CHEMISTRY AND BIOACTIVITY

OF STEM BARK OF

Artocarpus nobilis

A RESEARCH PROJECT REPORT PRESENTED

BY

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TO THE

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In partial fulfillment of the requirement for the award of the degree of

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Natural Products Laboratory
Institute of Fundamental Studies
Hantana Road
Kandy
Sri Lanka

DECLARATION

I do hereby declare that the work reported in this research project report was exclusively carried out by me under supervision of Dr. U.L.B. Jayasinghe and Dr. D.B.M. Wickramaratne. The research project, which carried out at the Institute of Fundamental Studies (IFS)-Kandy, as a partial fulfillment of the award of the degree of B.Sc.(Applied Sciences) of the Sabaragamuwa University of Sri Lanka.

.....

Signature of the Candidate

Date: 28.04.2003

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TO MY

SISTERS, BROTHERS AND TEACHERS

ACKNOWLEDGEMENT

I wish to express my deepest appreciation and gratitude to my supervisor Dr. U.L.B.Jayasinghe, Senior Research Fellow, Institute of Fundamental Studies, Kandy for his constant supervision, invaluable guidance and unfailing encouragement given to me during my final year project period.

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Special thanks are also due to all the person who helps me willingly in various ways during this period.

ABSTRACT

The dried, ground stem bark of *Artocarpus nobilis* was extracted with cold methanol. The extract was evaporated to dryness under reduced pressure using rotary evaporator. The methanol extract was partitioned with n-butanol and water. The n-butanol crude extract was subjected to antifungal activity test against *Cladosporium cladosporioides* by TLC bio-autography method The n-butanol extract was shown positive response; at the meantime the above n-butanol extract was indicating that absence of alkaloids. In order to isolate two antifungal active compounds and an inactive compound, the n-butanol extract was subjected to combination of chromatography such as normal silica gel, reversed phase C_{18} silica, Sephadex LH-20, and HPLC. The final purification of two antifungal active compounds and an inactive compound was achieved by high performance liquid chromatography (HPLC). Their NMR, IR, Mass, UV, Physical constants, and structure determination studies are also in progress.

CONTENTS

LIST OF FIGURES

LIST OF TABLE

LIST OF ABBREVIATIONS

EtOAc	-	ethyl acetate
CH_2Cl_2	-	dichloromethane
$CHCl_3$	-	chloroform
Me	-	methyl
TLC	-	thin layer chromatography
R_f	-	retardation factor
S_f	-	solvent front
UV	-	ultra violet
NMR	-	nuclear magnetic resonance
HPLC	-	high performance liquid chromatography
Mp	-	melting point
IR	-	Infra Red
MW	-	molecular weight
sp.	-	species
spp.	-	various species in the genus

1.0. INTRODUCTION

1.1. Importance of Medicinal Plants

Many of the natural products in plants of medicinal value offer us new sources of drugs, which have been used effectively for centuries in traditional medicine. There are many compounds used in medicine today whose original derivatives were of plant origin.[1]

Plants are sources of poisons, addictives drugs, and hallucinogens. These have importance in human medicine and in human social action and behavior. Plants provide us with thousands of novel compounds, which give us fragrances, flavorings, dyes, fibers, foods, beverages, building materials, heavy metal chelators (important in bioremediation), biocides and plant growth regulators.[1]

Sri Lanka is a tropical island situated in southern part of India subcontinent. Soil type and altitude have provided the basis for high plant population and habitat diversity. Flowering plants have high endemism 24% about 550 flowering plants and 7 pteridophytes have recognized as Medicinal Plants. [2]

Herbal medicine is heavily used in dry and wet zone and up to the mid country. Use of herbal medicine in high altitude area is somewhat less prevalent and number of used in herbal medicine is smaller in higher altitude areas. [2]

Branch of medical science, which deals with the drug plant most of drugs obtained from wild plants growing all parts of world. Medicinal value of drug plants is due to presence in the plant tissues of some chemical substances that produce a definite physiological action on human body. The most important of these substances are glycosides and alkaloids. Some of these chemicals are powerful poisons and therefore drugs should be prepared and prescribed only by expert physicians. Glycosides derived from carbohydrates and not from proteins. [3]

1.2. Introduction of the Plant *Artocarpus nobilis* [4]

Classification of the plant

Family	-	Moraceae
Genus	-	*Artocarpus*
Species	-	*nobilis*
Scientific name	-	*Artocarpus nobilis*
Local names	-	Arsini-pla (T); Del, Bedi-del (S)

1.2.1. Nutritional and Therapeutic value of *Artocarpus nobilis* [20]

Latex is used to treat warms. Seeds are good for Asthma patients. Seed oil is used in traditional medicine.

