumour and to nervous origin connected with neurovegetative disturbances [21] causing malabsorption syndrome [22,23].

1.3. Rationale for the Present Study

The current treatment of diarrhoea in Zambia includes the use of adsorbents such as Kaolin and Pectin [24]; anti-diarrhoeal agents such as Diphenoxylate [ethyl-1-(3-cyano -3,3-diphenylpropyl)-4-phenyl-4-piperidinecarboxylate],Loperamide [4-(4-chlorophenyl)-4-hydroxy-N,N-dimethyl-α,α-diphenyl-1-piperidinebutanamide hydrochloride], Lomotil [a mixture of diphenoxylate hydrochloride and atropine sulphate], and Imodium [25] which are basically piperidine derivatives; and a number of anti-biotics such as septrin,penicillin etc.[26,27]. Anti-diarrhoeal action of piperidine derivatives has been attributed to their anti-peristaltic action. These drugs act directly on the smooth muscle of the intestine.



Loperamide

However, most of these currently used drugs cause several side effects manifested in the forms of gastrointestinal upset, skin rashes, mucous membrane lesions, abnormalities in early formation of red blood cells, fever, elevation of serum iron, etc [28]. Moreover, many of these drugs such as Lomotil and Imodium cannot be given to children as they often cause

paralysis of the intestines. These drugs also induce grey syndrome in premature and new-born infants [28]. Hence, there is a need to develop more potent but less toxic anti-diarrhoeal agents/remedies.

An aqueous decoction prepared from stem bark of the plant **Cassia abbreviata-Oliv.** is traditionally used as anti-diarrhoeal remedy in Zambia by many people. Apart from three references that shall be referred to later, there are no other phytochemical or pharmacological reports on this plant in the reported literature.

Initial investigations carried out by Prakash et al in this laboratory confirmed the anti-diarrhoeal activity of the crude ethanolic extract of stem bark of **Cassia abbreviata-Oliv** [13(i),13(ii)]. Prakash et al reported that hot ethanolic extract was slightly more potent than aqueous and cold ethanolic extracts [13(i),13(ii)]. Hot ethanolic extract was therefore, chosen for the present study. They further reported that the ethanolic extract exhibited significant anti-cholinergic action on the rabbit ileum [13(i),13(ii)].

It is significant to note that a well known plant of the genus **Cassia**, popularly called **Senna**, has long been used as a purgative and pharmaceutical preparations from **Senna** leaves are incorporated in British Pharmacopoeia as official purgatives for treatment of constipation [24].

These observations stimulated considerable scientific interest and prompted us to undertake the present study on stem bark of **Cassia abbreviata-Oliv.** with a view to isolate the active principle(s) responsible for its anti-diarrhoeal activity.

1.4 Anti-diarrhoeal Medicinal Plants

Cassia abbreviata-Oliv is a native Zambian tree of the leguminosae family. It is distributed throughout the country plateaus especially in the eastern part of Zambia. It is also distributed in the eastern, central and southern regions of Africa. **Cassia abbreviata-Oliv.** is also known by a number of vernacular names, including Muleza (Chewa), Munsokansoka (Bemba) and Namayaka (Inamwanga). The species, abbreviata, is claimed to have considerable therapeutic potential and it is largely used as a traditional anti-diarrhoeal remedy [29] though in some parts of the country, it is also used to treat sexually transmitted diseases such as chancroid and candidiasis. The stem bark is preferentially used as an anti-diarrhoeal remedy by soaking it in water and then drinking the aqueous decoction. The aqueous decoction is bitter and reddish brown in colour. It is also reported that the Lamba and Lenje people make use of warmed up cold infusion of **Cassia abbreviata-Oliv.** roots for the relief of toothache by holding it in the mouth [29].

In South Africa, the plant is reportedly used as a remedy for dysentery and diarrhoea [29]. In East Africa, Raymond reports that roots and stem bark of **Cassia abbreviata-Oliv.** are used for treating diarrhoea and malaria and that they contain tannins [30].

Some other plants belonging to the **Cassia** genus are also claimed to be effective against diarrhoea. **Cassia horrida** is used by traditional practitioners of medicine in Zimbabwe as a remedy for diarrhoea [31]. **Cassia Singueana Del.** is used as an anti-diarrhoeal remedy in Erithrea because of its astringent property [29].

A few other plants that do not belong to the genus **Cassia** have also been reported to have anti-diarrhoeal therapeutic potential. For example **Acontum heterophyllum** of aconitum plant

species is reported to be used as a remedy for diarrhoea and dysentery in India [32].

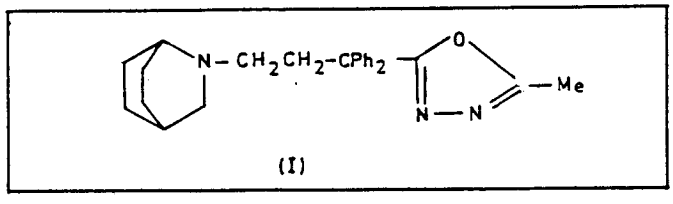
Aconus calamus L. is said to contain tannin as an astringent and it is, therefore, a useful diarrhoeal remedy [32]. **Aegle marmelos L. correa** has been claimed to be very useful in treating chronic diarrhoea and dysentery, particularly in patients having diarrhoea alternating with constipation [32].

Genn rivale is reported to be rich in tannin and it is claimed to be effective against dysentery, chronic diarrhoea, and most ailments of the digestive tract [33]. An infusion of **Anaphalium polycephalum** is also reported to be rich in tannin and it has been used successfully as an anti-diarrhoeal remedy on an emperical basis [33]

Fiscus is another important genus which is reported to provide both emollients and astringents [34]. The bark of **Fiscus vogeliana** of tropical Africa is said to be rich in tannins and it is said to be useful as an anti-diarrhoeal remedy, as a vermifuge and in wound dressing [34].

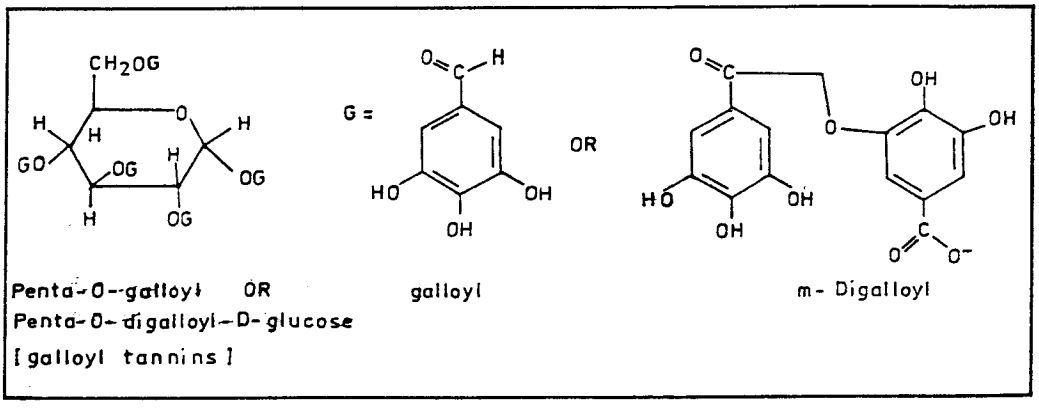
The unripe fruit, bark and leaves of a species of **Custard apple** are reported to contain tannin often associated with an inactive alkaloid methyl-tyrosine, and a resin. Extracts are emperically used as astringents in diarrhoea [34].

Chemical structure of a new anti-diarrhoeal drug, (I), has been elucidated by Mackerer et al [35].



(I)

Tannins example given below as well as their genins have been found to have the property of precipitating proteins and mucus and they also constrict blood vessels [34; 36]. It has been discovered that it is this astringent action that gives tannins value in controlling haemorrhage, checking diarrhoea and application to wounds, ulcers and deep burns which are thereby covered with an impervious protective coating [34]. Tannins are reported to be one of the more abundant bio-active principles and they are present in most trees, shrubs and in many herbs [34]. Among the more important of these are the thorn tree (Acacia), the mahogany tree (Diospyrosmelanoxylon), the cucumber tree (Kigeli), the mango tree (Mangefera indica), the cayor apples (Parinarium), the mangrove (Rhizophora mangle), the African tulip tree (Spathodea) and the artanot (Xanthoxylum senegalense) [34].

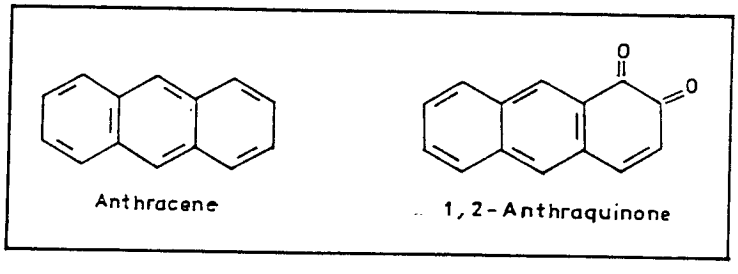


1.5 Secondary Metabolites Produced by Plant Species of the Genus Cassia

Apart from the work done in the authors laboratory by Prakash et al [13(i),13(ii)], there is no other phytochemical or pharmacological report on **Cassia abbreviata-Oliv** in the literature. However, a number of plant species of the genus **Cassia** have been investigated by many researchers and a wide range of compounds have been isolated. Some of these

compounds are reported to exhibit varied biological activities but there is no report on the isolation of any anti-diarrhoeal compound from any of the plant species genetically related to **Cassia abbreviata-Oliv**.

A number of plant species of the genus **Cassia** have been found to contain anthracene compounds or oxymethylanthraquinones as their active principles [29]. These compounds are irritant purgatives which stimulate the peristaltic movements of large intestine specifically.

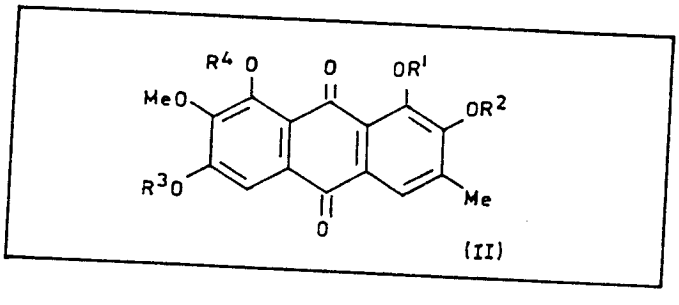


Githens has reported that the seeds of **Cassia absus L.** are used as purgative, an anthelmintic for round worms, and in ophthalmia in Southern Africa [39]. In East Africa, Erithrea, and West Africa, **Cassia aschrek fork** is reported to be used for treating stomach complaint and it is also used as a violent purgative on account of its anthracene and oxymethylanthraquinone contents [37]. The content of oxymethylanthraquinone was determined to be 1.1% in the leaf and 1.2% in the pod by Maurin [38]. The plant is reported to be one of the sources of Senna leaf used as a purgative. The active principle of Senna leaves was found to be cathartic acid, which causes purgation [29].

Yun-Choi et al, extracted, fractionated and characterized by NMR, mass spectrometry and m.ps, anthraquinones of **Cassia obtusifolia** seeds [40]. Their inhibitory effects on blood platelet aggregation were studied on rat cells stimulated by arachidonic acid, ADP,and

collagen [40].

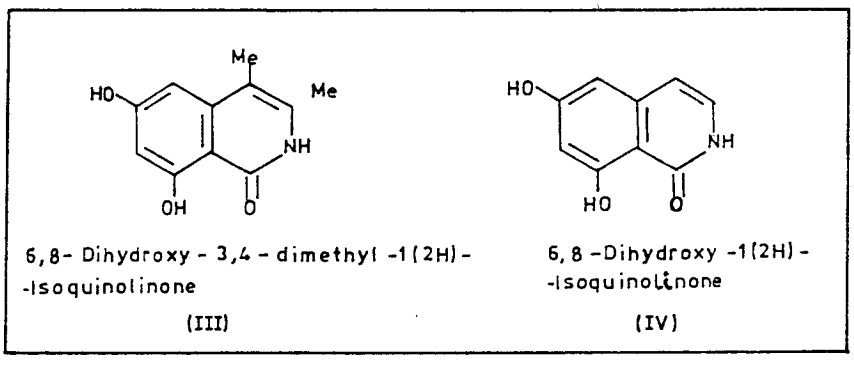
Nikaido et al, identified aurantio-obtusin and obtusin as inhibitors of adenosine 3′,5′-cyclic monophosphate phosphodiesterase in seeds of **Cassia obtusifolia** and **Cassia tora** [41]. Studies on the constituents of purgative crude drugs were carried out by Kitanaka et al. Three new antaraquinones, 1-desmethylchryso-obtusin (II: $R^1=R^2=H, R^3=R^4=Me$), 1-dimethylobtusin (II: $R^1=R^2=R^4=H$, $R^3=Me$), and 1-desmethylaurantio - Obtusin (II: R^1 to $R^4=H$), were isolated from the seeds of **Cassia obtusifolia** and their structures were established by spectral and chemical methods [42].



(II)

Gonzalez et al, isolated 7 new anthraquinones by dichloromethane extraction of **Cassia gregyii** roots, three of which were 5-hydroxy-1,4,6,7-tetramethoxy-2-methylanthraquinone, 1,5,7-trihydroxy-2-methylanthraquinone and 5,6-dihydroxy-1,4,7-trimethoxy-2-methylanthraquinone [43]. The structures of all the isolated anthraquinones were elucidated on the basis of chemical and spectral analysis.

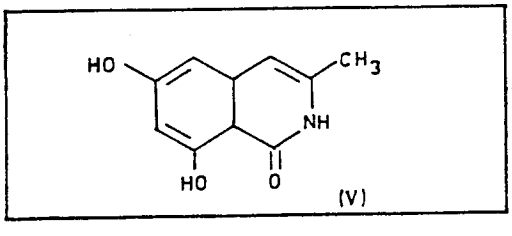
Two Isoquinoline alkaloids (III) and (IV), have been isolated from the leaves of **Cassia siamea** by EL-Sayed et al, and their chemical structures were determined by uv and IR spectrometry [44].



6,8- Dihydroxy - 3,4 - dimethyl -1(2H)- -Isoquinolinone (III)

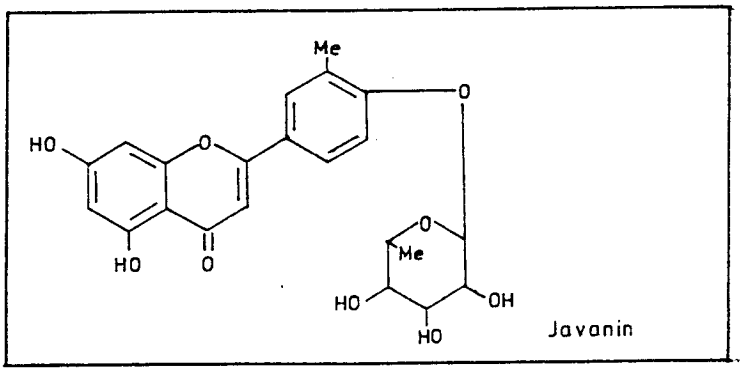
6,8 -Dihydroxy -1(2H)- -Isoquinolinone (IV)

Ahn isolated another alkaloid 6,8-dihydroxy-3-methyl-1(2H)-isoquinolinone, V, from the seeds and leaves of **Cassia siamea** [45].



(V)

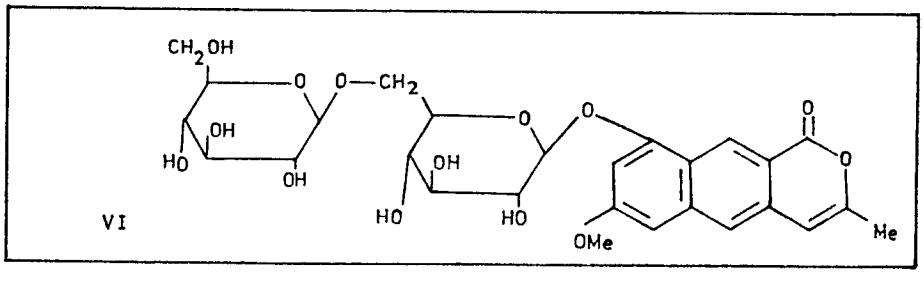
A flavone glycoside named Javanin was isolated from the **Cassia javanica** immature leaves by Chakraborty et al and its chemical structure was established as 5,7-dihydroxy-3-methyl flavon-4-0-rhamnopyranoside on the basis of spectral data and chemical studies [46].



Javanin

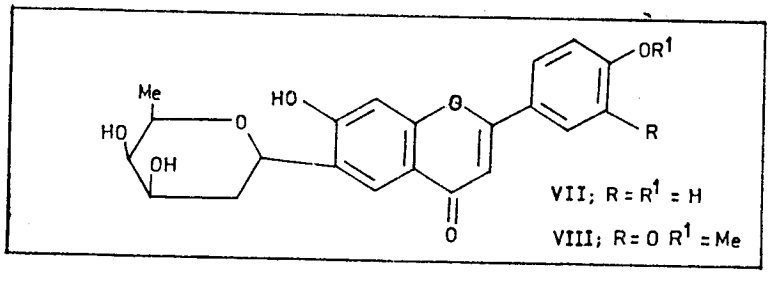
It was the first report on the natural occurrence of a flavone-O-glycoside having a C-Me grouping in ring B.

Wong et al, have isolated a naphtho-pyranoglycoside, 9-[(ß-O-glucopyranosyl-(1→6)-O-ß-D-glucopyranosyl)oxy]-10-hydroxy-7-methoxy-3-methyl-1H-naphtho [2,3-C] pyran-1-one, VI, from the seeds of **Cassia tora** [47].



VI

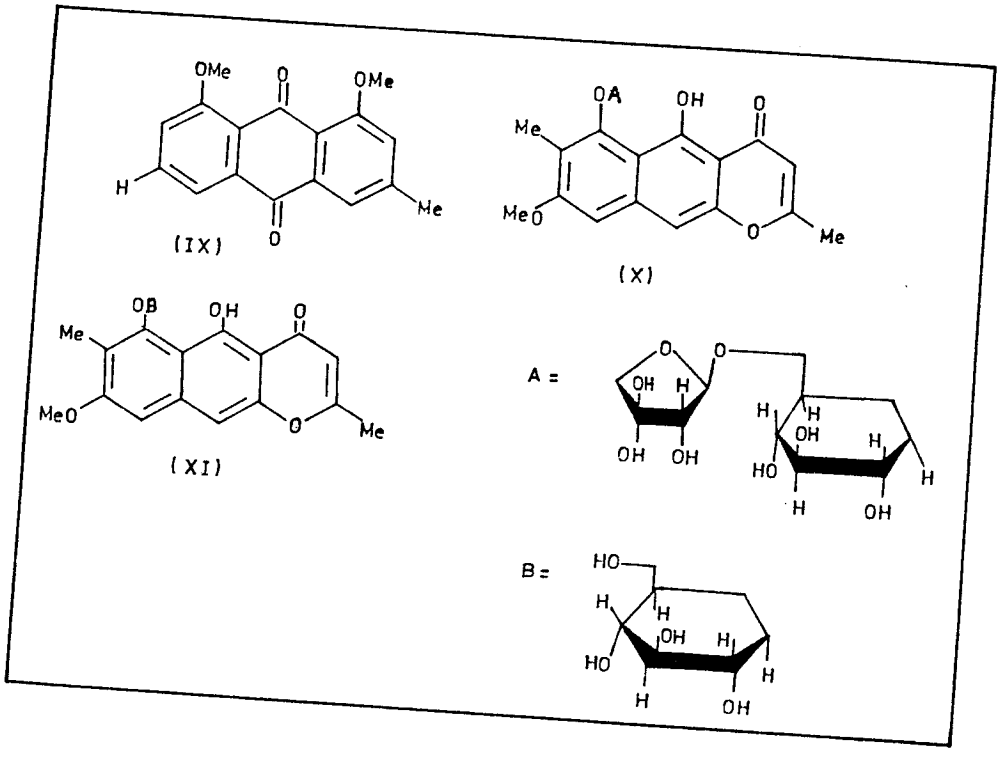
Structure of two glycosides, pegenin 6-C-ß-D- cloidise, VII, and diosmentin-6-C-ß-D-Ohoside, VIII, isolated from **Cassia torasa** leaves were elucidated by Kitanaka et al by X-ray and spectroscopic analysis [48].



In 1990, Kashiwada et al, isolated and identified a few flavan-3-ols and proanthrocyanidins from the pods of **Cassia fistula** and the bark of **Cassia javanica** [49].

A new flavone glycoside, 7,4′-dihydroxy flavone-5-O-ß-O-D-galacto pyranoside, was isolated from **Cassia auriculata** by Linn et al and its structure was confirmed by chemical and spectral studies (IR,uv,PMR, and MS) [50].

Phytochemical examination of methanolic extract of **Cassia pudibunda** roots by Messana et al, led to the isolation of three new compounds, ruborfusarin-6-0-ß-D-glucopyranoside, (IX), quinquangulin-6-0-ß-D-apiofuranosyl-(1→6)-O-ß-D-glucopyranoside (X), and quinquangulin-6-O-ß-D-glucopyranoside (XI), [51]. The antimicrobial activities of these compounds were also reported.



A new flavonol glycoside, kaempferol-7-0-[ß-D-galactopyranosyl-(1→4)-α-L-rhamnopyranoside], and two known flavonol glycosides, quercetin-3-0-[ß-D-glycopyranosyl-(1→6)-α-L-rhamnopyranoside], and myricetin -3-0-α-L-rhamnopyranoside were isolated from **Cassia biflora** leaves by Ahmad et al [52].

Tripathi et al isolated another new flavonoid glycoside, rhamnetrin-3-0-(6"-0-α-L-rhamnopyranosyl)-ß-D-galactopyranoside, from **Cassia siamea** stem bark [53].

The isolation and characterization of four flavonoids, kaempferol, quercetin, kaempferol-3-O-rutinoside and rutin from the heartwood of **Cassia montana** are reported [54].