Other uses: Timber is hard which can be used in many ways.

Food uses: Tender portion and seeds are boiled and eaten. Since the seed contain a higher percentage of oil, they are roasted and eaten as a snack.

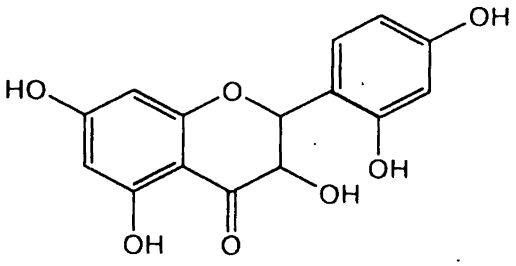
Family of Moraceae [5, 6]

The family Moraceae (mulberry family) consist of about 73 genera and over 1000 species, mostly of pan tropical. It is divided in to four sub families or tribes: Moroideae, Artocarpoideae, Concepha loideae and Cannaboideae. Some authors(Rendle,Hutchinson) the cannaboideae is treated as a separate family, Cannabiaceae. The family is characterized by the presence of milky latex and/or usually 2 stigmas and the usually pendulous single ovule. Economically, the family is important for the many edible fruits produced, Figs(*Ficus*), Mulberries(*Morus*), Breadfruit and Jack fruit (*Artocarpus*). The fibers of hemp(*Cannabis*) are used for cordage and the drug Marijuna is obtained from the staminate flowers of the same plant. The fruits of hops(*humulus*) are used in flavoring beer.

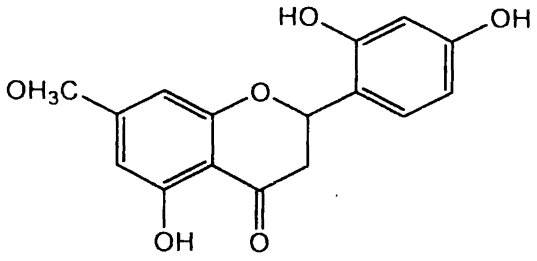
Thirty-four species of Moraceae belonging to 11 genera are represented in Sri Lanka. But only five species are endemic, namely:

1. *Allaeanthus zeylanicus* Thw.
2. *Artocarpus nobilis* Thw.
3. *Ficus caudiculata* Trim.
4. *Ficus mooniana* King.
5. *Ficus diversiformis* Miq.

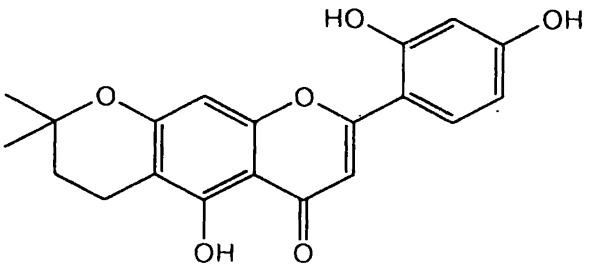
Large number of phenolic compounds had been reported from Moraceae.[6]



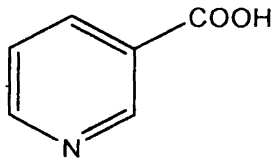
1. Morin



2. Artocapetin



3. Cycloartocarpesin



4. Nicotinic acid

Alkaloids and alkaloid-like substances had been reported and were found in a few of the species tested, including *Cannabis sative, Ficus carica, F. hispida, F. septica, Maclura pomifera(1/4) and Morus alba(1/8).*

Plant *Artocarpus nobilis*

Evergreen tree 25m high with heavy crown of large, stiff leaves; bark grey with orange scale-scars, fissured by lenticels. Twigs 10-12mm thick, hispid-scabrid. Stipules 3-12cm long, hairy. Lamina 14-32*8-23cm, usually obovate, shortly acuminate, base cuneate, margin strongly sinuate and inlrolled, smooth; lateral veins 10-13 pairs; petiole 2-3cm long; sapling leaves pinnately lobed. Inflorescences axillary, solitary or paired. Male head 7-13*1.5 cm, cylindric, covered by flowers and bracts; peduncle 3-7 cm long; perianth bilobed; stamen 1.8 mm long. Female head stouter, covered by peltate bracts; stigma simple, exserted to 1mm. Syncarp up to 20*10 cm, cylindric, covered by short; close, rigid, sybcylindric process 1*1-1.5 mm, with persistent peltate bracts; peduncle 10-15 cm long; fruiting perianthus not fleshy. Seeds 8*7 mm. [4]

Distribution: Sri Lanka; frequent in wet zone forest up to 700 m attitude and villages; flowering in June.