Three new anthraquinone glycosides, 2-0-ß-D-glucopyranosyl-1, 2, 4, 8-tetrahydroxy-6-methoxy-3-methylanthraquinone, 3-0-ß-D-glucopyranosyl-3-hydroxy-6, 8-dimethoxy-2-methylanthraquinone and 3-0-ß-D-glucopyranosyl-1, 3-dihydroxy-6, 7, 8-trimethoxy-2-methylanthraquinone, have been isolated from the seeds of **Cassia gradis** by Singh et al [55]. The structures were determined by spectroscopy and confirmed by synthesis.

A new anthraquinone glycoside, 1,5,8-trihydroxy-6-methoxy (or 7-methoxy)-2-methylanthraquinone-3-0-ß-D-galactopyranosyl (1→4)-0-ß-D- mannopyranoside, was isolated from **Cassia auriculata** seeds by Linn [56].

1-hydroxy-6, 8-dimethoxy-2-methylanthraquinone-3-O-rutinoside was isolated from roots of **Cassia siamea** by Tripathi et al [57].

An antifeedant and insecticidal steroid, 6-α-7-α-22, 26-24, 25-triepoxy-5, 26-dihydroxy - 17(13-18)-abeo-5-α-ergosta-2, 13, 15, 17-tetraen -1- one and a new hydroxyketone, 11-Hydroxyhexacosan-2-one, were isolated by Srivastava et al, from **Cassia siamea** bark [58]. A number of sterols were isolated and identified from the root and stem bark of **Cassia singueana** by Makasa et al, in Tanzania [59].

Chakraborty et al, have isolated sitosterol, behenic acid, palmitic acid, margaric acid, eupol and 3,3′,4,5,5′,8-hexahydroxyflavone from **Cassia tora** leaves and characterized them by

spectroscopic and chemical methods [60]. The hexahydroxyflavone was isolated for the first time while the other compounds were being recorded from **Cassia tora** leaves for the first time.

EL- Sayed et al, have isolated a few Teflavonoids from **Cassia italica**, including a new compound tamarixetin-3-rutinoside-7-rhamnoside which was reported for the first time [61].

From **Cassia petersiana** leaves, diterpenoids, colensenone, and colensanone have been isolated by Msonthi [62].

A single phytoalexin was isolated and purified from 12 to 14 day old leaves of **Cassia obtusi folia** inoculated with **Albernaria cassia** by Sharon et al [63]. Its structure was elucidated by NMR and mass spectrometry as 2-(ß-hydroxy phenoxy)-5, 7-dihydroxy chromone.

Giuliano et al, isolated two compounds from **Cassia semicordata** roots and assigned the structures 6,8-dihydroxy-7-acetyl-1-keto-3-methoxy-4,4-dimethyl-1,4-dihydronaphthalene and di-2-(3,6,8-trihydroxy-7-acetyl-4,4-dimethyl-1,4-dihydro)-naphthyl-methane on the basis of 2D-NMR and mass spectral data [64].

It is clear from the literature reports that the plant genus **Cassia** produces a wide variety of secondary metabolites such as anthracenes, anthraquinones, flavonoids, sterols, flavone-glycosides, tannins, quercetin, rutin, etc., some of which have potent bio-activities. It is also clear that anthraquinone and anthracene derivatives are primarily responsible for the purgative action of a number of plant species of the genus **Cassia** and tannins, due to their astringent

action could be responsible for claimed anti-diarrhoeal efffects of certain plants. In view of the anti-cholinergic action of **Cassia abbreviata Oliv.** stem bark reported by Prakash et al [13(i),13(ii)], it is possible that claimed anti-diarrhoeal activity of the plant material could be due to secondary metabolites other than tannins. It would, therefore, be interesting to investigate the bio-active principle(s) of **Cassia abbreviata-Oliv.** as it could provide some novel structural lead(s) responsible for its anti-diarrhoeal effects.

1.6 Objectives

The broader aim of the research was to acquire a reasonable training in some of the scientific techniques used for the development of drugs from plant materials which could be practiced in future for scientific investigation of plant materials traditionally used as medicine in Zambia. The specific objectives of the research were:

- To obtain gross extract from stem bark of **Cassia abbreviata-Oliv.** and evaluate its anti-diarrhoeal activity;
- To isolate active components/fractions responsible for the anti-diarrhoeal activity of the gross ethanolic extract;
- To purify the most potent anti-diarrhoeal fraction with a view to isolate at least one pure compound;
- To study the pure compound by spectroscopic and other methods with a view to comment on its chemical structure, if possible.

In order to come up with these objectives, the following hypothesis were taken into consideration:

- That the stem bark of **Cassia abbreviata-Oliv.**, traditionally used for treatment of diarrhoea in Zambia, has demonstrable anti-diarrhoeal activity in-vitro;
- That the components/fractions responsible for anti-diarrhoeal activity can be chemically separated and isolated;
- That the most unpredictable phenomenon of artifact formation during the course of chemical separation and purification of the plant material can be kept to a minimum;
- That the compound(s) responsible for anti-diarrhoeal activity act(s) as single entity or entities in controlling diarrhoea.
- That at least one pure compound can be isolated from the potent anti-diarrhoeal fraction through chromatographic separation which could be studied by spectroscopic and chemical methods;

1.7. Plan of Work

In order to achieve our aforesaid aims, samples of plant material were collected from Chongwe area in Lusaka, botanically identified by Dr P.S.M. Phiri, Department of Biology, School of Natural Sciences, University of Zambia, and anti-diarrhoeal activity of the ethanolic extract prepared from the plant material was evaluated in-vitro by the method of Perry et al [67].

Increased intenstinal motility is widely considered to be the cause of diarrhoea because in any conduit or tube containing fluid, movement of the wall directly corresponds to the flow rate of fluid [68]. It is also well established that the intestine from any small animal continues to

give responses for many hours if kept in a suitable medium such as Tyrode's solution in an organ bath at 37°C [68]. The test extracts were, therefore, expected to inhibit the motility of the rabbit ileum to signify any demonstrable anti-diarrhoeal activity in-vitro.

The ethanolic extract was systematically fractionated by acid-base and solvent extraction procedures [69]. The components of the ethanolic extract were seperated on the basis of their differences in polarity, solubility, pH and partition characteristics. Similar fractionation schemes involving the use of bases, acids and organic solvents have also been used by many researchers, for example, Rahman et al [70,71] and Griffin [72]. The most potent anti-diarrhoeal fraction was identified by evaluating the anti-diarrhoeal activities of each of the thirteen fractions.

The modes of anti-diarrhoeal actions of NPOO1Z and extract 13 were studied by monitoring and recording the pharmacological effects of the solutions of test extracts in propylene glycol on certain receptor sites such as parasympathetic ganglia and postganglionic acetylcholine receptors on smooth muscle of the rabbit ileum suspended in Tyrode's solution in an organ bath at 37°C with the aid of a Kymograph/electronic transducer. In addition, the relationship between the dose and the anti-diarrhoeal response, i.e., Dose-Response curve of the most potent anti-diarrhoeal fraction, extract 13, was also studied.

In order to get some useful information for further separation and purification of the most potent fraction, extract 13, the ethanolic extract, NPOO1Z, and extract 13 were tested for the presence of nitrogen, sulphur, tannins and alkaloids. The Rf characteristics of extract 13 were also studied in a variety of solvent systems by thin layer chromatography.

The most potent anti-diarrhoeal fraction, extract 13, was subjected to bioassay directed separation and purification with a view to isolate its anti-diarrhoeal component(s) in a pure state. Extract 13 was progressively seprated into less complex mixtures by a combination of thin layer chromatograohy [69,70,71] and preparative chromatography [73 to 76]. Anti-diarrhoeal activity was monitored at each stage of separation/purification and only those eluates/fractions which demonstrated appreciable anti-diarrhoeal activity were chosen for further purification.

Silica gel-60 Merck type 44784 and a solvent system MeOH:Me_2CO:EtOAc:CHF (1:2.5:1:7) were used as stationary phase and mobile phase, respectively. The eluting solvent system was arrived at by trial and error.

A potent anti-diarrhoeal compound LAMM, m.p. 59-4-60°C, was isolated from extract 13 by bio-assay directed chromatographic separation and purification.

The compound LAMM was analysed by ^{1}H NMR and H-H cosy-45 experiments so as to get some information on its structure. Analysis of the spectrum showed that it was not sufficiently pure because the peaks on the H-H cosy spectrum did not correlate well. LAMM was, therefore, purified further by HPLC and the pure compound isolated in the largest quantity, 1.2 mg, was analysed by 500 MHz ^{1}H NMR spectroscopy with a view to comment on its structure. The methods used for detailed analysis are described in the subsequent chapter two.

CHAPTER TWO

EXPERIMENTAL

2:1 Collection of Authentic Sample of the Plant Material

In March, 1992, a native higher plant traditionally used to treat diarrhoea and whose vernacular names are Muleza (Chewa), Munsokansoka (Bemba), and Namayaka (Inamwanga) was located in Chongwe area 40 Km east of Lusaka city in Zambia. Stems and branches of the plant were collected using an axe, a panga, and a knife. The stems were chopped to 20 cm long pieces and placed in a large polyethylene sack.

The plant materials were brought to the authors laboratory and the branches with leaves were taken to Dr. P.S.M. Phiri, Department of Biology, School of Natural Sciences, University of Zambia, for botanical identification. Upon thorough investigation the plant was botanically identified as **Cassia abbreviata-Oliv.**

2:2 Preparation of Gross Ethanolic Extract of the Stem Bark of Cassia abbreviata-Oliv.

2:2:1 Preparation of Dry Powdered Stem Bark of Cassia abbreviata-Oliv.

The stem bark of **Cassia abbreviata-Oliv.** was removed and chopped to small pieces of about 6-9 cm long by use of a panga and a knife. These pieces were spread on four large sheets (87 cm x 68 cm) of paper on bench tops in a room away from sunlight. It took two weeks for the pieces to be sufficiently dry for grinding.

The shade dried stem bark was pounded and subsequently ground to a fine powder in a heavy duty grinding mill, Retsch GmbH, Model No. 5657, Germany. Sieves of 0.5, 0.25 and, 0.05 mesh were successively used to

ensure that the bark was ground to a fine powder.

1.730 kg of the fine bark powder was thus collected and stored in a large air-tight amber coloured bottle in a cool dark drawer. The powder was light brown in colour and had a slight choking smell.

2:2:2 **Extraction of Powdered Stem Bark of Cassia abbreviata-Oliv.**

The powdered stem bark of **Cassia abbreviata-Oliv.** was extracted with 95% ethanol by Soxhlet extraction procedure [73].

Three Soxhlet extractors were set up in a series as shown in Figure 1. For each Soxhlet extraction assembly, boiling chips and 150 ml of extracting solvent, ethanol, were placed into a 250 ml round bottomed flask. 25.28 g of the stem bark powder was placed into a cellulose porous extracting thimble of internal diameter (30 mm) and external length (110 mm). The thimble was loosely plugged with cotton wool at the top to prevent the powder from spilling into the flask during extraction.

Soxhlet extraction was carried on for six hours to ensure that all the ethanol extractibles of the plant material were fully extracted.

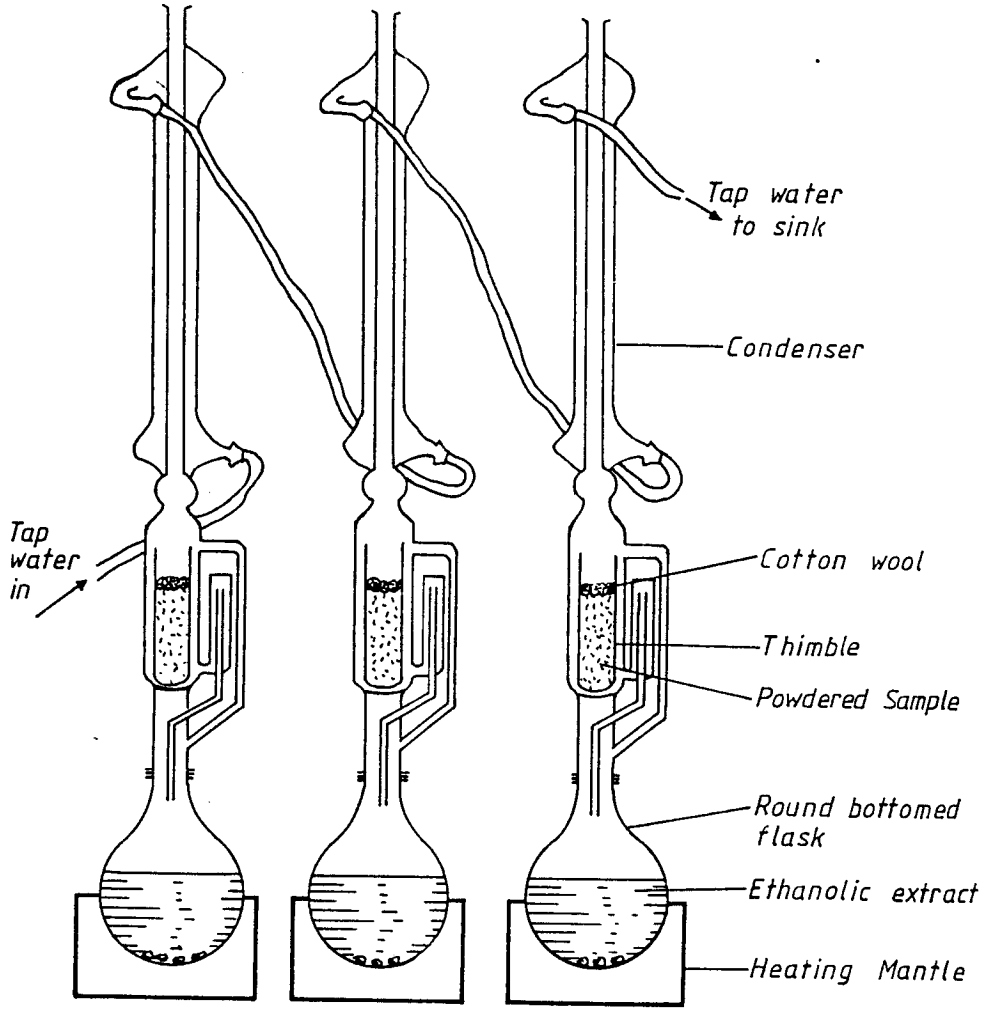


Figure 1: Soxhlet Extraction.

The experiment was done repeatedly till all the 1.73 kg of stem bark powder of **Cassia abbreviata-Oliv.** sample, was extracted.

Small dark brown particles were observed in the hot ethanolic extract which were collected by suction filtration using Whatman No. 41 filter paper while the ethanolic extract was hot. The clear filtrate was poured into a clean 3 litre flat bottomed flask and kept in a cool dark place while the residue was transferred to a petri-dish, dried over anhydrous calcium chloride in a vacuum desiccator for 24 hours and finally stored in an air-tight container labelled *NPM2*.

After Soxhlet extraction, the thimble containing the exhausted plant material was carefully removed from the extraction chamber using a pair of tongs and it was allowed to stand in a clean dry beaker, 300 ml, for 2-3 days, at the end of which the dry exhausted plant material was easily removed from the thimble with a spatula without damaging the thimble. The exhausted plant material was pooled and stored in a large air-tight bottle labelled exhausted plant material. The thimbles were carefully washed with ethanol, dried and reused for extraction.

A light milk yellow precipitate was formed as the clear hot filtrate cooled. The precipitate was collected by suction filtration, using Whatman No. 41 filter paper, transferred to a petri dish, dried over anhydrous calcium chloride in a vacuum desiccator for 24 hours and stored in an air tight container

labelled NP001X. On keeping, the colour of the residue changed from light milk yellow to deep dark brown. The clear filtrate obtained from suction filtration was evaporated to dryness under reduced pressure at 40 °C using a Büchi Model No. RE120 rotary evaporator. A brown solid remained as residue which was collected with the help of a spatula and stored in an air-tight bottle labelled NP001Z.

The anti-diarrhoeal activities of NP001X and NP001Z were evaluated as out lined in 2:3. Amounts of NP001X and NP001Z obtained are shown in Table 2.

Extract	Weight (g)
NP001X	20.1635
NPOO1Z	281.1889
Total Gross Ethanolic	301.3524

Table 2: Weights of Extracts

2:3 Evaluation of Anti-diarrhoeal Activity

Anti-diarrhoeal activity of the test extract was evaluated by the method of Perry et al, [67] at School of Medicine of the University of Zambia in the Pharmacology Laboratory. Subsequently, the effect of test extracts in propylene glycol (1 mg/ml) on certain receptor sites such as para-sympathetic ganglia and post-ganglionic acetylcholine receptors on smooth muscle of the rabbit ileum suspended in Tyrode's solution in an organ bath were monitored with the aid of a kymograph/electronic transducer.

Increased intestinal motility is widely considered to be the cause of diarrhoea because in any conduit or tube containing fluid, movement of the wall directly corresponds to the flow rate of fluid [68].

The test extracts were dissolved in propylene glycol making 20% solution (W/V) while the required chemical solutions were made using deionised water as presented in 2:5. Deionised water was used, even to rinse the apparatus because it has a lower concentration of ions than ordinary distilled water that would have undesirable effect on the highly sensitive living tissue [67]. The apparatus was set up as presented in Figure 2 and connected to either a kymograph as shown in Figure 3 or to an electronic transducer as shown in Figure 4. The glass chamber was filled with deionised water and a thermostat heater was used to ensure that the temperature of the organ bath was constant at 37 °C.

2:3:1 Preparation of Rabbit Ileum

The rabbit was starved and only allowed to drink water for 24 hours. The animal was sacrificed by stunning and cutting the throat. The abdomen was opened and the caecum lifted forward to find the ileum to the back of caecum. The ileum was cut at a point 5 to 10 cm below the stomach and a length was

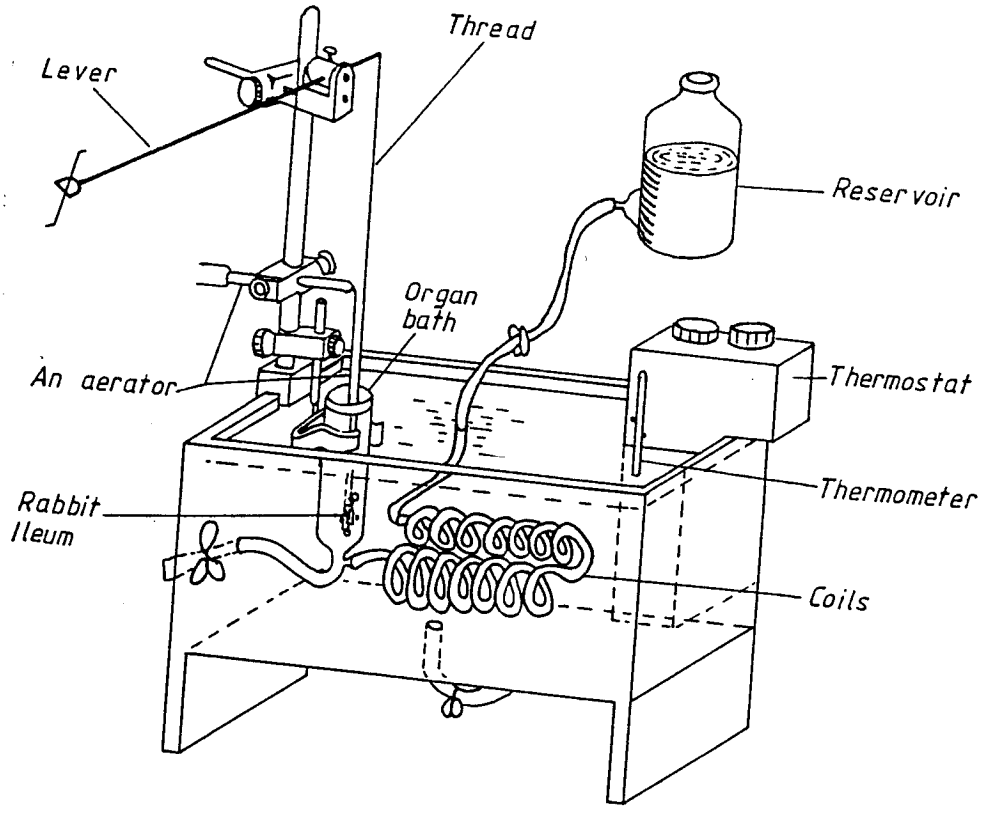


Figure 2: Apparatus for Anti-diarrhoeal Activity Test with an Isolated Tissue

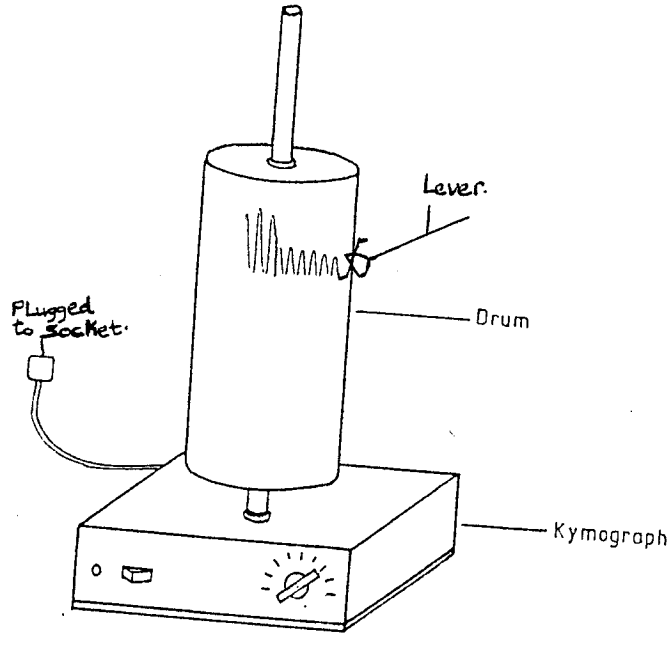


Figure 3: The Kymograph

taken from there downward towards caecum. The ileum was then removed and placed in a 300 ml beaker containing warm air-aerated Tyrode's solution at a constant temperature of 37 °C. In order to avoid damaging the gut muscle, all handling was done with fingers rather than gripped with forceps. The mesentery was trimmed away and pieces of required length were cut from the ileum and placed in a petri-dish containing Tyrode's solution at 37 °C. Since the rabbit ileum is wide and clears itself because of its spontaneous activity, it was not washed through.

2:3:2 Setting up the Preparation by the Method of Magnus

A thread was attached at each end of the ileum tissue by inserting a needle from inside of the gut outwards, the lumen of the gut was not closed off. The preparation was placed in a 50ml organ bath. One thread was tied to a fixed pin which was hollow and served as an aerator while the thread at the other end of the tissue was attached to a lever/transducer, mounted uprightly as shown in Figure 4. The thermostat heater ensured that temperature of the bath was constant at 37 °C and the temperature was monitored with the help of a thermometer (0-100 °C). The Tyrode's solution in the organ bath was changed at frequent intervals during investigation while fresh Tyrode's solution from the reservoir stood in the coils so that temperature of the preparation in the organ bath was not altered even when it was washed.

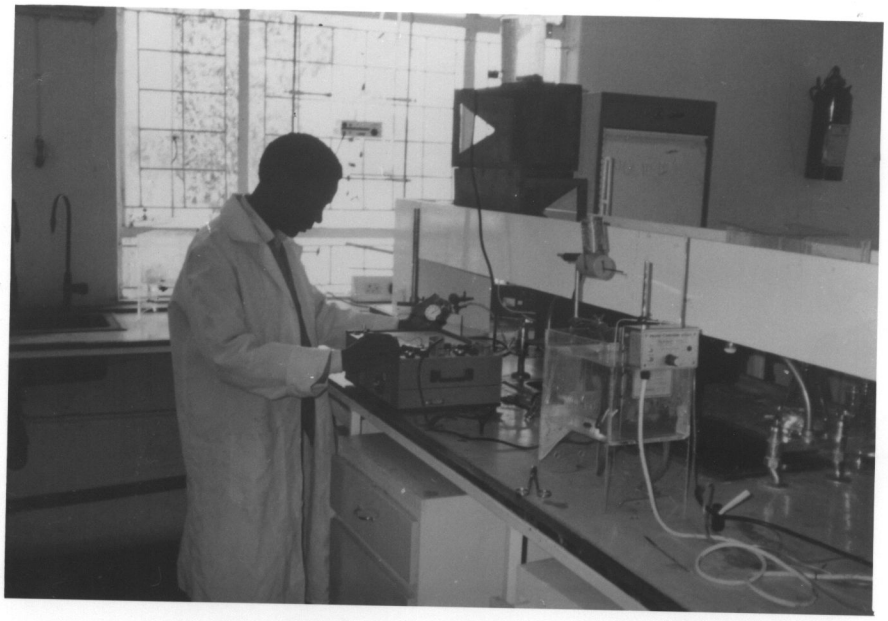


FIGURE 4: Test for anti-diarrhoeal activity using an electronic transducer

2:3:3 Parameters of the Organ Bath

Parameters of the organ bath that was used are described in Table 3 given below:

Parameter	Description
Load	1.0 - 0.5 g
Magnification	5 fold
Organ bath Length	11.0 cm
Volume of Organ bath	50 ml
Length of Ileum when relaxed	2 - 3 cm
Aerator	Air
Medium	Tyrode's Solution

Table 3: Organ Bath Parameters

2:3:4 Anti-diarrhoeal Investigation of NP001X and NP001Z

NP001X and NP001Z solutions were prepared as out lined in 2:5. A piece of ileum, 2-3 cm long, was prepared as described in 2:3:1 and suspended in the air-aerated 50 ml organ bath containing Tyrode's solution as described in 2:3:2. The recording papers were glued on the rotating cylinders or drums of the kymograph. As the drums rotated slowly on kymograph, the response, rhythmic pendular movement responses, ie continuous contraction and relaxation of the tissue was recorded as the pointer produced a tracing of the responses on the paper. Effects of distortion were minimised by adjusting the magnification imparted by transducer and also by use of a frontal writing lever. The frontal writing lever recorded with constant friction and its point fell slightly against the paper and traced linear/longitudinal contractions as straight lines and not as arcs.

The normal rhythmic pendular movement responses were followed for sixty seconds. The kymograph was then stopped and using a syringe, 0.15 ml of NP001X (0.2 g/ml) solution was added to the 50 ml organ bath. Concentration of NPOO1X in the organ bath was, therefore, 600 μg/ml. The air bubbles facilitated homogeneous mixing of NPOO1X and Tyrode's solutions. After 30 seconds stabilization, the kymograph was switched on and responses of the tissue to NPOO1X solution were recorded for 60 seconds. The kymograph was then switched off. It was preferred to wash the tissue by draining and refilling because this type of washing is a more convenient method than washing by overflow. Moreover, the ileum tissue is robust enough to tolerate washing by draining and refilling.

After washing and refilling with Tyrode's solution twice, the tissue was allowed to stabilize for thirty seconds after which its normal response was again recorded for 60 seconds. The normal rhythmic pendular movement responses of the tissue were finally compared with the rhythmic pendular movement responses of the tissue to the test solution. Two parameters, amplitude and tone, were considered for comparison.

Following the same procedure, the rhythmic pendular movement responses of ileum tissue to NP001Z, 600 μg/ml. were also monitored and subsequently compared with the normal rhythmic pendular movement responses of the tissue in an organ bath.

2:4 Investigation of the Effect of NP001Z on Para-Sympathetic Ganglia, Post-Ganglionic Acetylcholine Receptors and other Receptors on Smooth Muscle of the Rabbit Ileum

Effects of NP001Z on para-sympathetic ganglia, post-ganglionic acetylcholine receptor and other receptors on smooth muscle of the rabbit ileum were evaluated against a number of stimulants such as 2 μg/ml acetylcholine, 1 μg/ml histamine, $2x10^{4}$ μg/ml barium chloride, $2x10^{4}$ μg/ml magnesium chloride and 0.2 μg/ml nicotine induced spasms by monitoring and comparing the rhythmic pendular movement responses of the ileum tissue to NP001Z, appropriate stimulant, and to the mixture of NP001Z and appropriate stimulant by the Method of Perry et al [67].

A piece of rabbit ileum was prepared and placed in an organ bath as described in 2:3:1 - 2:3:3. Appropriate dilutions of NP001Z and other test chemical solutions, acetylcholine, histamine, barium chloride, magnesium chloride and nicotine, were prepared as outlined in 2:5.

Effect of NP001Z against Acetylcholine

The normal rhythmic pendular movement responses of rabbit ileum were recorded for 60 seconds. 0.1 ml of acetylcholine, 1 mg/ml, in deionised water was added to the organ bath using a 1 ml syringe and the response of the tissue was recorded for 60 seconds. The tissue was washed with Tyrode's solution and its normal response was recorded again after stabilization.

0.1 ml of the above acetylcholine solution and 0.15 ml of NP001Z in propylene glycol were added to the organ bath beginning with acetylcholine

and the response of ileum tissue to the mixture was recorded for 60 seconds. The tissue was then washed with Tyrode's solution and its normal response was again recorded. The rhythmic pendular movement response of the tissue to acetylcholine, was compared with the rhythmic pendular movement response of the tissue to the mixture of acetylcholine and NP001Z solution.

Effects of NP001Z against other stimulants were similarly evaluated.

2:5 Preparation of Solutions

(a) NP001X in propylene glycol (0.2 g/ml)

1 g of NP001X was dissolved in 3 ml of propylene glycol in a 5ml volumetric flask and the volume of solution was raised to the mark with propylene glycol.

(b) NP001Z in propylene glycol (0.2 g/ml)

1 g of NP001Z was dissolved in 3 ml of propylene glycol in a 5 ml volumetric flask and the volume of solution was raised to the mark with propylene glycol.

(c) $BaCl_2$ in deionised water (0.01 g/ml)

0.5 g of $BaCl_2$ was dissolved in deionised water in a 50 ml volumetric flask and the volume of solution was raised to the mark with deionised water.

(d) $MgCl_2$ in deionised water (0.01 g/ml)

0.5 g of $MgCl_2$ was dissolved in 10 ml of deionised water in a 50 ml volumetric flask and the volume of solution was raised to the mark with deionised water.

(e) Histamine in deionised water (0.5 mg/ml)

0.125 g of histamine was dissolved in deionised water in a 250ml volumetric flask and the volume of solution was raised to the mark with deionised water.

(f) Nicotine in deionised water (0.1 mg/ml)

Tobacco collected from 20 cigarettes of Consulate brand manufactured by Rothmans and Pall Mall Zambia Limited was placed in a 300 ml beaker, containing 200 ml of hot deionised water for 3 - 5 minutes. The aqueous decoction was filtered and the filtrate was collected in a stoppered flask and kept in a refrigerator ready for use.

Nicotine extraction from the cigarettes was based on the information obtained from my co-supervisor, Dr. Khare, that 1 mg of nicotine can be extracted from one cigarette of Consulate brand made by Rothmans and Pall Mall Zambia Limited by the method employed.

(g) Tyrode's solution

A mixture of 40.0 g of NaCl, 5.0 g of D-Glucose, 1.3 g of $CaCl_2 .2H_20$, 5.0 g of $NaHCO_3$, 1.5 g of $MgCl_2 .6H_20$, 0.15 g of $NaH_2 PO_4.H_2O$ and 0.1 g of KCl was dissolved in 1 litre deionised water in a 5 litre volumetric flask and volume of the solution was raised to the mark with deionised water.

(h) Acetylcholine in deionised water (1 mg/ml)

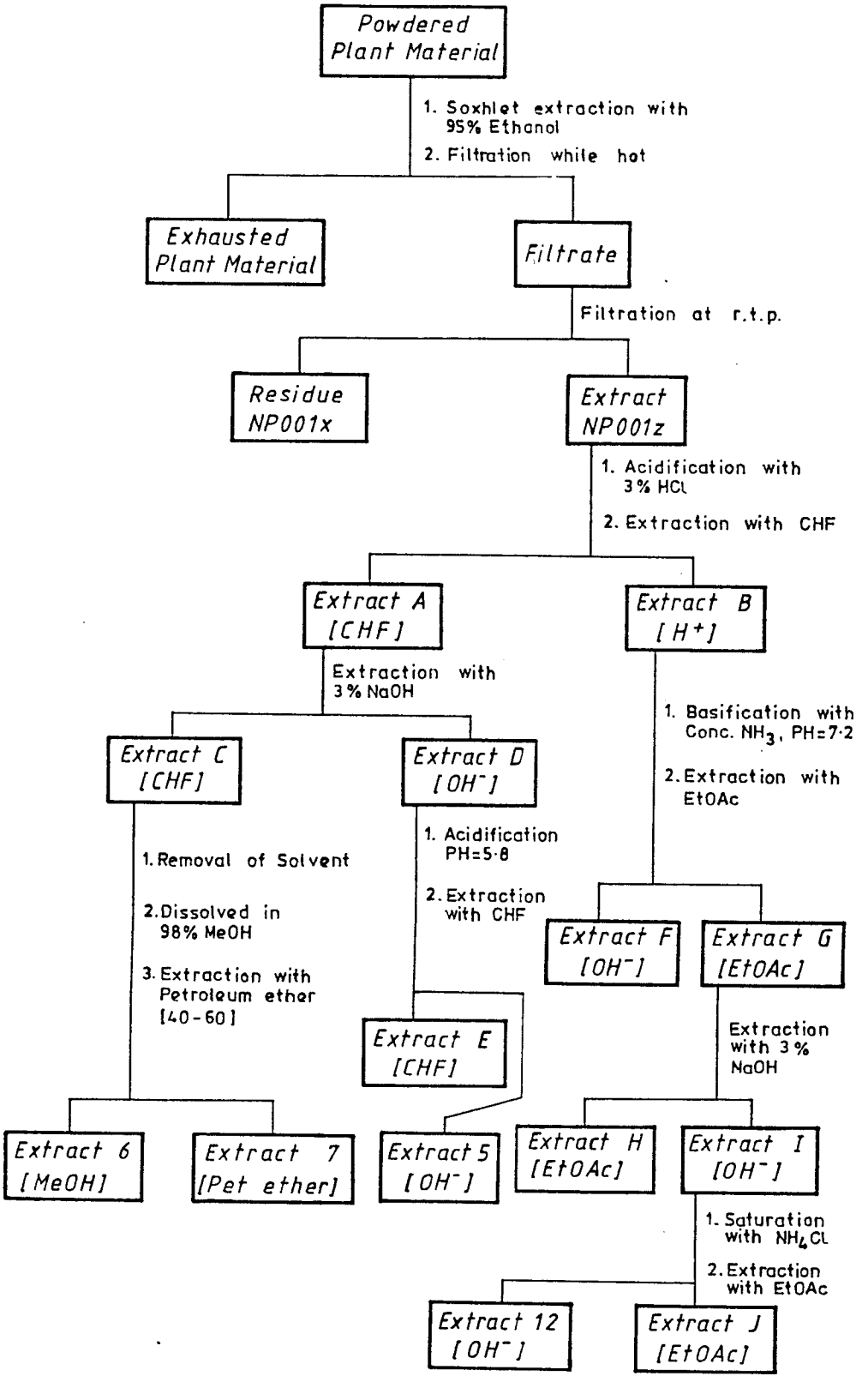
0.1 g of acetylcholine was poured into a 100ml volumetric flask and then diluted to the mark with deionised water.

2:6 Bioassay Directed Fractionation of NP001Z

NP001Z was partitioned between a variety of solvents into a number of acidic, basic and neutral fractions and anti-diarrhoeal activity of various fractions so obtained was evaluated in-vitro by the method of Perry et al described in 2:3. The partitioning and separation of NPOO1Z was essentially based on the differences in polarity, pH, solubility and partition characteristics of its components.

2.6.1 Fractionation of NP001Z

Using a slightly modified scheme of Mitscher et al [69], componets of extract NPOO1Z were seperated into 13 fractions on the basis of their differences in polarity, pH, sulubility and partition characteristics as presented in Scheme 1 on page 37. 150.3 g of NP001Z was transferred to a 5 litre conical flask. 600ml of 3% aqueous HCl (V/V) was poured into the flask slowly and the mixture was stirred using a magnetic stirrer for 20 minutes. The mixture was then filtered by suction filtration using Whatman No. 41 filter paper and the residue was successively washed with 20 ml of 3% aqueous HCl and 50 ml of distilled water.



Scheme 1. Fractionation of Gross Ethanolic Extract.

A deep dark reddish brown clear filtrate was obtained. The dark brown residue (42.1 g), which turned even darker on keeping, was dried over anhydrous calcium chloride in a vacuum desiccator, and stored in an air-tight container and labelled IPO1.

The clear filtrate, 670 ml. was exhaustively extracted with chloroform in small aliquots. In a typical experiment 100 ml of the filtrate was extracted with 30 ml of chloroform for three times. The clear yellow chloroform layers were pooled together and labelled extract A. The deep dark reddish brown acidic aqueous layers were pooled together separately and labelled extract B.

2:6:2 **Treatment of Extract A**

25 g of anhydrous magnesium sulphate was placed in a 2000 ml stoppered conical flask and 630 ml of extract A were added to it. The mixture was carefully swirled and allowed to stand for 3 hours. Magnesium sulphate was filtered off and the clear filtrate was collected in a 1 litre stoppered conical flask.

A small portion, 60 ml, of the filtrate was evaporated to dryness at 35°C under reduced pressure using a Büchi rotary evaporator Model No. RE120. An orange yellow solid was obtained which was dried over anhydrous calcium chloride in a vacuum desiccator for 24 hours, stored in an air-tight container and labelled extract 2 (0.1735 g).

The remaining 570 ml of the filtrate, was extracted with 3% aqueous NaOH in small aliquots. In a typical experiment 100ml of extract A was extracted with 25ml of 3% aqueous NaOH in a 250 ml separatory funnel for two times. The pale yellow chloroform layers were pooled together and labelled extract C. The light reddish aqueous layers were pooled together and labelled extract D.

2:6:3 **Treatment of Extract C**

25 g of anhydrous magnesium sulphate and the light pale yellow extract C were placed in a conical flask, the mixture was carefully swirled and then left to stand for 3 hours. Magnesium sulphate was filtered off and the clear filtrate was collected in a 1 litre stoppered conical flask. The filtrate was evaporated to dryness at 35°C under reduced pressure using a Büchi rotary evaporator. 0.1826 g of an orange yellow solid was obtained which was dried over anhydrous calcium chloride in a vacuum desiccator, stored in an air-tight container and labelled extract 4.

0.1352 g of extract 4 was dissolved in 2 ml methanol and the solution was partitioned between equal volumes of methanol and petroleum ether (40-60°C) in a separatory funnel. The solution was shaken for 15 minutes and separation was allowed to be visible after which the yellow methanol layer was collected in a flask and the solvent was evaporated under reduced pressure at 40°C using a Büchi rotary evaporator. A yellow orange solid was obtained which was

dried over anhydrous calcium chloride in a vacuum desiccator and labelled extract 6 (0.04 g). The light pale yellow ether layer was collected in another round bottomed flask, and the solvent was evaporated under reduced pressure at room temperature using a Büchi rotary evaporator. A yellow solid was obtained which was dried over anhydrous calcium chloride, stored in an air-tight container and labelled extract 7 (0.0952 g).

2:6:4 **Treatment of Extract D**

300 ml of extract D obtained in 2:6:2 was acidified to pH 5.8 at 5-7°C using concentrated A.R hydrochloric acid and a pH meter Model HM-5ES, Japan. On acidification, the reddish colour of extract D turned yellow.

The acidified extract D was extracted with chloroform in small aliquots. In a typical experiment 100 ml of acidified extract D was extracted with 30 ml of chloroform for three times. The pale orange chloroform layers were pooled together and labelled extract E. The aqueous layers were pooled separately and labelled extract 5.

2:6:5 **Treatment of Extract E**

Extract E was swirled with 20 g of anhydrous magnesium sulphate in a conical flask for few minutes and left to stand for 3 hours. Magnesium sulphate was filtered off and the clear filtrate was evaporated to dryness at 35°C under reduced pressure using a Büchi rotary evaporator. An orange brown solid was obtained, which was dried over calcium chloride in a vacuum desiccator,

stored in an air-tight container and labelled extract 8 (0.0925 g).

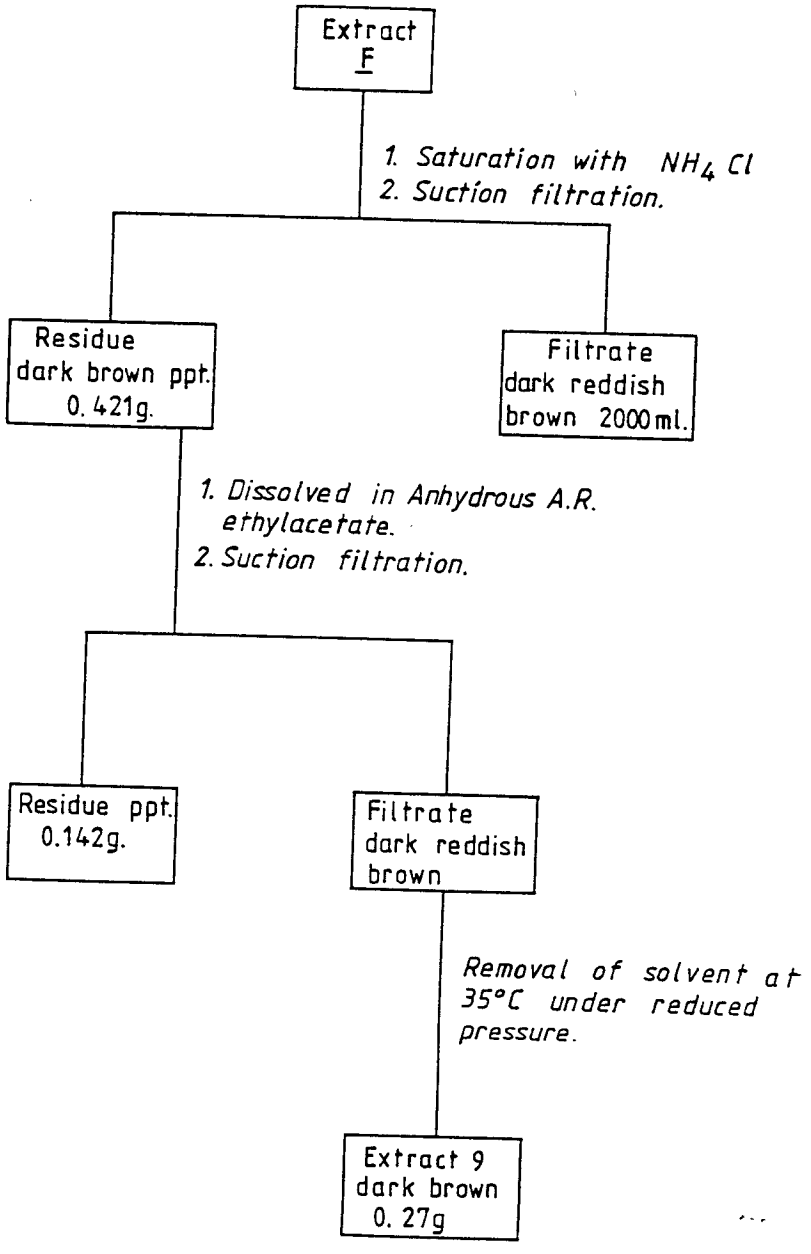
2:6:6 **Treatment of Extract B**

Extract B obtained in 2:6:1 was neutralised to pH 7.2 with concentrated NH_3 (sp. gr. 0.88) at 5 - 7°C using a pH meter Model HM-5ES. The neutral solution was dark reddish brown in colour.

The neutral solution, was exhaustively extracted with ethylacetate in small aliquots. In a typical experiment 150 ml of the neutral solution was extracted with 50 ml of ethylacetate for three times. The aqueous layer, deep dark reddish brown in colour, was collected in a separate stoppered conical flask and labelled extract F. The pale reddish orange ethylacetate fractions were pooled together in a 2000 ml flat bottomed stoppered flask and labelled extract G.

2:6:7 Treatment of Extract F

Extract F was treated according to Scheme 2 shown below.



Scheme 2: Treatment of Extract F.

2:6:8 Treatment of Extract G

25 g of anhydrous magnesium sulphate was placed in a 1000 ml stoppered conical flask and then 675 ml of extract G were added. The mixture was swirled for 1-2 minutes and left to stand for 3 hours. The drying agent was filtered off and the filtrate was collected in a 1000 ml flat bottomed stoppered flask.