1.3 Medicinal uses of some plants from family Moraceae [7]

(1) *Antiaris toxicaria* (pers.)

Local names: Ali, Arandali(T); Riti(S)

In small quantities, the juice of this tree is a mild cardiac and circulatory stimulant and in large doses it act as a myocardial poison. It stimulates intestinal and uterine contractions.

(2) *Artocarpus heterophylls* Lam.Encyl.

Local names: Pala,Tagar(T); Herali, Waraka (S);

The tender leaves minced fine and roasted with scraped coconut is a specific for insomnia, while the juice of the tender fruit with coconut milk and jaggery is a miraculous antidote for narcotic poisoning. The leaves are used in skin diseases and the root for diarrhoea and fever. The roots are also used for skin diseases and as an antiasthmatic.

(3) ***Ficus benghalensis*** Linn. Sp. Pl.

Local names: Ala, Kadavam(T); Nuga, Mahanuga(S).

An infusion of the bark is considered a specific diabetes. The milky juice is applied externally on pains, bruises, rheumatism, lumbago, and on cracked and inflamed soles of the feet.

(4) ***Ficus hispita*** Linn.f.,Suppl.

Local names: Ottamalam, Peytti(T); Kotadimbula(S).

All parts are astringent to the bowels, antidysenteric and useful for ulcers, biliousness, psoriasis, nose and mouth and diseases of the blood. The fruit is an aphrodisiac, tonic, lactagogue and emetic.

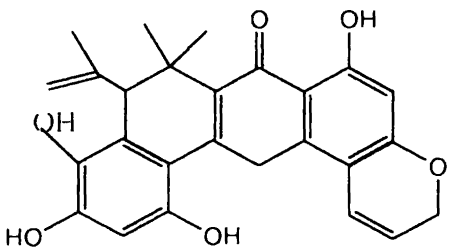
(5) ***Ficus racemosa*** Linn. Sp.Pl.

Local names: Atti, Asuvattam(T); Attikka(S).

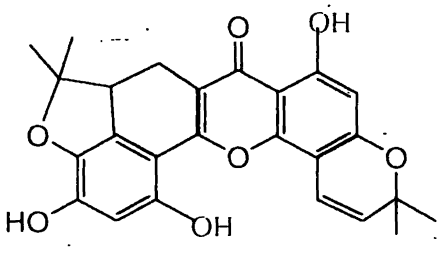
An infusion of the bark or the expressed juice of the unripe fruits is given for menorrhagia, hacmoptysis and urinary diseases. The milky juice is a administered for piles and diarrhoea. The juice of the root is a tonic and given for gonorrhoea.

Compounds previously reported from *Artocarpus nobilis* [6]

Two chromenodihydrobenzoxanthones have been reported from the bark of *A. nobilis*



5. Xanthone(high polar)



6. Xanthone(less polar)

1.3 Primary Metabolites

The synthesis of primary metabolites is always growth-linked ie linked to biomass production. The biosynthesis of all primary metabolites branch off from either the glycolytic pathway of the Citric (TCA) cycle. One broadly differentiates three main product classes: Organic acids such as citric acid, lactic acid, gluconic acid etc. Aminoacids such as L-glutamic acid, L-lysine, L-aspartate, L-tryptophan, D-arylglycin. Organic solvents such as butanol, isopropanol, butylene-glycol. [8]

The primary metabolites, in contrast, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident. [9]

1.5. Secondary Metabolites

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances traditionally referred to as secondary metabolites. [9]

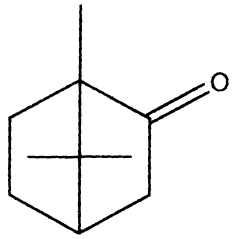
e.g.: Alkaloids, Terpenes, Steroids, Glycosides, etc.

1.5.1. Terpenes (Terpenoids)

The divers, widespread, and exceedingly numerous family of natural products constructed from five carbon building units and so comprising compounds with C_5, C_{10}, C_{15}, C_{20},.... C_{40} skeletons are synonymously termed terpenoids, terpenes, or isoprenoids; with the important sub group of steroids. These compounds are typically found in all parts (i.e., seed, flowers, roots, wood, foliage) of higher plants and also occur in mosses, liver worts, algae, and lichens, although some are of insect or microbial origin. Members of the class, as compounds of oils or in extracts, have been used since antiquity as ingredients of flavours, preservatives, perfumes, medicines, narcotics, soap,