A small portion of extract G, 175 ml, was evaporated to dryness at 35°C under reduced pressure using a Büchi rotary evaporator. A light orange solid was obtained which was dried over anhydrous calcium chloride in a vacuum desiccator and labelled extract 10 (3.02 g).

The remaining 500 ml of extract G was extracted with 3% aqueous NaOH in small aliquots. In a typical experiment 150 ml of extract G was extracted with 30 ml of 3% NaOH for two times.

The yellow ethylacetate layers were pooled together in a 1 litre stoppered conical flask and labelled extract H while the deep dark reddish brown aqueous layers were pooled together in a 1 litre stoppered conical flask and labelled extract I.

2:6:9 Treatment of Extract H

Extract H was added to a 1 litre conical flask containing 25 g of anhydrous magnesium sulphate. The mixture was swirled for 1-2 minutes and allowed

to stand for 3 hours. The drying agent was filtered off and the clear filtrate was evaporated to dryness under reduced pressure at 35 °C using a Büchi rotary evaporator. An orange yellow solid was obtained which was dried over anhydrous calcium chloride in a vacuum desiccator and labelled extract 11 (0.243 g).

2:6:10 Treatment of Extract I

Extract I was saturated with solid NH_4Cl and stirred vigorously for 30 minutes. A thick brown precipitate was observed. The precipitate was filtered off and the deep dark brown filtrate, 300 ml, was extracted with ethylacetate in small aliquots. In a typical experiment 100 ml of the filtrate was extracted with 30 ml of ethylacetate for three times. The orange yellow ethylacetate layers were pooled together and labelled extract J. The deep dark reddish brown aqueous layers were pooled separately into another 1 litre conical flask and labelled extract 12.

2:6:11 Treatment of Extract J

25 g of anhydrous magnesium sulphate was added to 270 ml of extract J. The mixture was swirled for 1-2 minutes and then left to stand in a stoppered flask for 3 hours. Magnesium sulphate was filtered off and the clear filtrate was evaporated to dryness under reduced pressure at 35 °C using a Büchi rotary evaporator. A reddish brown solid was obtained, which was dried over anhydrous calcium chloride in a vacuum desiccator, stored in an air-tight container and labelled extract 13 (7.8 g).

Extract 13 was tested for the presence of free NH_4^+ ions [77], and it was found to be completely free of NH_4^+ ions.

2.7 Selection of Potent Fraction

The most potent anti-diarrhoeal fraction was selected by evaluating and comparing the effects of each fraction on normal rhythmic pendular movement responses of rabbit ileum by the method of Perry et al as described in 2:3.

Solutions of test extracts ($5.0x10^3$ μg/ml) were prepared in propylene glycol as shown in Table 4. A solution of ammonium chloride in propylene glycol ($5.0x10^3$ μg/ml) was also prepared.

1.0 ml of the test solution was added to a 50 ml organ bath containing Tyrode's solution and its effect on the rhythmic pendular movement responses of the rabbit ileum was recorded using a kymograph. Effects of 100 μg/ml of each of the test extracts were, thus, monitored.

Extract Number	Mass of Extracts in Grammes	Volume of Propylene Glycol in mls.
2	0.0400	7.84
4	0.0474	9.29
6	0.0400	7.84
7	0.0952	18.67
8	0.0703	13.78
9	0.0400	7.84
10	0.0412	8.08
11	0.0249	4.88
12	0.1505	29.51
13	0.041	8.04

Table 4: Preparation of Test Extract Solutions

Effect of varying the concentration of test extracts on the motility of rabbit ileum was also monitored by varying the volume of test extract solution added to the organ bath and recording the rhythmic pendular movement responses of the tissue for each concentration using a kymograph. Any increase or decrease in the response of the tissue caused by change in concentration of the test extract was carefully noted. Volumes of the test extracts used and their corresponding concentrations in the organ bath are shown in Table 5.

Test Extract	Volume of Test Extract Solution Added to 50ml Organ Bath (ml)	Concentration of Test Extract in the Organ Bath (μg/ml)
Extract 13	1.0 2.0 3.0	100 200 300
NP001Z	0.15 0.20 0.25 0.30	600 800 1,000 1,200

Table 5: Volumes of Test Solutions and Corresponding Concentrations

Extract 13 was found to exhibit significant anti-diarrhoeal activity and it was, therefore, chosen for further separation and purification by a combination of analytical and preparative chromatographic techniques.

2.8 Bioassay Directed Fractionation of Anti-diarrhoeal Extract 13

4.0 g of extract 13 was fractionated by gravity column chromatography over silica gel 60, Merck type 4478 in small aliquots.

In a typical experiment a 20% solution of 1.3 g of extract 13 was made in a mixture of CHF:MeOH(5:1) and applied to a column of 2.5 cm diameter and 56 cm height packed with 170 g of silica gel. The mixture was successively eluted with 100 ml each of hexane, dichloromethane, ethylacetate, ethylacetate:methanol mixture in varying proportions up to 20% methanol and finally with methanol. Eluates of 50 ml each were collected in 100 ml Erlenmeyer flasks.

The solvent was removed under reduced pressure from each of these eluates at 37°C using a Büchi rotary evaporator. Each residue was dried over anhydrous calcium chloride in a vacuum desiccator. Anti-diarrhoeal activity of each of the residues was evaluated by the method of Perry et al, described earlier in 2:3. Anti-diarrhoeal activity was found to be concentrated in residues obtained from ethylacetate-methanol (5:1) and in methanol fractions. Bioactive residues were combined together for the purpose of further separation and purification.

2.8.1 Further Separation of Bioactive Fraction

Analytical thin layer chromatography on the combined bioactive fraction was performed on precoated thin layer aluminium sheets Merck type 5735 coated with silica gel 60 F_{254} of 0.20 mm thickness using a variety of solvent systems. A large number of solvent systems were tried so as to obtain reasonable differences in Rf values of the components of the bioactive fraction which could permit their separation by liquid-solid chromatography either on a gravity column or on a flash column. Observation of the chromatogram was done under uv lamp, Betrchter, at wavelengths 254 nm and 366 nm and by spraying the plates with Dragendorff's and Vanillin reagents. Since there are no literature reports on the plant under investigation, a suitable solvent system for separation had to be established experimentally. Solvent system CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1) was found to be the most suitable.

In view of the Rf values of not more than 0.5 and differences in Rf values of more than 0.1-0.15, Flash Column chromatography was employed for separation because of its obvious time advantage.

Flash column chromatography was set up as described by W. Clark Still et al, [76]. A Flash column of diameter 16 mm was packed with a high grade of silica gel 60 (40-63 μm particle size) Merck type 9385 (9 cm) and then sand (3.1 mm). A 20% solution of 160 mg of sample in CHF:MeOH (5:1) was loaded onto the column. In operation, the eluting solvent was driven down the column by nitrogen pressure. To obtain best results, the eluent flow rate was maintained at 5 cm/min. The parameters used in flash chromatography are summarized in Table 6.

Parameters	
Column diameter (mm)	16
Volume of eluent (ml)	200
Typical sample loading (mg)	160
Flow rate (cm/min)	5.0
Height of sand (mm)	3.1
Height of silica gel (cm)	9.0
Typical fraction size, eluent (ml)	10

Table 6: Flash Column Chromatography Parameters

After elution, silica gel was removed from the column and washed with a more polar solvent, methanol. Methanol was subsequently removed from the washings by evaporation under reduced pressure and the residue was labelled 13SG.

Since anti-diarrhoeal activity had to be evaluated at the School of Medicine using the facilities of University Teaching Hospital laboratories, it was not possible to evaluate the pharmacological activity of each of the eluates due to the limited period of time

for which such facilities were made available. The eluates were, therefore, combined together on the basis of analytical TLC and the solvent was removed from the combined eluates under reduced pressure at 35°C using a Büchi rotary evaporator. The obtained solids were dried over anhydrous calcium chloride in a vacuum desiccator and labelled as shown in Table 7. Thus, a total of 2.88 g of Bioactive Fraction was separated by flash column chromatograpy.

Eluates Combined	Weight (mg)	Label of solid
1 to 6	47.52	131006
7 to 13	47.16	137013
14 to 20	47.16	131420
Material in the gel	506.16	13SG

Table 7: Random Combination of Eluates

The potent anti-diarrhoeal eluate was selected by evaluating and comparing the effects of solutions of 131006, 137013, 131420 and 13SG in propylene glycol (250 μg/ml) on normal rhythmic pendular movement responses of rabbit ileum by the method of Perry et al described earlier in 2:3.

Test solutions were prepared by dissolving 2.5 mg of each of the test materials in 10 ml of propylene glycol.

0.1 ml of the test solution was added to a 50 ml organ bath containing Tyrode's solution and its effect on the normal rhythmic pendular movement responses of the rabbit ileum was recorded using a kymograph/electronic transducer. Effects of

0.5 μg/ml of each combined eluates were, thus, monitored.

Fractions 131006, 131420 and 13SG were found to have demonstrable anti-diarrhoeal activity in-vitro. However, 131006 was most potent of the three fractions and it was, therefore, selected for further purification to isolate at least one pure compound from it. A total of 47.52 mg of 131006 was obtained.

2.9 Purification of Fraction 131006

8.1 mg of a pure compound, LAMM, was isolated from 45.02 mg of 131006 by Gravity Column chromatography using the solvent systems CHF:Me_2CO:MeOH:EtOAc (7:5:2.5:1:1) and (8:2.5:1:1). Analytical thin layer chromatography and two dimensional thin layer chromatography were employed in different solvent systems to determine the purity of the isolated compound.

Gravity Column chromatography was set up. A column of diameter of 5 mm was packed with 3.04 g (21 cm) of silica gel-60 Merck type 44784. The slurry was made in chloroform, the least polar solvent of the eluting solvent system. A 20% solution of 9.0 mg of 131006 in chloroform-methanol mixture in the ratio of 7:1 was applied onto the column and subsequently eluted using the solvent system CHF:Me_2CO:MeOH:EtOAc (7.5:2.5:1:1). 13 eluates of 2 ml volume each were collected. The eluates were concentrated at 35°C under reduced pressure using a *Büchi rotary evaporator. Each concentrated eluate* was subsequently examined by analytical thin layer chromatography in four different solvent systems, CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1), Petroleum ether:Ethylacetate (3:7),

Dichloromethane:Ethylacetate (2:3) and Chloroform:Ethylacetate (2:5). Chromatograms were observed under uv lamp at wavelengths 254 nm and 366 nm as shown in Figure 5.

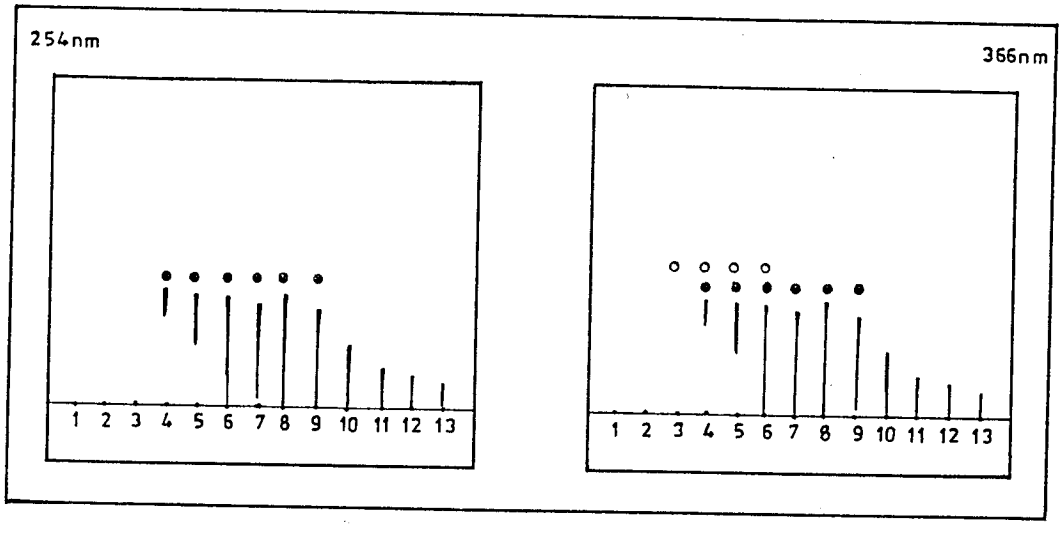


Figure 5: Analytical Thin Layer Plates Observed under uv Lamp.

Eluate 3 showed a single spot. Upon calculations of the Rf values, the compound in eluate 3 was noted to be present in the eluates 4. 5, and 6 as well. Eluate 3 was kept aside and labelled I while eluates 4, 5, and 6 were poured together for repeating Gravity Column chromatography to recover more of the same compound.

The size of eluates were this time reduced from 2 ml to 1.5 ml maintaining the same solvent system CHF:Me_2CO:MeOH:EtOAc (7.5:2.5:1:1). 10 eluates were collected, concentrated and then subjected to analytical thin layer chromatography in four different solvent systems as done earlier. The chromatogram was observed under uv lamp at 254 nm and 366 nm as shown in Figure 6

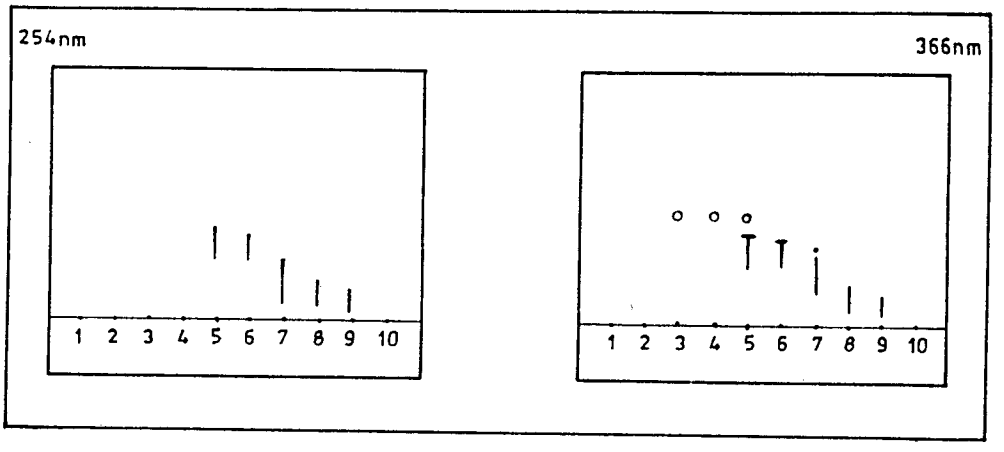


Figure 6: Analytical Thin Layer Plates Observed under uv Lamp.

Eluates 3 and 4 each showed one spot whose Rf values were identical in all the four different solvent systems. These eluates were combined and labelled II.

Rf values of I and II, in four different solvent systems CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1), Petroleum ether:Ethylacetate (3:7), Dichloromethane:Ethylacetate (2:3), and Chloroform:Ethylacelate (2:5) were found to be identical.

A little of I and II, were poured together and two dimensional thin layer chromatography was performed on the mixture in different solvent systems to confirm the purity of the compound isolated. In a typical experiment, a 4cm x 4cm square precoated thin aluminium sheet Merck type 5735 coated with silica gel 60 F_{254} of 0.2 mm thickness was cut. The sample, mixture of I and II , was applied about 1cm from two ends of the sheet as shown in Figure 7. The square plate was then developed by allowing the solvent system CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1) to ascend its length in a chromatography tank. The plate was carefully removed using a clean forcep and air dried. The plate was subsequently developed at right angles by

allowing another solvent system Petroleum ether:Ethylacetate (3:7), to ascend its width in another chromatography tank. The square plate was carefully removed using a clean forcep and air-dried. The plate was finally observed under uv lamp at wavelengths 254 nm and 366 nm. Only one spot was observed to have developed. Two similar square plates were cut and the same procedure as outlined above was followed. One plate was first developed with the solvent system Dichloromethane:Ethylacetate (2:3) followed by Petroleum ether:Ethylacetate (3:7) while the other plate was developed beginning with the solvent system Chloroform:Ethylacetate (2:5) followed by solvent system Petroleum ether:Ethylacetate (3:7). The plates were observed under uv lamp at wavelengths 254 nm and 366 nm. Only one spot was observed in each case.

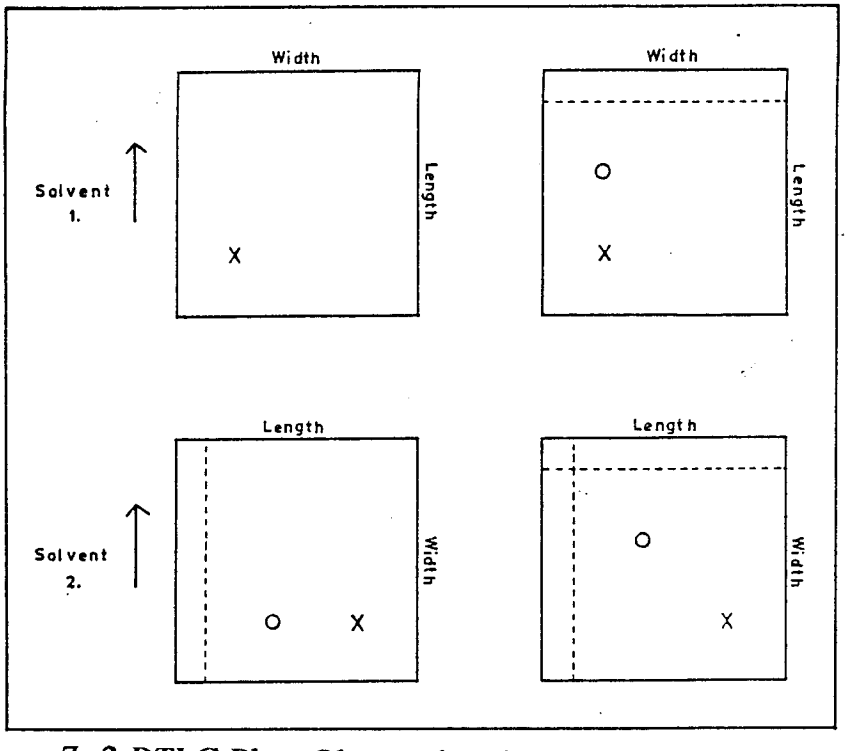
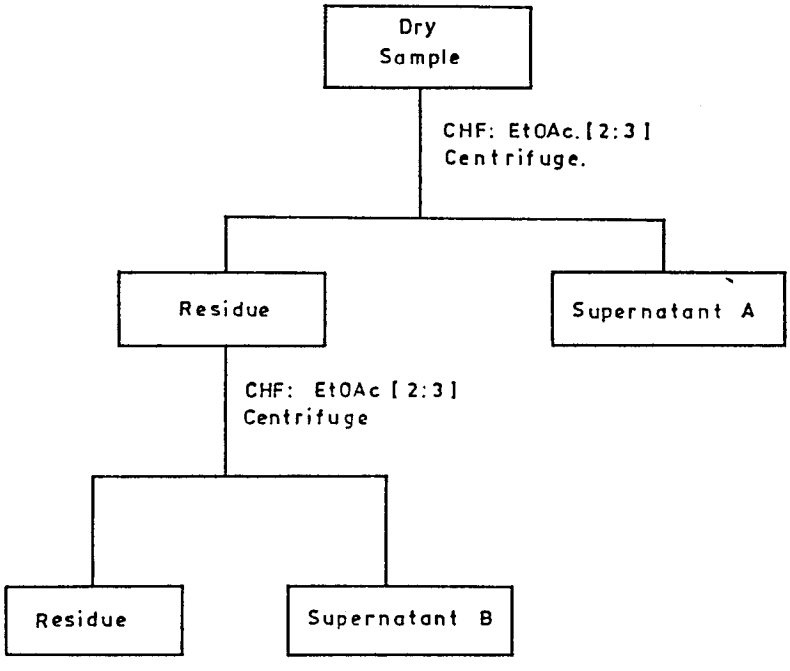


Figure 7: 2-DTLC Plate Observed under uv Lamp.

Combined eluates, I and II, were pooled together and the solvent was evaporated under reduced pressure at 35°C using a Büchi rotary evaporator. The residue was

dried over anhydrous calcium chloride in a vacuum desiccator, and labelled LAMM. Remaining quantity of 131006 was similarly processed to obtain a total of 8.1 mg of LAMM.

In order to remove any traces of silica gel that may have been dissolved in the eluent during elution, Scheme 3, outlined below, was followed. The sample was found to be insoluble in chloroform but soluble in ethylacetate.A mixture of chloroform and ethylacetate was, therefore, used.



Scheme 3: Removal of Silica gel from the Sample

The two supernatants A and B were pooled together and the mixture was concentrated under reduced pressure at 35°C by the use of a Büchi rotary evaporator.

Two dimensional thin layer chromatography was carried out as described earlier. After the purity had been reconfirmed, the sample was transferred to a previously weighed test-tube. The solvent was removed under reduced pressure at 35°C using a Büchi rotary evaporator and the residue dried over anhydrous calcium chloride in a vacuum desiccator for 24 hours. 8.1 mg of a solid, LAMM, m.p. 59.4 - 60°C was thus isolated.

The anti-diarrhoeal activity of LAMM was determined by the method of Perry et al, [67] as described earlier in 2:3. 0.25 mg of LAMM was dissolved in 1.0 ml of propylene glycol, 0.1 ml of the solution was added to a 50 ml organ bath containing Tyrode's solution and its effect on the normal rhythmic pendular movement response of the rabbit ileum was recorded using a kymograph/electronic transducer. Effect of 0.5 μg/ml of LAMM was, thus, monitored.

LAMM was also analysed for the presence of tannins using the method commended by J.B. Harborne [86] as in **2.11.2(b)**.

2.10 High Performance Liquid Chromatography (HPLC) Analysis of LAMM

The compound LAMM was analyzed by 400 MHz ^{1}H NMR and H-H cosy 45 experiments. The results showed that the compound, LAMM, was not pure because the peaks on the H-H cosy spectrum did not correlate.

The sample LAMM was purified by HPLC using a Binary Water 501 HPLC pumping system with automated gradient controller and Waters 991 photodiode array detector at the Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, U.K., with the help of my supervisor, Dr. S. Prakash.

Since the sample was non-ionic and soluble in high polarity solvents, the reverse phase partition chromatography mode was used.

Analytical HPLC was performed on 5 μ Spherisorb ODS(2) Column (10 mm x 250 mm).

3.6 mg of LAMM was dissolved in 1.8 ml of HPLC grade methanol (BDH HiPer solvent methanol). The sample was loaded onto the column by injecting 10 μl and 20 μl of the above solution of LAMM for analytical and preparative chromatography, respectively, directly into the eluent stream via an injection valve with the aid of a syringe. BDH HiPer solvent methanol and double distilled water were used for elution.

Analytical HPLC was performed by use of isocratic elution to determine the eluting solvent systems. After the eluting solvent systems had been established, preparative HPLC was performed utilizing Gradient elution. The programme followed is outlined in Table 8 below.

Time (min)	Flow (ml/min)	%MeOH	$\%H_20$
Initial	1.00	40	60
10.0	1.00	50	50
20.0	1.00	65	35
25.0	1.00	95	5
30.0	1.00	95	5

Table 8: Gradient Programme

The compound LAMM was found to constitute 3 components which disintegrated into 5 components whilst on the column. Five fractions were collected each emerging from the column at a characteristic retention time/peak, in pre-weighed containers. Eluates were freeze dried and quantities of the residues are presented in Table 9.

Fraction	Weight of sample tube (g)	Weight of tube and residue (g)	Weight of residue (mg)
1	1.627	1.62732	0.32
2	1.6421	1.6433	1.2
3	1.7311	1.7315	0.4
4	1.6501	1.6504	0.3
5	1.6621	1.66251	0.41

Table 9: Weights of Sample Tubes and Residues

The major fraction 2 was analyzed by 500 MHz ^1H NMR at JEOL laboratories, U.K., with the kind help of Professor R. D. Waigh, University of Strathclyde, U.K., and the data obtained is presented under Results and Discussion chapter.

In view of the extremely small quantities of the pure samples obtained, other fractions could not be analyzed by NMR and other spectroscopic methods.

2.11 Other Chemical and Biological Tests

2.11.1 Evaluation of Anti-bacterial Activity

Anti-bacterial activities of the gross ethanolic extract of stem bark of **Cassia abbreviata-Oliv** and extract 13 were evaluated in-vitro against a test system of six micro-organisms by Agar Streak Dilution Method by comparing the growth of micro-organisms in the presence and in the absence of test extract under identical conditions. Streptomycin sulphate (10 mg/ml) was used as standard for comparison purposes. Clear zones of inhibition were taken as indicators of anti-bacterial activity. The minimum inhibitory concentrations (MICs) were also determined. The evaluation of anti-bacterial activity and determination of MIC were carried out at Disease Control Laboratory, School of Veterinary Medicine, University of Zambia.

Pure cultures of six micro-organisms **Staphylococcus aureus, Escherichia coli, Salmonella gallinarium, Shigella dysentrie, Bacillus spp.** and **Proteus spp.** were obtained from the Microbiology Laboratory of the Department of Pathology and Microbiology, University Teaching Hospital, Lusaka. Bacterial slants of each of these micro-organisms were prepared as described in 2:11:1:1 and stored at 0-4 $^{\circ}$C.

Bacterial suspensions of each of the above six micro-organisms were subsequently prepared by diluting one loopful of the respective bacterial slant with Trypticase Soy Broth.

3 Petridishes labelled Test, Standard and Blank were packed in canisters and sterilized at 110°C for 1 hour. Each petridish was then divided into six sectors labelled I, II, III, IV, V, and VI. 10 ml of Trypticase Soy Agar, T.S.A. (15 mg/ml) was transferred into 3 stoppered tubes labelled 1, 2, and 3 with the aid of an automatic syringe. The tubes were autoclaved at 121°C for 15 minutes and their contents were then allowed to cool to 55°C in a thermostatic water bath. The following operations were performed under strict aseptic conditions.

0.1 ml of streptomycin sulphate (10 mg/ml) was added to tube 1 and the mixture was poured into a petridish labelled Standard. The dish was then swirled to achieve homogeneity.

0.1 ml of DMSO was added to tube 2 and the mixture was poured aseptically into the petridish labelled Blank. The dish was then swirled to achieve homogeneity.

0.1 ml of NP001Z sample in DMSO ($1.5x10^5$ μg/ml) was added aseptically to tube 3 and the mixture was poured into petridish labelled Test. The dish was then swirled to achieve homogeneity.

The petridishes were first left to stand on a flat surface to allow the agar to congeal. The petridishes were turned upside down after the agar had congealed and kept at room temperature for overnight. By streaking in a radial pattern, each micro-organism was inoculated aseptically in a separate sector in all the three petridishes labelled Test, Standard and Blank as shown in Table 10 below.

Micro-organism	Sector
Staphylococcus aureus	I
Escherichia coli	II
Salmonella gallinarium	III
Shigella dysentrie	IV
Bacillus spp.	V
Proteus spp.	VI

Table 10: Micro-organisms in separate sectors

The petri dishes were incubated at 37°C for 24 hours, and then examined for any clear zones of inhibition. The results obtained are shown in 3:1:11.

Determination of MIC.

The MICs of NP001Z and extract 13 were determined as follows:

1.50 g of test extract was dissolved in 10 ml of DMSO and the solution was serially diluted to 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions.

Test: A set of seven sterilized test tubes numbered 1 to 7 was set up and 10 ml of sterilized T.S.A. at 50°C was transferred aseptically to each of these tubes. 0.1 ml of undiluted solution of NPOO1Z was aseptically added to tube 1. 0.1 ml of 1:2 dilution of NPOO1Z was aseptically added to tube 2, etc., until 1:64 dilution was aseptically added to tube 7. The contents of the tubes were shaken and poured

aseptically into a set of 7 sterile petridishes such that contents of tube 1 were poured into petridish 1, contents of tube 2 were poured in petridish 2, etc., and the petridishes were swirled to achieve homogeneity. The agar was allowed to congeal and petridishes were subsequently turned upside down.

Standard: A stock solution of Gentamycin (80,000 I.U/2 ml) was made up to 100 ml with sterilized deionized water. This stock solution was diluted to give a concentration of 100 I.U/ml to make the working standard.

10 ml of sterile T.S.A. at 50°C was transferred aseptically to a sterile test tube labelled Standard and 0.1 ml of working Gentamycin standard solution was added to it. The contents of the tube were shaken and aseptically poured into a sterile petridish labelled Standard. The petridish was swirled to ensure homogeneity and the agar was allowed to congeal. After agar had congealed, petridish was turned upside down.

Blank: 0.1 ml of DMSO was transferred aseptically to a sterile petridish labelled Blank and 10 ml of sterile TSA at 50°C was added to it. The dish was then swirled to ensure homogeneity, the agar was allowed to congeal and the petridish was subsequently turned upside down.

Each of the 9 petridishes, seven labelled Test, one Standard and one Blank, was then divided into six sectors labelled I, II, III, IV, V, and VI. By streaking in a radial pattern, each of the six selected micro-organisms was inoculated aseptically in a separate sector in all the nine petridishes as shown in Table 10.

Petridishes were incubated at 37°C for 24 hours and then examined for any clear zones of inhibition. The lowest dilution at which a clear zone of inhibition was observed was taken as the MIC.

MIC of extract 13 was similarly evaluated.

The observed MICs of extract NPOO1Z against **Staphylococcus aureus, Shigella dysentrie, Proteus spp.** and **Bacillus spp.** and the observed MICs of extract 13 against **Staphylococcus aureus** and **Proteus spp.** are shown in Table 15.

Further experiments were set up to narrow down the MIC ranges of extracts NPOO1Z and 13. Several dilutions of NPOO1Z at concentrations of 90 μg/ml, 100 μg/ml, 110 μg/ml, 130 μg/ml, 150 μg/ml, 170 μg/ml and 190 μg/ml were prepared and a number of dilutions of extract 13 at concentrations of 45 μg/ml, 55 μg/ml, 65 μg/ml, 75 μg/ml, 85 μg/ml, 95 μg/ml, 100 μg/ml, 105 μg/ml and 110 μg/ml were also prepared. MICs of NPOO1Z and extract 13 were subsequently evaluated as described above and the results are presented in Table 16.

2.11.1.1 Preparation of Bacterial Slants and Solutions

(a) Preparation of Bacterial Slants

5 ml of T.S.A. was transferred into each of the six tubes labelled A, B, C, D, E and F. The tubes were stoppered and auto-claved at 121°C for 15 minutes. The tubes were then allowed to cool to room temperature with one end elevated by 15 degrees from the table top so that the available surface area in the tube will be as large as possible. The agar was, however, not allowed to

flow into the lids of the tubes. The tubes were left to stand undisturbed in this position for overnight where upon the agar congealed.

Each tube was aseptically inoculated with one loopful of a pure culture of one micro organism using a sterile platinum loop as shown in Table 11.

Micro-organism	Set
Staphylococcus aureus	A
Escherichia coli	B
Salmonella gallinarium	C
Shigella dysentrie	D
Bacillus spp.	E
Proteus spp.	F

Table 11: Micro-organism and Corresponding Sets

The tubes were incubated in an air-dry oven at 37°C for 24 hours and stored at 0-4°C.

(b) Preparation of Bacterial Suspension

10 ml of Trypticase Soy Broth, T.S.B, (3 mg/ml) was transferred into each of the six stoppered 15 ml sterile tubes labelled 1 to 6 with the aid of an automatic syringe and the tubes were autoclaved at 121°C for 15 minutes. Each tube was then inoculated with two loops full of one test micro-organism obtained from the above slants under sterile conditions using a standard

platinum loop as shown in Table 12. The tubes were incubated at 37°C for 24 hours.

Micro-organism	Tube No.
Staphylococcus aureus	1
Escherichia coli	2
Salmonella gallinarium	3
Shigella dysentrie	4
Bacillus spp.	5
Proteus spp.	6

Table 12: Micro-organism and Corresponding Tubes

(c) Streptomycin Sulphate (10 mg/ml)

1.0 g of streptomycin sulphate was dissolved in 40 ml of deionized water in a 100 ml volumetric flask and volume of the solution was raised to the mark with deionized water. The solution was stored at 4°C.

(d) Trypticase Soy Broth, T.S.B (3 mg/ml)

1.5 g of Trypticase Soy Broth was dissolved in 500 ml of boiling deionized water. The solution was heated further for 10 minutes until it became clear.

(e) Trypticase Soy Agar, T.S.A. Solution

7.5 g of Trypticase Soy Agar was dissolved in 500 ml of hot deionized water.

To this solution, 15.0 g of T.S.B. was added and the mixture was gently heated on a hot plate for a few minutes.

(f) Sodium Chloride (8.5 mg/ml)

4.25 g of analytical reagent grade sodium chloride was dissolved in 40 ml of deionized water in a 500 ml volumetric flask and then diluted to the mark with deionized water.

(g) NP001Z ($1.5x10^5$ μg/ml)

300 mg of NP001Z was dissolved in 2.0 ml of DMSO.

2.11.2 Detection of the Presence of Tannins

(a) Extracts NPOO1Z and 13 were tested for the presence of Tannins by the use of 5% aqueous ferric chloride reagent [1].However, 0.5 g of extract 13 was used instead of 1 g. A blue black greenish precipitate was observed confirming the presence of Tannins in the test extract.

(b) The extracts, NPOO1Z and 13, were again investigated for the presence of Tannins by the method recommended by J.B. Harborne [86] as well. Production of a red colour extractable into amyl/butyl alcohol confirmed the presence of Tannins. Following 2-D paper chromatography using top phase of Butan -2-ol: Acetic acid: Water (14:1:15) followed by 6% acetic acid, Tannins were detected as dark purple spots under uv light.

2.11.3 Detection of Nitrogen and Sulphur in Extract 13

Extract 13 was tested for the presence of nitrogen and sulphur by fusing the test extract with metallic sodium (Lassaignes test), a detection technique prescribed by Vogel [87].

(a) Detection of the Presence of Nitrogen

To 1 ml of sodium extract, 0.1 g of ferrous sulphate crystals were added and the mixture was heated to boiling. 3 drops of dilute sulphuric acid (2 M) were then added to the hot mixture.

A dirty green bluish precipitate of ferric ferrocynide was observed, confirming the presence of Nitrogen in extract 13.

(b) Detection of the Presence of Sulphur

(i) To 1 ml of sodium extract, a small crystal of sodium nitroprusside was added. No purple colouration was observed. confirming the absence of sulphur in extract 13.

(ii) To 1ml of sodium extract, 3ml of dilute 5% aqueous acetic acid and 1ml of 5% aqueous lead acetate solutions were added. No black precipitate of lead (II) sulphide was observed, confirming the absence of sulphur in extract 13.

Sodium extract was prepared by the method described by Vogel [87].

2.11.4 Detection of Alkaloids

(a) Walls procedure [65] was used to detect the presence of alkaloids in NP001Z and extract 13.

0.5 g of test extract was dissolved in 10 ml of distilled water, filtered, and the filtrate was acidified with 1% hydrochloric acid to pH 5.8. A few drops of Meyer's reagent were then added to a small portion, 2.5 ml, of the acidified filtrate.

An orange yellow crystalline precipitate was observed indicating the presence of alkaloids in the test extracts.

(b) The test extracts were chromatographed in duplicate on precoated analytical T.L.C plates, silica gel - G60, Merk type 5735, using MeOH:Me_2CO:EtOAc:CHF in the ratio of 1:2.5:1:7 as the solvent system. One chromatogram was sprayed with Dragendorff's reagent and the chromatogram was observed for any colour changes. Orange yellow spots, indicating the presence of alkaloids, were observed.

The other chromatogram was sprayed with vanillin reagent, allowed to dry, and then heated at 120°C for 15 minutes. No specific colour development was observed.

2.11.4.1 Preparation of Reagents

(a) Preparation of Meyer's Reagent

1.36 g of mercuric chloride and 5.00 g of A.R. potassium iodide were dissolved in a small quantity of distilled water and volume of the solution was then raised to 100 ml with distilled water [65].

(b) Preparation of Dragendorff's Reagent

8.0 g of bismuth nitrate, 27.2 g of potassium iodide and 20 ml of concentrated nitric acid were diluted into a small quantity of distilled water and volume of the solution was then raised to 100 ml with distilled water [65].

(c) Preparation of Vanillin Reagent

1 g of vanillin was dissolved in 100 ml of 50% aqueous phosphoric acid [65].

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Authentic Plant Material

Family - leguminosae

Genus - Cassia

Species - abbreviata

Author - Oliver

hence, **Cassia abbreviata-Oliv.**

Effects of various extracts and compounds under investigation on the motility, normal rhythmic pendular movement responses, of the smooth muscle of rabbit ileum are presented in sections 3.1.2 to 3.1.7, Figures 8 to 27.

3.1.2 Anti-diarrhoeal Activities of Extracts NP001X and NP001Z

At a concentration of 600μg/ml, NP001Z was found to exhibit demonstrable anti-diarrhoeal activity in-vitro as it decreased the normal rhythmic pendular movement renponses of smooth muscle of rabbit ileum as shown in Figure 9 while NP001X did not exhibit any observable anti-diarrhoeal effect at the same concentration as shown in Figure 8. NP001Z was, therefore, selected for further investigation.

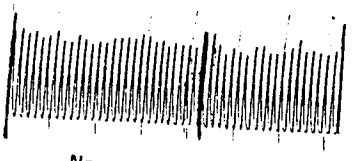


Figure 8: Effect of NP001X on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

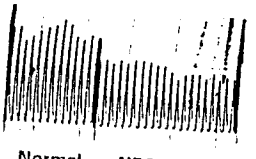


Figure 9: Effect of NP001Z on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

3.1.3 Dose-Response Relationship of Ethanolic Extract NPOOIZ

The degree of inhibition of the motility, normal rhythmic pendular movement responses, of rabbit ileum was found to increase with an increase in dose of NP001Z as shown in Figure 10 and Graph 1. These results indicate that anti-diarrhoeal potency of extract NP001Z increased with an increase in its dose. The slope of the dose-response curve, Graph 1, is negative because an increase in dose of NP001Z resulted in a decrease in the amplitude of normal rhythmic pendular movement response of the rabbit ileum.

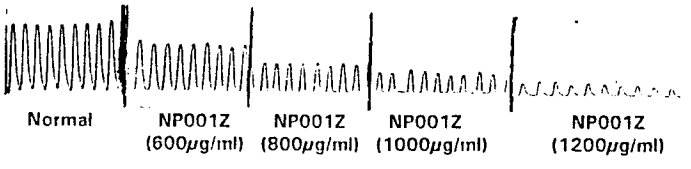
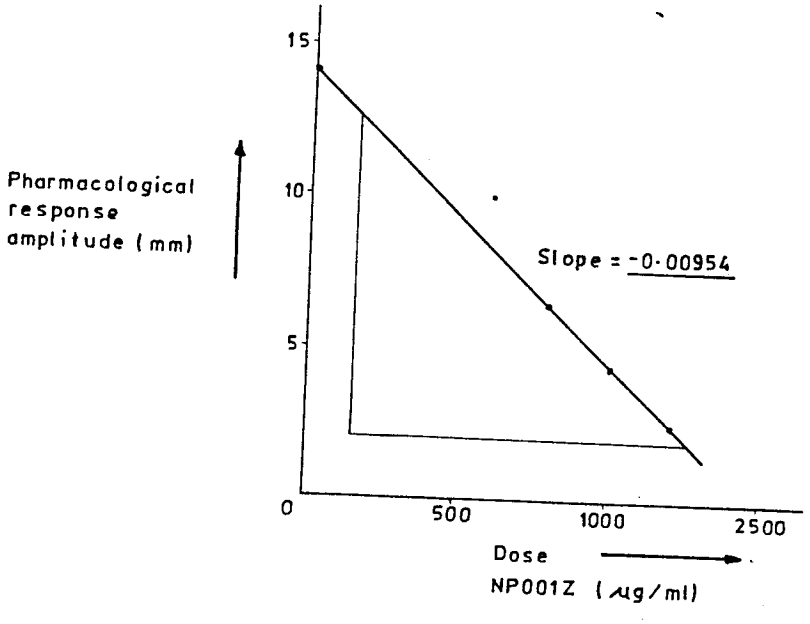


Figure 10: Pharmacological responses of smooth muscle of rabbit ileum to the graded doses of NPOOIZ



Graph 1.

Dose - Response Curve of NPOOIZ

3.1.4 Effect of NPOOIZ on Para-sympathetic Ganglia, Post-ganglionic Acetylcholine Receptors and Other Receptors on Smooth Muscle of the Rabbit Ileum

Figures 11 to 15 show the effects of extract NP001Z on the increase in motility of rabbit ileum induced by five known stimulants. At a concentration of 600 μg/ml, extract NP001Z either fully or partly abolished the spasms induced by acetylcholine, histamine, nicotine, barium chloride and magnesium chloride.

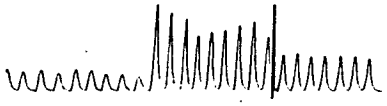


Figure 11: Effect of NP001Z on smooth muscle of rabbit ileum against acetylcholine (Ach)induced spasm

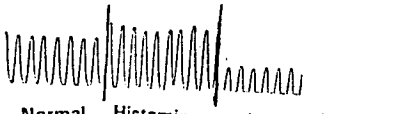


Figure 12: Effect of NP001Z on smooth muscle of rabbit ileum against histamine (Hist)induced spasm

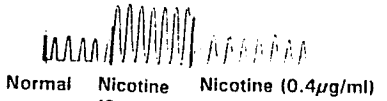


Figure 13: Effect of NP001Z on smooth muscle of rabbit ileum against nicotine induced spasm

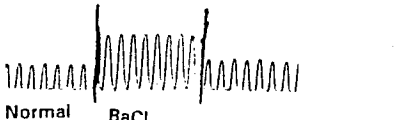


Figure 14: Effect of NP001Z on smooth muscle of rabbit ileum against barium chloride ($BaCl_2$)induced spasm

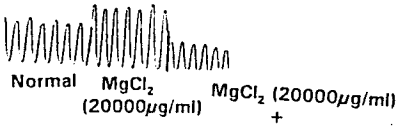


Figure 15: Effect of NP001Z on smooth muscle of rabbit ileum against magnesium chloride ($MgCl_2$)induced spasm

3.1.5 Anti-diarrhoeal Activities of Various Fractions Isolated from NPOOIZ

Figure 16 shows the effects of various fractions obtained from fractionation of the anti-diarrhoeal extract NP001Z on the motility of the rabbit ileum. Extracts 9 and 13 have significant anti-diarrhoeal activity because these extracts reduced the motility of rabbit ileum. Extracts 2,4,6,7,8,10,11 and 12 were found to be stimulants because they increased the motility, normal rhythmic pendular movement responses, of rabbit ileum. Extract 10 was found to be the most potent stimulant and extract 13 was more potent anti-diarrhoeal agent.

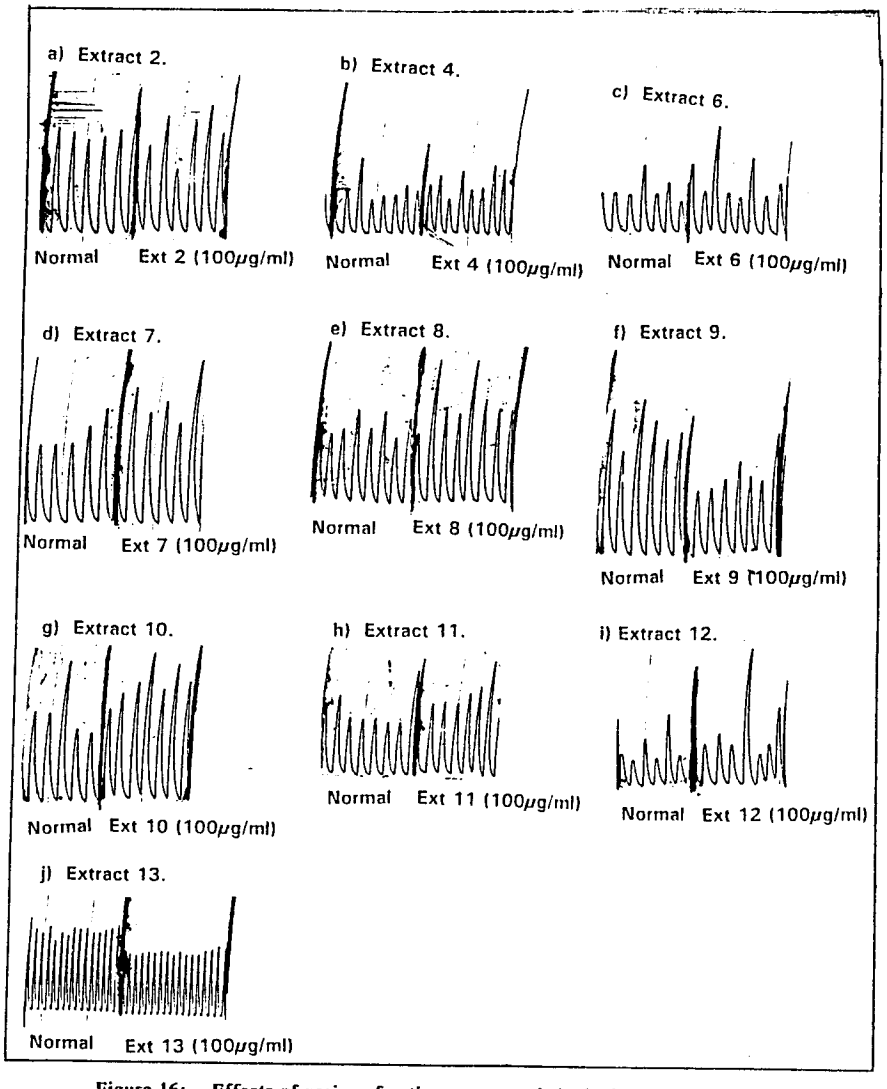


Figure 16: Effects of various fractions on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

3.1.6 Confirmation of Anti-diarrhoeal Activity of the Selected Extract 13

Anti-diarrhoeal activity of extract 13 was found to be reproducible because it demonstrated significant reduction in the motility of rabbit ileum when the experiment was repeated as shown in Figure 17.

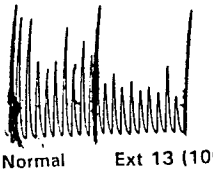


Figure 17: Effect of extract 13 on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

3.1.7 Dose-Response Relationship of Extract 13

Effects of graded doses of extract 13 on the motility of rabbit ileum are shown in Figure 18 and Graph 2. The results indicate that anti-diarrhoeal activity of extract 13 increased with an increase in its dose because the degree of inhibition of normal rhythmic pendular movement responses of rabbit ileum decreased with an increase in dose of extract 13. The slope of the dose-response curve, Graph 2, is negative because an increase in dose of extract 13 resulted in a decrease in the amplitude of normal rhythmic pendular movement response of the rabbit ileum.

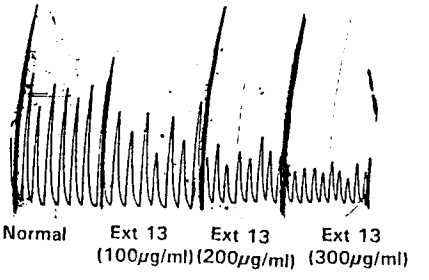
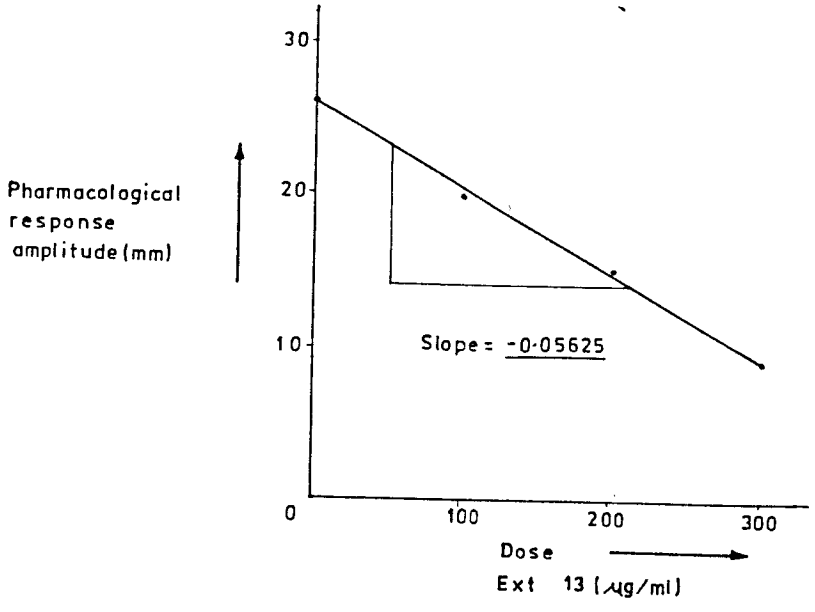


Figure 18: Phamacological responses of smoth muscle of rabbit ileum to the graded doses of extract 13.



Graph 2.

Dose - Response Curve of Extract 13

3.1.8 Pharmacological Effects of Extract 13 on Para-sympathetic Ganglia, Post-ganglionic Acetylcholine Receptors and Other Receptors on Smooth Muscle of the Rabbit Ileum

Effects of extract 13 on the increased motility of rabbit ileum induced by a number of stimulants, acetylcholine, histamine, nicotine and barium chloride are shown in Figures 19 to 22.

At a concentration of 100 µg/ml, extract 13 was found to antagonize the spasms induced by nicotine and barium chloride as shown in Figures 21 and 22. Extract 13 did not reduce acetylcholine and histamine induced spasms as shown in Figures 19 and 20.

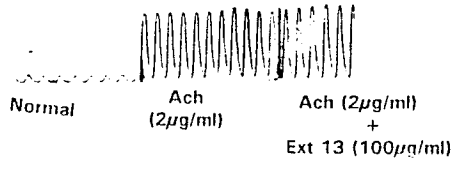


Figure 19: Effect of extract 13 on smooth muscle of rabbit ileum against acetylcholine (Ach)induced spasm

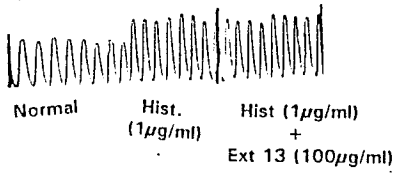


Figure 20: Effect of extract 13 on smooth muscle of rabbit ileum against histamine (Hist)induced spasm

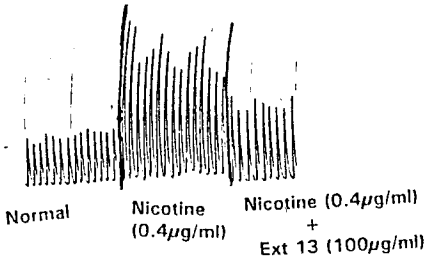


Figure 21: Effect of extract 13 on smooth muscle of rabbit ileum against nicotine induced spasm

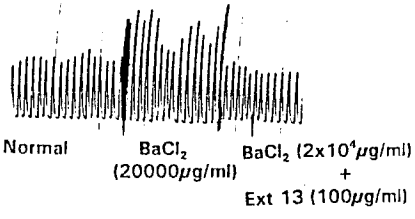


Figure 22: Effect of extract 13 on smooth muscle of rabbit ileum against barium chloride ($BaCl_2$)induced spasm

3.1.9 Rf Values of LAMM

Rf values of the compound LAMM indicate that it is highly polar.

Solvent system	Rf Values
CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1)	0.5362
CH_2Cl_2:EtOAc (2:3)	0.5185
CHF:EtOAc (2:5)	0.4883
PE:EtOAc (3:7)	0.5016

Table 13: Rf values of LAMM in a number of solvent systems

3.1.10 A Effects of Various Fractions Isolated from Chromatographic Separation of Extract 13 on the Motility of Rabbit Ileum

Observed effects of various fractions obtaianed from chromatographic separation and purification of extract 13 on the motility of rabbit ileum are

shown in Figures 23 to 26.

At a concentration of 0.5 μg/ml, fractions 131006, 131420 and 13 SG were found to exhibit appreciable anti-diarrhoeal activity because they decreased the motility of rabbit ileum as shown in Figures 23,25 and 26. Fraction 131006 was clearly the most potent anti-diarrhoeal fraction.

At a concentration of 0.5 μg/ml, fraction 137013 was found to be stimulant because it increased the motility of rabbit ileum as shown in Figure 24.

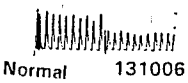


Figure 23: Effect of 131006 on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

Figure 24: Effect of 137013 on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

Figure 25: Effect of 131420 on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

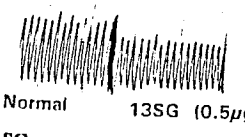


Figure 26: Effect of 13SG on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum

3.1.10 B Effects of Compound LAMM on the Motility of Rabbit Ileum

Effect of a compound LAMM isolated from the most potent anti-diarrhoeal fraction, 131006, on the motility of rabbit ileum is shown in Figure 27.

At a concentration of 0.5 μg/ml, compound LAMM demonstrated considerable anti-diarrhoeal activity as it reduced the motility of rabbit ileum to a large extent.

Figure 27: Effect of LAMM on normal rhythmic pendular movement responses of smooth muscle of rabbit ileum.

3.1.11 Evaluation of In-Vitro Anti-bacterial Activity (Agar-Streak Dilution Method)

In-vitro anti-bacterial activities of extracts NP001Z and 13 against six pathogenic micro-organisms are presented in Table 14.

At a concentration of 1500 μg/ml, extract NP001Z was found to be active against **Staphylococcus aureus, shigella dysentrie, Proteus spp.** and **Bacillus spp.** because NP001Z inhibited the growth of these mico-organisms. NP001Z did not show any observable anti-bacterial action against **Escherichia coli** and **Salmonella gallinarium.**

Extract 13 was found to be active only against two of the six micro-organisms, **Shigella dysentrie** and **Proteus spp.**

Test Extract at a concentration of 1500μg/ml	MICRO-ORGANISMS					
	1	2	3	4	5	6
NPOO1Z	(+)	(+)	(-)	(-)	(+)	(+)
Extract 13	(-)	(+)	(-)	(-)	(+)	(-)

Key: 1. Staphylococcus aureus
2. Shigella dysentrie
3. Escherichia coli
4. Salmonella gallinarium
5. Proteus spp.
6. Bacillus spp.

\+ = Active, Test extract inhibits the growth of the bacteria.
\- = Inactive, Test extract does not inhibit the growth of the bacteria.

Table 14: Anti-bacterial Activities of NPOO1Z and Extract 13.

3.1.12 Determination of Minimum Inhibitory Concentrations (MICs)

Effects of graded doses of extracts NP001Z and 13 on the growth of six selected micro-organisms are shown in Tables 15 and 16.

The MICs of NP001Z against **Staphylococus aureus, Shigella dysentrie, Proteus spp.** and **Bacillus spp.** were found to be 150 μg/ml, 100 μg/ml, 150 μg/ml and 150 μg/ml respectively. The MICs of extract 13 against **Shigella dysentrie** and **Proteus spp.** were found to be 75 μg/ml and 100 μg/ml respectively.

Test Extract	Dilution	Conc. (µg/ml)	MICRO-ORGANISMS					
			1	2	3	4	5	6
NPOO1Z	NEAT	1500.00	(+)	(+)	(-)	(-)	(+)	(+)
	1:2	750.00	(+)	(+)	(-)	(-)	(+)	(+)
	1:4	375.00	(+)	(+)	(-)	(-)	(+)	(+)
	1:8	187.50	(+)	(+)	(-)	(-)	(+)	(+)
	1:16	93.75	(-)	(-)	(-)	(-)	(-)	(-)
	1:32	46.88	(-)	(-)	(-)	(-)	(-)	(-)
	1:64	23.44	(-)	(-)	(-)	(-)	(-)	(-)
Extract 13	NEAT	1500.00	(-)	(+)	(-)	(-)	(+)	(-)
	1:2	750.00	(-)	(+)	(-)	(-)	(+)	(-)
	1:4	375.00	(-)	(+)	(-)	(-)	(+)	(-)
	1:8	187.50	(-)	(+)	(-)	(-)	(+)	(-)
	1:16	93.75	(-)	(-)	(-)	(-)	(-)	(-)
	1:32	46.88	(-)	(-)	(-)	(-)	(-)	(-)
	1:64	23.44	(-)	(-)	(-)	(-)	(-)	(-)
DMSO			(-)	(-)	(-)	(-)	(-)	(-)
Strepto-mycin sulphate		100	S	S	S	R	S	S
Genta-mycin		1I.U/ml	S	S	S	S	S	S

Key:
1. Staphylococus aureus
2. Shigella dysentrie
3. Escherichia coli
4. Salmonella gallinarium
5. Proteus spp.
6. Bacillus spp.

+ = Active
- = Inactive
S = Sensitive (bacteria does not grow in the presence of test material)
R = Resistant (bacteria grows in the presence of test material)

Table 15: In-Vitro Anti-bacterial Activities of Graded Doses of NPOO1Z and Extra 13.

Test Extract	MIC (μg/ml)					
	MICRO-ORGANISMS					
	1	2	3	4	5	6
NPOO1Z	150	100	(-)	(-)	150	150
13	(-)	75	(-)	(-)	100	(-)

Table 16: Minimum Inhibitory Concentrations of NPOO1Z and Extract 13

Key: 1. Staphylococcus aureus
2. Shigella dysentrie
3. Escherichia coli
4. Salmonella gallinarium
5. Proteus spp.
6. Bacillus spp.

\+ = Active, Test extract inhibits the growth of the bacteria.
\- = Inactive, Test extract does not inhibit the growth of the bacteria.

3.1.13 Other Chemical Qualitative Tests

Extracts NP001Z and 13 gave positive tests for the presence of tannins, nitrogen and alkaloids. Sulphur compounds and ammonium ions were found to be absent in these extracts as shown in Table 17.

Test Extract	Tannins	Nitrogen	Sulphur	Alkaloids	Free NH_4^+ions
NPOO1Z	Present	Present	Absent	Present	Absent
Extract 13	Present	Present	Absent	Present	Absent

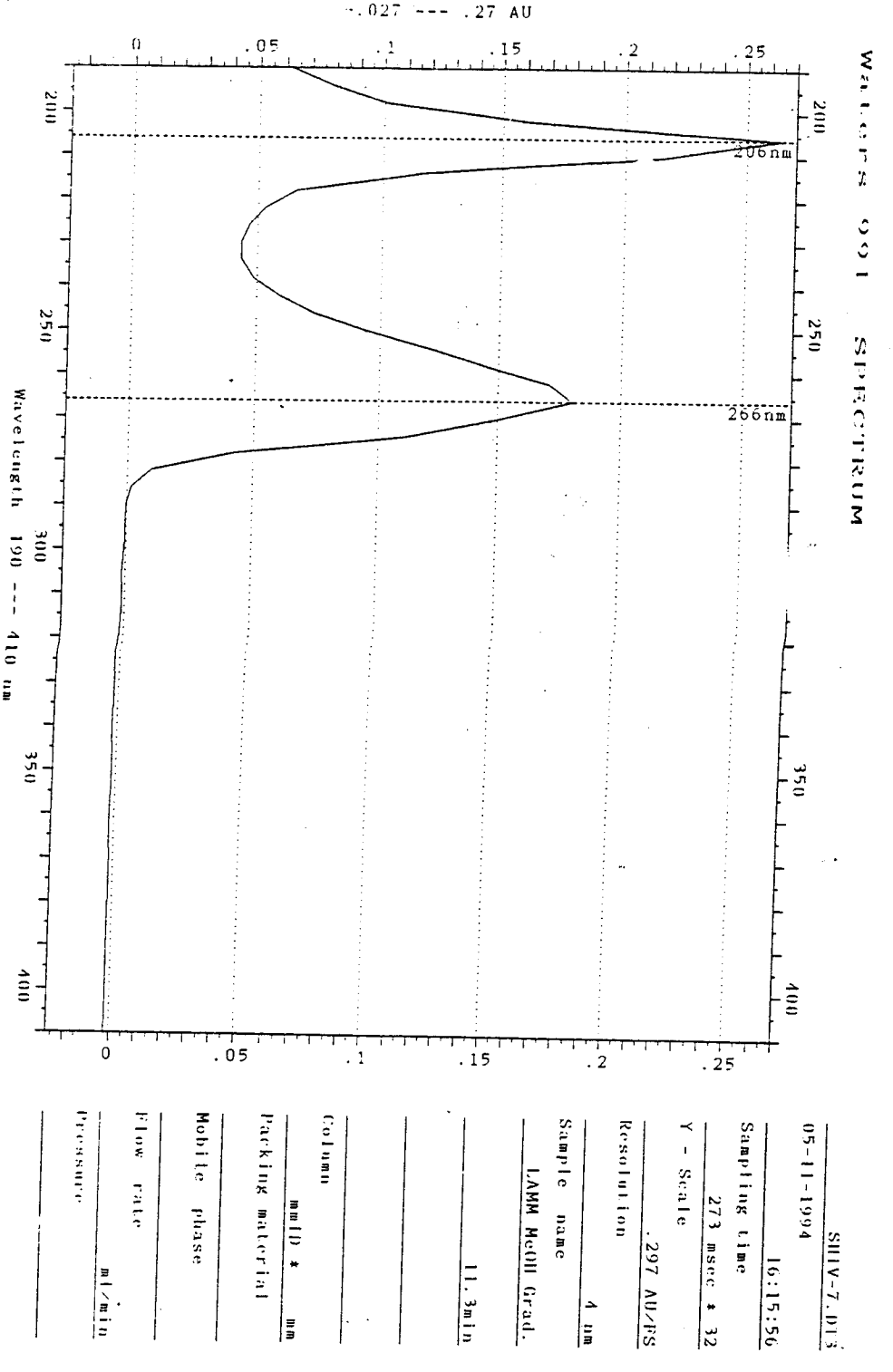
Table 17: Qualitative Analysis of NPOOIZ and Extract 13

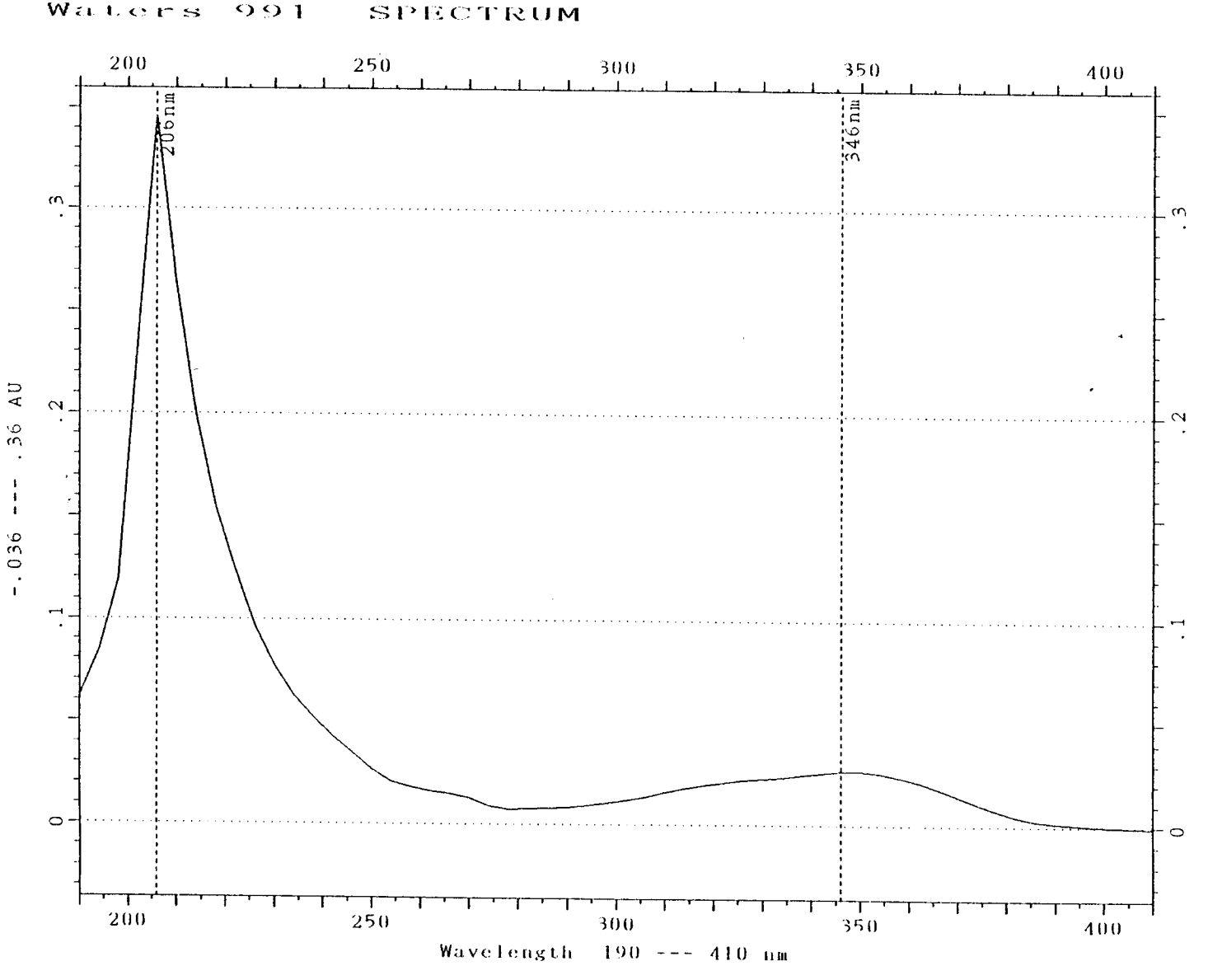
3.1.14 HPLC Purification of LAMM

HPLC purification of LAMM was done using Binary Waters 501 HPLC pumping system with automated gradient controller and Waters 991 photodiode array detector.

3.1.14.1 Analytical HPLC Data

The analytical HPLC data on the sample LAMM using methanol gradient is presented on pages 83,84 and 85. The data shows that the sample LAMM is a mixture of three compounds as three eluates with different uv spectra emerged at the characteristic retention times of 11.3, 24.7 and 37.4 minutes.

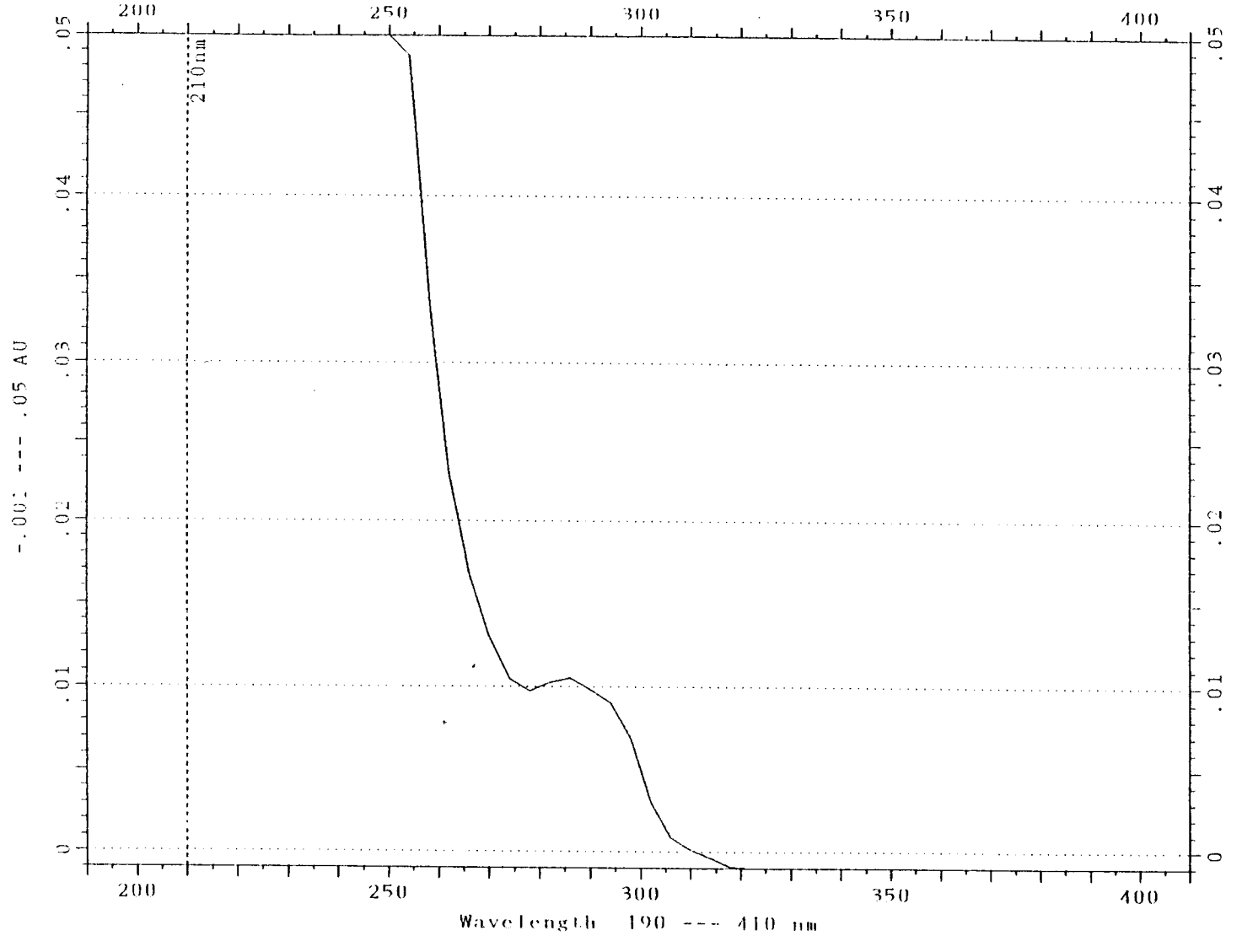




SHIV-7.DT3
05-11-1994
16:15:56
Sampling time
273 msec * 32
Y - Scale
.396 AU/FS
Resolution
4 nm
Sample name
I.AMM MeOH Grad.
24.35min
Column
mmID * mm
Packing material
Mobile phase
Flow rate
ml/min
Pressure

Waters

Waters 991 SPECTRUM



SHIV-7.DT3

05-11-1994

16:15:56

Sampling time

273 msec * 32

Y - Scale

.051 AU/FS

Resolution

4 nm

Sample name

LAMM MeOH Grad.

37.4min

Column

.mmID * mm

Packing material

Mobile phase

Flow rate

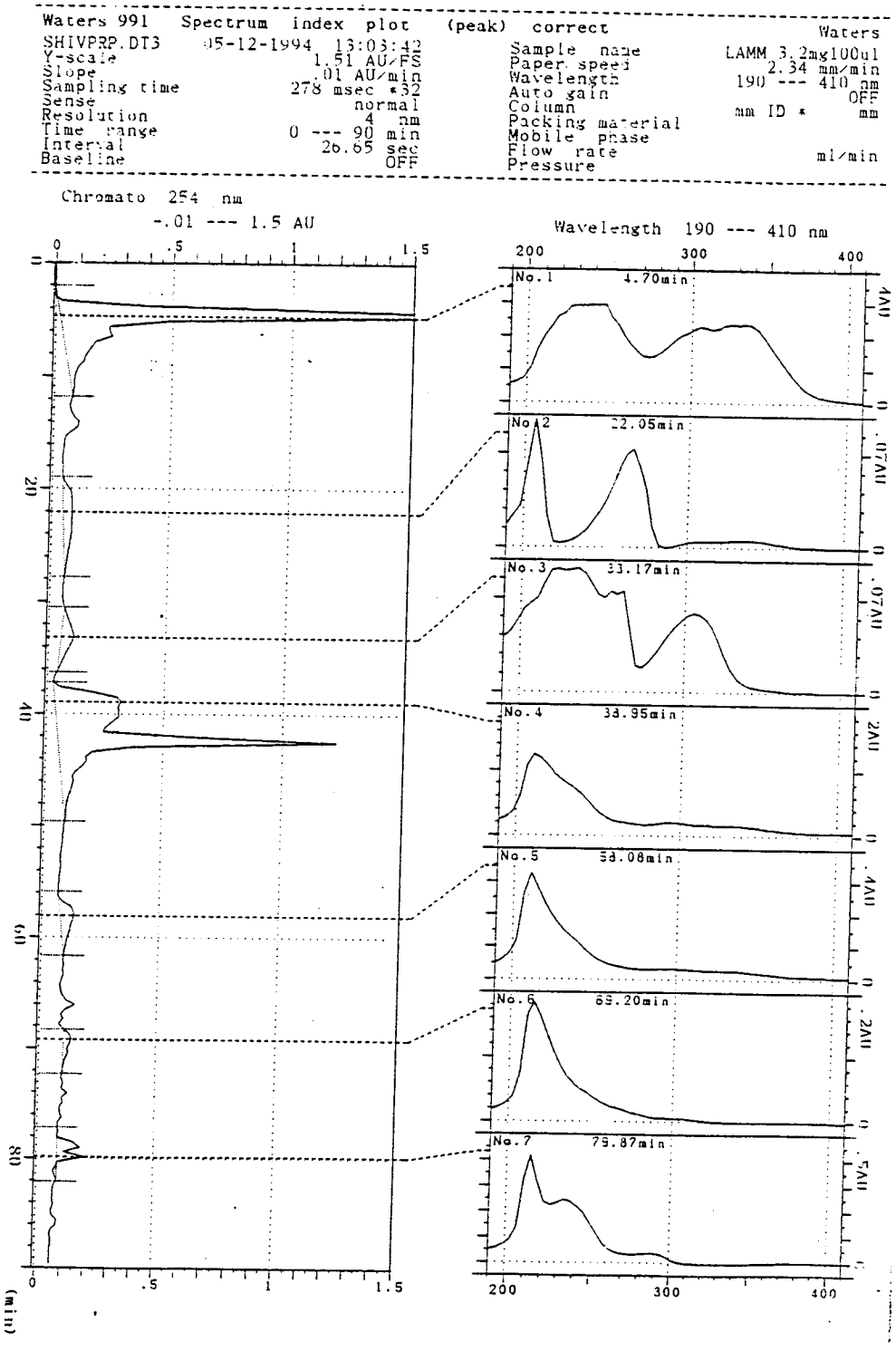
ml/min

Pressure

Waters

3.1.14.2 Preparative HPLC Data

Preparative HPLC data on the sample LAMM using methanol gradient is shown below. The data clearly shows that the sample LAMM is a mixture of 5 compounds because eluates collected at characteristic retention times of 4.07, 22.05, 33.17, 38.95 and 58.08 minutes had different uv spectra as shown below.

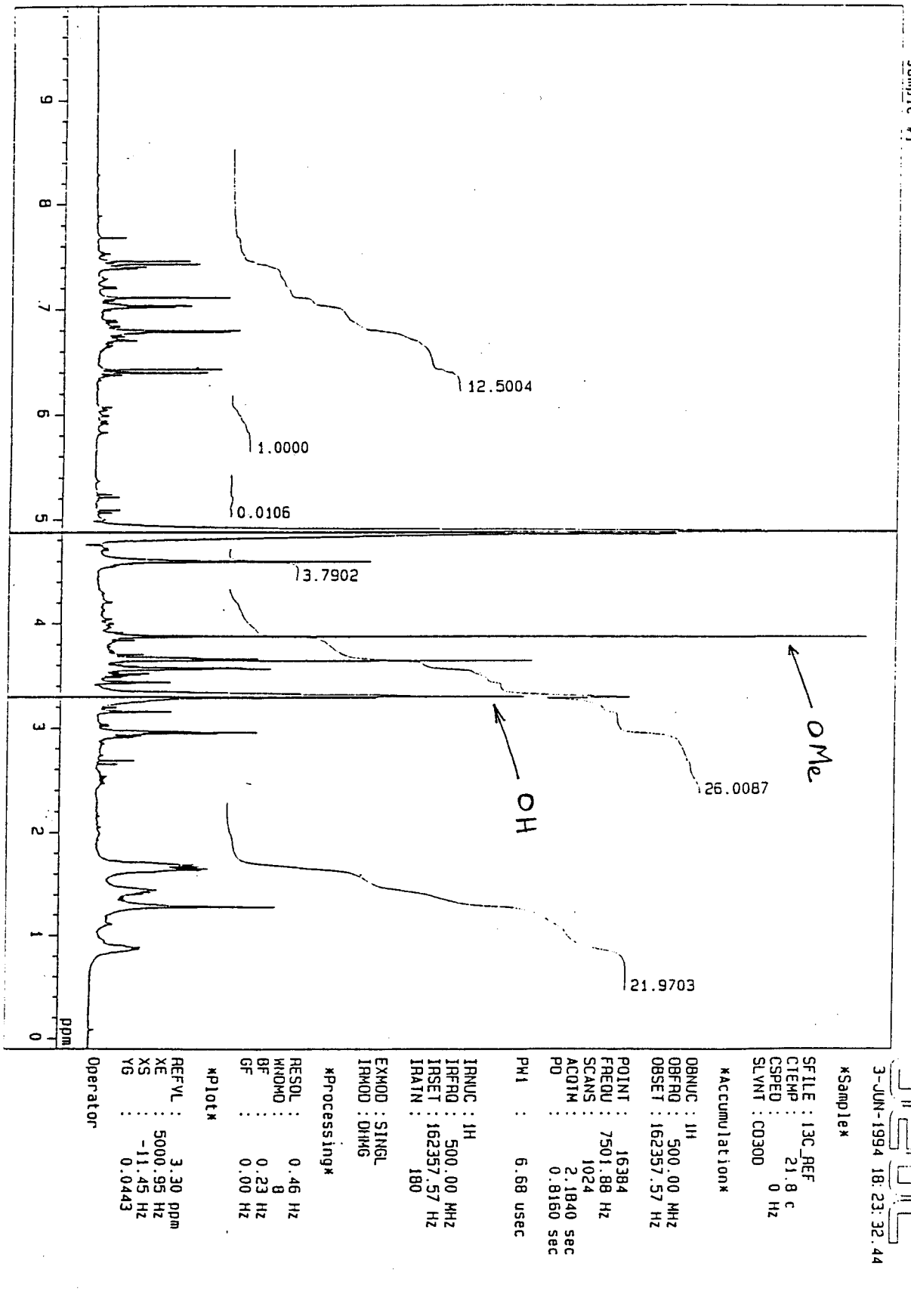


These resuslts indicate that the sample LAMM was a mixture of three compounds, two of which disintegrated whilst on the HPLC column to give a total of five compounds. One of these five compounds, fraction 2, was isolated in 1.2 mg quantity. The quantities of other four compounds ranged from 0.3 to 0.4 mg as presented in Table 9 on page 52.

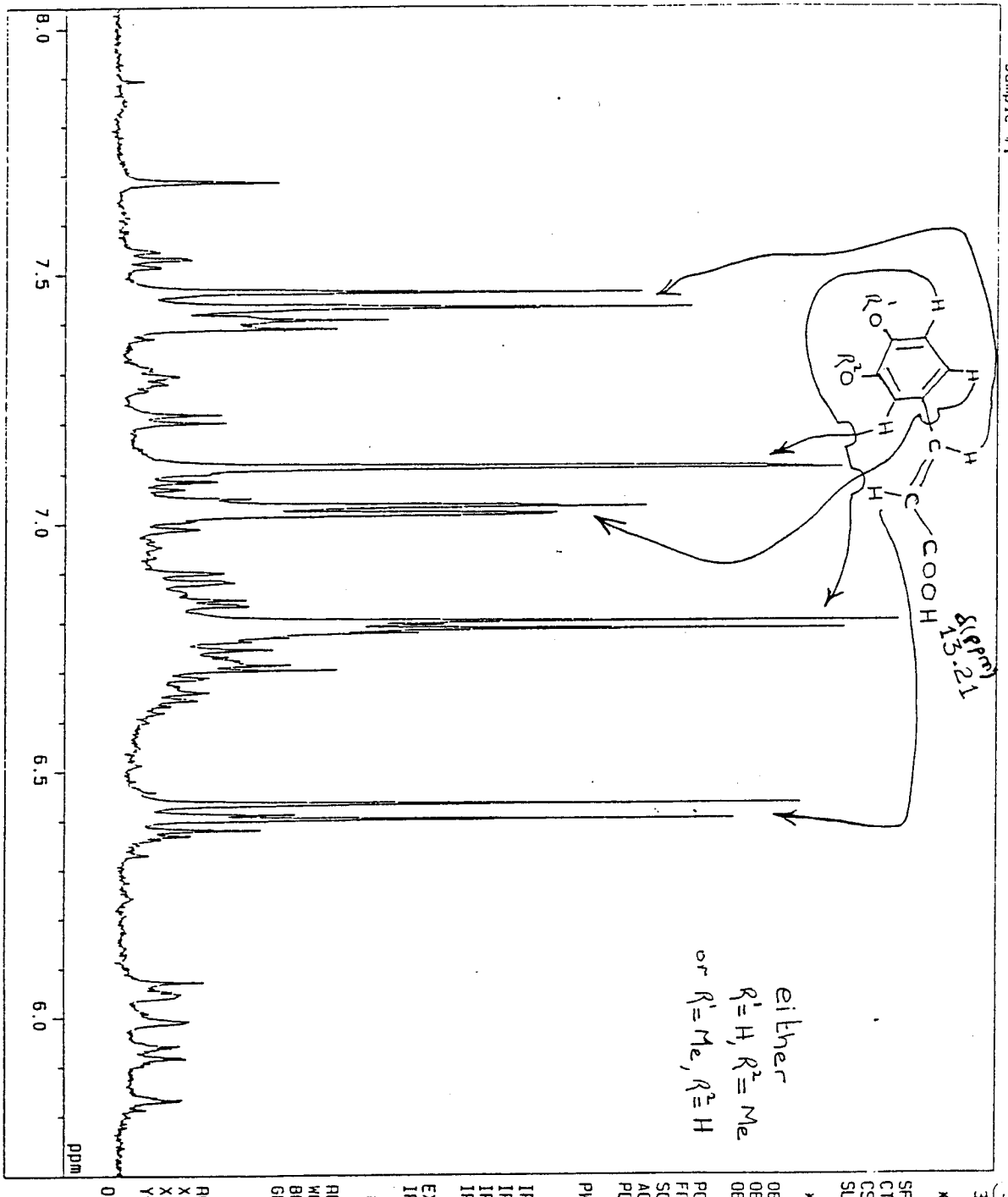
3.1.15 500 MHz ^{1}H NMR Spectrum of Pure Compound.

500 MHz ^{1}H NMR spectrum of the major fraction obtained from HPLC purification of the sample LAMM is presented on pages 88 and 89. The spectrum shows a number of NMR signals in the δ regions of :1-5 ppm and 6-8 ppm.

500MHz ^{1}H NMR spectrum



500MHz ^{1}H NMR spectrum expanded in the δ region 6 to 8 ppm.



3.1.15.1 500 MHz ^{1}HNMR Chemical Shifts, Multiplicities and Coupling Constants, J Values.

Description of significant signals appearing on the ^{1}H NMR spectra shown on pages 88 and 89 is given below:

^{1}HNMR (CCl_4:TMS) δ (ppm):

13.21(s,1H); 7.45(d,1H,J=17Hz);

7.12(dd,1H,J=3Hz,1.5Hz); 7.05(dd,1H,J=8Hz,3Hz);

7.0(m); 6.8(dd,1H,J=8Hz,1.5Hz);

6.43(d,1H,J=17Hz);3.9(s,3H); 3.3(s,1H).

Key: s = singlet

d = doublet

m = multiplet

dd = doublet of doublets

The probable structures of the compound and the assignment of various peaks are shown on pages 88 and 89. Detailed analysis of the ^{1}H NMR spectra is presented under discussion section on pages 99 to 104.

3.2 DISCUSSION

Initial investigations carried out in this laboratory by Prakash et al revealed that aqueous decoction of the stem bark of **Cassia abbreviata-Oliv.** has demonstrable anti-diarrhoeal activity in-vitro. It was further reported that hot ethanolic extract was slightly more potent than aqueous and cold ethanolic extracts [13(i),13(ii)]. Therefore, hot ethanolic extract was chosen for the present study. .

In order to isolate anti-diarrhoeal constituent(s) of the stem bark of **Cassia abbreviata-Oliv.**, an authentic sample of the plant was collected, botanically identified by Dr. P. S. M. Phiri of Biology Department (UNZA) and an ethanolic extract of the

dried powdered stem bark was prepared by Soxhlet extraction.

During Soxhlet extraction, the clear ethanolic extract turned turbid on standing indicating fractional precipitation of some of the components of the total ethanol extractibles present in **Cassia abbreviata-Oliv.** stem bark. The suspended particles, NPOO1X, were collected separately and these were found to constitute 1.2% of the dry stem bark powder while the clear filtrate yielded 281.1899 g of a solid, NPOO1Z, representing 16% of the dry powdered plant material. The total ethanol extractibles were, therefore, 301.3524 g which is 17.4% of the dry stem bark powder.

In order to evaluate the anti-diarrhoeal activity of test extracts NPOO1X and NPOO1Z, their effects on normal rhythmic pendular movement responses of rabbit ileum were monitored in an isolated organ bath in-vitro by the method of Perry et al [67]. Inhibition of the motility, normal rhythmic pendular movement responses, of the smooth muscle of rabbit ileum was taken as the principal indicator of anti-diarrhoeal effect because increased intestinal motility is widely considered to be the cause of diarrhoea [64].

At a concentration of 600 μg/ml, NPOO1Z was found to exhibit demonstrable anti-diarrhoeal activity in-vitro as shown in Figure 9 while NPOO1X did not exhibit any observable anti-diarrhoeal effect at the same concentration as shown in Figure 8. Extract NP001Z was therefore selected for further investigation

Anti-diarrhoeal activity of NP001Z was further confirmed by monitoring and recording the pharmacological responses of rabbit ileum to the graded doses of NPOO1Z. The Dose-Response curve, Graph 1, clearly demonstrates that NPOO1Z has a definite in-vitro inhibitory effect on the smooth muscle of rabbit ileum as the degree of inhibition of motility of the rabbit ileum increased with increase in dose of NPOO1Z.

Observed inhibitory effect of NP001Z inplies that one or more constituents of NP001Z either interact(s) with or bind(s) to one or more receptor sites on the ileum tissue cell because a drug molecule must approach one or more receptor sites on the cell sufficiently close in order to alter the functioning of the receptor sites and cellular processes [81].

To determine the mode of inhibitory action of NPOO1Z, its effects on smooth muscle of rabbit ileum against chemical agonists, 2 μg/ml acetylcholine, 1 μg/ml histamine, 0.4 μg/ml nicotine, $2x10^{4}$ μg/ml barium chloride, and $2x10^{4}$ μg/ml magnesium chloride induced spasms were evaluated and the results are shown in Figures 11, 12, 13, 14 and 15, respectively. Acetylcholine has two types of actions, muscarinic and nicotinic action. Muscarinic action involves the stimulation of sympathetic and parasympathetic receptors and it is characterized by the fact that such effects can easily be abolished by small doses of atropine (1 μg/ml) [81,82]. After muscarinic effects have been blocked by atropine, larger doses of acetylcholine (4-5 μg/ml) produce another set of pharmacological actions which are similar to those of nicotine. Nicotine acts on ganglion cells, autonomic ganglia and parasympathetic ganglia

receptors. Metallic salts, magnesium chloride and barium chloride are used when direct inhibitory action of the test extract is being investigated [82]. 600 μg/ml of NPOO1Z was found to either partly or fully abolish acetylcholine, histamine, barium chloride, magnesium chloride, and nicotine induced spasms. These results show that NPOO1Z reduces the effect of sympathetic, parasympathetic and autonomic activity because it antagonizes acetylcholine, histamine and nocotine induced spasms. NP001Z is also a direct inhibitor as it antagonizes magnesium chloride and barium chloride induced spasms as well.

The ethanolic extract, NPOO1Z, of **Cassia abbreviata-Oliv.** stem bark was subjected to bio-assay directed fractionation, a widely used current methodology for isolation of bio-active components from natural products. Less complex anti-diarrhoeal fractions were obtained by fractionating NPOO1Z systematically by bulk transfer methodology as illustrated in Scheme 1 on page 37. Anti-diarrhoeal activities of all the fractions were evaluated by the method of Perry et al [67]. This type of fractionation where acids and bases are used besides organic solvents has been employed by many other researchers [70,71,72]. The risk of artifact formation due to hydrolytic reactions, molecular rearrangements, etc. was minimised by using 3% aqueous solutions of acids and bases in place of 5% aqueous solutions used by Mitscher et al and by maintaining low temperatures during neutralization [69].

At a concentration of 100 μg/ml, extracts 2, 4, 6, 7, 8, 10, 11, and 12, obtained by aforesaid fractionation of NPOO1Z, were found to be stimulants and the results were reproducible. Since the aim of the present study was to investigate anti-diarrhoeal

components, these extracts were not investigated any further. However, it may be interesting to undertake a separate study in future to investigate some of these stimulant fractions.

Extracts 9 and 13 were found to have anti-diarrhoeal activity on account of their inhibitory action on the motility of rabbit ileum.

In order to confirm the reproducibility of these results, experiments were repeated several times and it was found that responses of rabbit ileum to extract 9 were less consistent where as extract 13 elicited consistent inhibitory effect on the rabbit ileum in all the experiments. Moreover, the pharmacological responses of smooth muscle of rabbit ileum to the graded doses of extract 13 further confirmed that the extract 13 has definite anti-diarrhoeal activity and that its anti-diarrhoeal potency increases with an increase in its dose. Extract 13 was, therefore, selected for further investigations.

The observed pattern of bio-activities of extract NP001Z and 13 fractions isolated from it is very interesting. It is clear from these results that ethanolic extract, NPOO1Z, prepared from **Cassia abbreviata-Oliv**. stem bark has two sets of bio-active constituents with opposing pharmacological effects on the motility of rabbit ileum in-vitro. It may be interesting to undertake a separate study in future to investigate whether the observed opposing pharmacological effects are due to mutual antagonism or due to some other mechanism.

Irrespective of the mechanism(s) of opposing pharmacological effects, it is probable that stimulant and inhibitory constituents are present in NPOO1Z and in the stem bark of **Cassia abbreviata-Oliv.** in such a balanced proportion that an aqueous decoction prepared from **Cassia abbreviata-Oliv**. /solution of NPOO1Z in propylene glycol, produces an optimum anti-diarrhoeal effect. Alternatively, it may also be possible that NPOO1Z did not contain any stimulant compounds originally, and compounds responsible for the observed stimulant action of fractions 2, 4, 6, 7, 8, 10, 11 and 12 could be artifacts arising from chemical transformation(s) during fractionation process. However, in the light of literature reports, this later possibility is highly unlikely because a number of plant species of the genus **Cassia** are known to produce a number of anthraquinone and anthracene derivatives which are well known for their purgative action [29]. It is, therefore, safe to conclude that the plant **Cassia abbreviata-Oliv**. also produces some secondary metabolites which could have purgative or stimulant action.

To determine the mode of inhibitory action of extract 13, its effects on the smooth muscle of rabbit ileum against chemical agonists 2 μg/ml acetylcholine, 1 μg/ml histamine, 0.4 μg/ml nicotine and $2x10^4$ μg/ml barium chloride induced spasms were evaluated. 100μg/ml of extract 13 was found to antagonize nicotine and barium chloride induced spasms (Figures 21 and 22). Unlike NPOO1Z, extract 13 did not antagonize acetylcholine and histamine induced spasms (Figures 19 and 20). These findings imply that extract 13 is an autonomic ganglion and neuromuscular blocking drug because it inhibited the nicotine induced spasms. In addition, extract 13 is also a direct inhibitor of the motility of the rabbit ileum because it inhibited the spasms

induced by barium chloride. It is also evident from the results that extract 13 does not act through sympathetic and parasympathetic receptors because it did not inhibit acetylcholine and histamine induced spasms. NPOO1Z exhibited a wider spectrum of pharmacological responses than extract 13, which is not surprising because during fractionation, the bio-active principles of NPOO1Z were distributed into various fractions, including extracts 9 and 13. Thus, extract 13 showed a narrower range of pharmacological responses than NPOO1Z.

Although extracts NPOO1Z and 13 were found to give a positive reaction for the presence of tannins, their inhibitory effects on the smooth muscle of rabbit ileum can not be ascribed to tannins because tannins are not known to affect the motility of the rabbit ileum. Moreover, the observed modes of anti-diarrhoeal actions (inhibition of the motility of rabbit-ileum) of extracts NPOO1Z and 13 cannot be explained in terms of tannins which act on the mucous membrane of gastrointestinal tract. Hence, extracts NPOO1Z and 13 are likely to contain some secondary metabolite(s), other than tannins, produced by **Cassia abbreviata-Oliv**. which is/are responsible for their in-vitro anti-diarrhoeal activity. This could be very interesting in view of the available literature on the plant species of the genus **Cassia**. Apart from tannins there is no report on anti-diarrhoeal activity of any other secondary metabolite (compounds) isolated from any of the plant species of the genus **Cassia**.

Extracts NPOO1Z and 13 were also found to give positive tests for alkaloids. Alkaloids are known to exhibit a wide range of bio-activities and a few isoquinoline alkaloids have been reported to be isolated from a couple of plant species of the genus **Cassia** [44] but there are no reports on their anti-diarrhoeal activity.

So far, all the experimental results discussed are on the basis of regarding diarrhoea as a consequence of functional disorder. However, diarrhoea can also be associated with a number of infections caused by bacteria and viruses [19,20]. In order to investigate the therapeutic potential of NPOO1Z and extract 13 against diarrhoea caused by microbial infections, particularly those associated with food poisoning in Zambia, anti-bacterial activities of the two extracts, NPOO1Z and 13, were evaluated in-vitro against a test system of six micro-organisms by Agar Streak Dilution Method [78,79].

Extract NPOO1Z was found to have demonstrable anti-bacterial activity in-vitro against **Staphylococcus aureus** (MIC = 150 μg/ml), **Shigella dysentrie** (MIC = 100 μg/ml), **Proteus spp.**(MIC = 150 μg/ml) **and Bacillus spp.** (MIC = 150 μg/ml). Extract 13 was also found to have significant anti-bacterial activity in-vitro *against* ***Shigella dysentrie*** *(MIC = 75 μg/ml)* and **Proteus spp.** *(MIC = 100 μg/ml).*

These results indicate that extract NPOO1Z has a broad anti-bacterial spectrum where as extract 13 has comparatively narrow anti-bacterial spectrum. It is possible that during the process of fractionation of extract NPOO1Z, its anti-bacterial constituents were distributed into a number of fractions including extract 13 because of their structural features. It must be pointed out that in-vitro inhibition of certain micro-organisms demonstrated by extracts NPOO1Z and 13 does not necessarily imply that these extracts would also be effective anti-bacterial agents in-vivo because of the problems associated with ADME (absorption, distribution, metabolism and excretion) characteristics of anti-bacterial compounds present in extracts NPOO1Z and 13. Although in-vivo experiments were not carried out, the in-vitro MIC values of extracts NPOO1Z and 13 suggest that the two extracts are quite potent anti-bacterial agents which could possibly be potential candidates for future studies.

To isolate the anti-diarrhoeal constituent(s), extract 13 was subjected to chromatographic separation on a silica column, silica gel G-60, Merck type 44784, using the solvent system CHF:Me_2CO:MeOH:EtOAc (7:2.5:1:1) and effects of various fractions on the motility of the rabbit ileum were evaluated by the method of Perry et al [67].

Since a highly polar solvent system was needed for separation, extract 13 must be a mixture of highly polar compounds. The use of reverse phase silica gel could have been better. However, availability of materials and facilities had to be taken into consideration. Moreover, highly polar solvent systems in conjuction with normal silica gel have been used by many researchers as well [65,71,85].

Fractions 131006, 131420 and 13SG were found to be inhibitory because each of these fractions decreased the normal rhythmic pendular movement responses of rabbit ileum. Fraction 131006 was the most potent inhibitor of the motility of the rabbit ileum. Fraction 137013 increased the motility of the rabbit ileum implying that it was a stimulant. These results indicated that extract 13 was a mixture of two sets of components, one that was inhibitory and the other which was stimulant. The presence of a stimulant in extract 13 could be attributed to a spill over during fractionation of extract NPOO1Z. It is also possible that the particular stimulant(s) was/were fractionated into extract 13 on account of their structural features.

Repeated purification of the most potent anti-diarrhoeal fraction, 131006, by gravity column chromatography linked with evaluation of anti-diarrhoeal activities of the fractions obtained yielded a compound LAMM. One and two dimensional TLCs of LAMM in a number of solvent systems showed only a single spot suggesting that it was a pure compound. At a concentration of 0.5 μg/ml, LAMM exhibited significant anti-diarrhoeal activity in-vitro (Figure 27).

The compound, LAMM, could not be analyzed by spectroscopic methods in Zambia due to the non-availability of the necessary instrumentation.

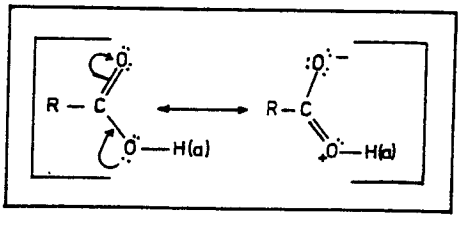
LAMM was analyzed by 400 MHz ^{1}H NMR and H-H cosy-45 experiments at the Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, U.K., with the help of Dr. S. Prakash and Professor R. D. Waigh. The ^{1}H NMR data was found to be inconclusive and could not be interpreted while the peaks on H-H cosy-

45 spectrum did not correlate. These results implied that LAMM was not pure.

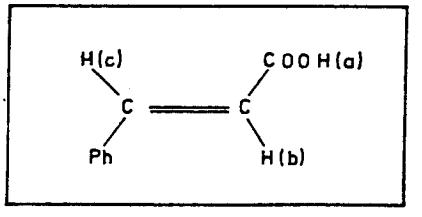
HPLC purification of LAMM was done at the Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, U.K., with the help of Dr. S. Prakash. LAMM was found to be a mixture of 3 different compounds, two of which disintegrated whilst on the column to give an overall total of 5 different compounds. The major fraction, 2, whose weight was 1.2 mg was analyzed by 500 MHz ^{1}H NMR at JOEL laboratories, U.K., with the help of Professor R. D. Waigh, of the University of Strathclyde, U.K.

From the 500 MHz ^{1}H NMR spectrum the compound could either be A, 3-(3′hydroxy -4′-methoxyphenyl)-2-propenoic acid or B,3-(4′-hydroxy-3′-methoxyphenyl)-2-propenoic acid. The multiplet at δ:7 ppm signifies aromatic protons, which do not have resonance at identically the same frequency implying that the aromatic ring is substituted by other groups besides the protons.

The proton appearing off scale at δ:13.21 ppm as a singlet must be a carboxylic proton labelled H(a). Carboxylic protons are least shielded protons because both resonance and the electronegativity effects of oxygen withdraw electrons from the acid protons and hence their resonance appear farther down field.



Two doublet peaks, one centred at δ:7.45 ppm to the left of the phenyl resonance and the other at δ:6.43 ppm to the right of the phenyl resonance, represent two chemically distinct types of protons. The areas under the two peaks were the same and when compared to the areas under other peaks on the same spectrum, the two peaks corresponded to 1 proton each. The coupling constants, J values, for the two doublets were also found to be the same, 17 Hz, implying that the two protons coupled each other. Since each of the two peaks is a doublet, these protons are on adjacent carbon atoms and spin-spin split each other. This implies that each of the adjacent carbon atoms is bearing only one proton and there must be a double bond between the two carbon atoms because of the chemical shift position. The chemical shifts of vinylic protons are larger than those of protons attached to sp^3 carbon atoms due to change in hybridization and deshielding due to the magnetic field generated by the π-electrons of the double bond fall into this region on the spectrum. In addition, the coupling constant, J value, of 17 Hz, is a common value for trans proton-proton coupling across a double bond. The cis isomer would be expected to exhibit a small splitting (J=6-12 Hz). The two protons which can be labelled H_b and H_c must, therefore, be vinyl protons, splitting one another into two doublets. The proton assigned with the larger chemical shift at δ:7.45 ppm must be attached to the carbon bearing the phenyl ring because it is in a deshielding area of the anisotropic field generated by the π-electrons of the aromatic ring. The proton assigned with the smaller chemical shift at δ:6.43 ppm must be attached to the carbon bearing the carboxyl group.



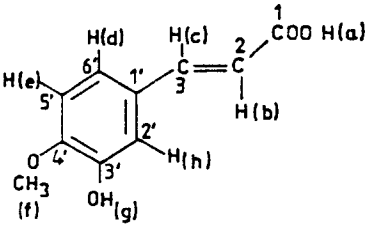
The strong peak at δ:3.9 ppm corresponded to 3 protons. The peak was a singlet implying that all the three protons exhibited the same chemical shift and must, therefore, be in magnetically identical environment. Protons attached to purely sp^3 carbon atoms have resonances in the range of δ:(0-2) ppm, however, protons on an sp^3 carbon which is attached to an electronegative or hetero-atom substituent or unsaturated carbon have greater chemical shifts. The peak at δ:3.9ppm must signify a CH_3 group highly deshielded not just by anisotropic field generated by π-electrons of the aromatic ring but also by an electronegative atom and so the chemical shift at δ:3.9 ppm should be due to a methoxy, OCH_3, substituent on the aromatic ring.

The singlet at δ:3.3 ppm corresponded to one proton. This proton is obviously highly deshielded and this deshielding must be due to not only anisotropic field generated by the π-electrons of the aromatic ring but also due to an electronegative atom, e.g., oxygen or nitrogen, and since the peak at δ: 3.3 ppm is a singlet, oxygen is the most likely electronegative atom because its valency is 2 and that of nitrogen is 3. Moreover, any other group, e.g., CH_3, attached to nitrogen atom does not fit into the data obtained. Thus, the proton at δ:3.3 ppm must be a hydroxy, OH, substituent on the aromatic ring, i.e., phenolic proton.

In order to come up with a clear picture about the pattern of substitution in the aromatic ring, the multiplet peak at δ:7.0 ppm was magnified and 3 chemically distinct types of aromatic protons which could be labelled h, d and e appeared at chemical shifts, δ, values of 7.12 ppm, 7.05 ppm and 6.8 ppm, respectively, implying that there were 3 aromatic protons in different chemical environments. The

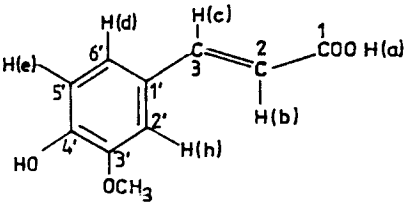
magnitude of coupling constant for protons d and e, Jde, was found to be 8 Hz implying that the protons d and e must be ortho to each other because a typical J value for aromatic protons at ortho positions is in the range of 6-10 Hz. For protons d and h, the coupling constant, Jdh, was found to be 3 Hz implying that the protons d and h must have been meta to each other because a typical J value for aromatic protons at meta positions is in the range of 1-4 Hz. For protons e and h, the coupling constant, Jeh, was 1.5 Hz implying that they must be para to each other because a typical J value for aromatic protons at para positions is in the range of 0-2 Hz.

Tentatively, the chemical structure of the compound under investigation should, therefore, be either A, 3-(3′-hydroxy-4′-methoxy phenyl)-2-propenoic acid (3-hydroxy-4-methoxy cinnamic acid), or B, 3- (4′-hydoxy-3′-methoxy-phenyl)-2-propenoic acid (4-hydroxy-3-methoxy cinnamic acid).



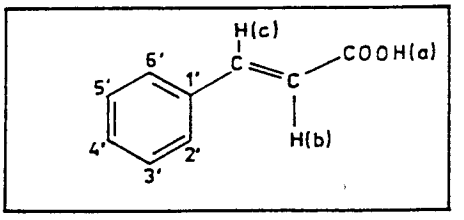
A

3-(3'-hydroxy-4'-methoxyphenyl)-2-propenoic acid.



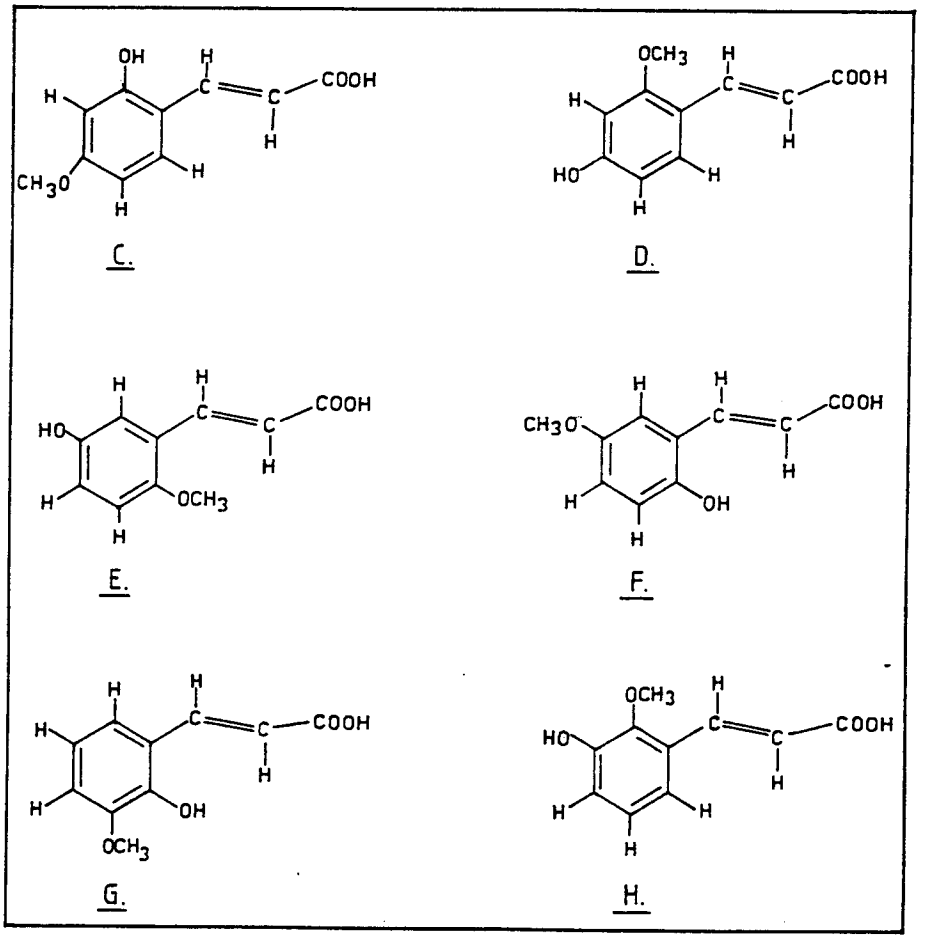
B

3-(4'-hydroxy-3'-methoxyphenyl)-2-propenoic acid.



Peaks corresponding to H_b and H_c were distinctly doublets but these peaks could not be doublets if carbon atoms $2'$ and $6'$ were substituted by either OCH_3 or by OH group due to the long range coupling between H_b/H_c and protons on these groups.

This implies that structures C, D, E, F, G, and H are not likely to be the possible structures of the compound.



The proposed structures A and B are purely tentative. This compound could not be pursued further due to the extremely small quantity of the sample at hand and the time constraint.

It may be interesting to point out that a number of cinnamic acid derivatives have been reported to exhibit varied inhibitory effects including the inhibition of mitochondrial transport across plasma and mitochondrial membrane, the growth of intraerythrocytic plasmodium falciparum in-vivo, the translocation of carbohydrates and amino acids across new permeability pathways induced in the host cell membrane by the parasite and the ATP production in the parasite and its utilization by the host cell in correlation with their hydrophobic character [83]. 3-hydroxy-4-methoxy cinnamic acid and 4-hydroxy-3-methoxy cinnamic acid have been reported to be responsible for anti-tumor activities of **Ixora javanica** extracts [84]. However, anti-diarrhoeal effects of methoxy cinamic acids have not been reported.

CHAPTER FOUR

CONCLUSION

CONCLUSION

The stem bark of **Cassia abbreviata-Oliv.** has potent anti-diarrhoeal and anti-bacterial activities in-vitro.

An ethanolic extract, NP001Z, prepared from **Cassia abbreviata-Oliv.** stem bark was found to exert anti-diarrhoeal action through sympathetic, parasympathetic and autonomic ganglia receptors implying that it is an anti-cholinergic and a neuromuscular blocking drug. It was found to be a direct inhibitor of the motility of rabbit ileum as well. These findings clearly indicate that the anti-diarrhoeal action of the stem bark extract under investigation is not due to tannins but due to some other secondary metabolite(s).

The findings of in-vitro anti-bacterial studies on NP001Z strongly suggest that the stem bark extract could be potentially useful in treating diarrhoea associated with **Shigella dysentrie, Proteus spp and Bacillus spp.** and it could also be effective against **Staphylococcus aureus** infection as well.

The stem bark extract, NP001Z, was found to contain two kinds of bio-active principles, one stimulant and the other inhibitory, with an overall inhibitory effect on the motility of the rabbit ileum.

The most potent anti-diarrhoeal fraction, extract 13, exerts its anti-diarrhoeal action through autonomic ganglia and neuromuscular blockage. It was also found to be a direct inhibitor of the motility of the rabbit ileum. Moreover, this fraction could be

potentially useful in controlling diarrhoea associated with **Shigella dysentrie** and **Proteus spp.**

The structure of a compound isolated from HPLC purification of a potent anti-diarrhoeal compound LAMM is tentatively proposed to be either 4-hydroxy-3-methoxycinnamic acid (Ferulic acid) or 3-hydroxy-4-methoxycinnamic acid.

This study has added to our scientific knowledge of some of the pharmacological effects of the plant **Cassia abbreviata-Oliv**. widely used by herbalists as a remedy in Zambia for treatment of diarrhoea. It has also contributed to our understanding of some of its secondary metabolites. The findings strongly support the folkloric usage of the stem bark of **Cassia abbreviata-Oliv**. in Zambia for treating diarrhoea.

These findings strongly suggest that further phytochemical, pharmacological and toxicological studies on this plant should be undertaken as there is a high probability of finding a novel structural lead which could be developed into a potent but less toxic anti-diarrhoeal drug in future.

In the author's opinion, investigation of this nature linked with toxicological studies could provide a source of less expensive but safe alternative source of medicine which may be important particularly to many third world countries where due to poor economic situation and perpetual shortages of drugs, a large proportion of the populace does not have an easy access to modern drugs.

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