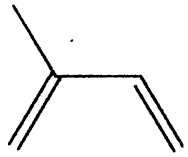
and pigments. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes or ketones are also found. Their building block is the hydrocarbon isoprene, $CH_2=C(CH_3)-CH=CH_2$. Terpene hydrocarbons therefore have molecular formulas $(C_5H_8)_n$,they are classified according to the number of isoprene.[10]

Camphor (**7**), easily obtainable virtually pure as a crystalline and very aromatic solid,



7. Camphor

Consequently, the lower terpenoids (C_{10} and C_{15} compounds) especially have been the subject of study since the dawn of modern chemistry. Many mono and a few sesqui- and diterpenoids (C_{10}, C15, C_{20} compounds respectively) were isolated and studied in the last century and by 1887 Wallach could propose on 'isoprene rule' that the monoterpenoids were hypothetically constructed by the linkage of isoprene [fig.5.2: 2-methyl buta-1,3-diene] units. [10]



8. Isoprene unit

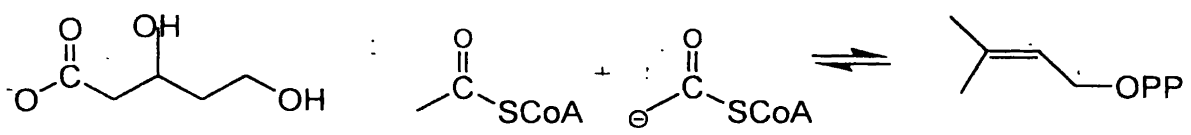
The carotenoids (C_{40} compounds; strictly tetraterpenoids, but historically named as the former), which occur as plant pigments, were also studied in the last century, although again their kinship with the lower terpenoids was not appreciated until much later. [10]

Table 1. Class of terpenoids [1]

C_n	Name	Parents	Occurrence in plants
C_5	Hemiterpenoid	IPP, DMAP	emissions, oils
C_{10}	Monoterpenoid	GPP	oils, petals
C_{15}	Sesquiterterpenoi	FPP	oils, resin, petals
C_{20}	Diterpenoid	GGPP	oils, resin, heartwood (rare)
C_{25}	Sesterterpenoid	GFPP	oils, resin, heartwood (rare)
C_{30}	Triterpenoid	Squalene	resin, heartwood, leafwax
C_{40}	Carotenoid	Phytoene	all green tissue, roots, petals
$C_{n\ (n=45\ to\ 10^5)}$	Polyisoprenoid	GGPP	latex, leafwax

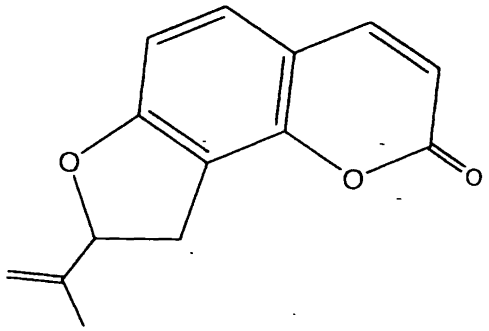
Hemiterpenoids- C_5 componds

The parent of the terpenoids is the C_6 compound mevalonic acid, which was isolated as a metabolite of a *Lactobacterium* species and was found to be a potent growth factor for yeast. Isoprene itself, although a known product of pyrolysis of monoterpenes, was never seriously considered as no remotely likely biochemical routes utilizing this compound could be suggested. [1]



9. Mechanism of mavalonic acid

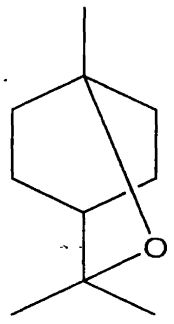
Not surprisingly, few true hemiterpenoids are known. Isoprene is emitted from leaves of many plant species and may play a role as a plant hormone similer to ethylene. Isopentenol and 3,3-dimethylallyl alcohols occur in some oils either free or as esters and may be more widespread in view of their ready loss during work-up due to volatility. The C5 unit is more concealedin Oroselone. [1]



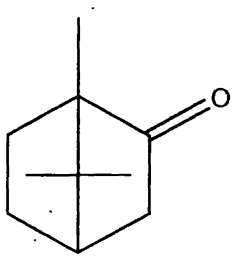
10. Oroselone

Monoterpenoids- C_{10} compounds

Often both enantiomers of a monoterpenoid occurs naturally and sometimes they differ in odour and taste: thus (+) and (-) carvone are characteristic of caraway and spearmint. During the last decade it has become apparent that monoterpenoids play an important role in chemical ecology. Some monoterpenoids like; 1,8.-cineol and Camphor are emitted by assorted plants like eucalyptus and sagebrush and inhibit germination and development of seeds of competing species. [3]

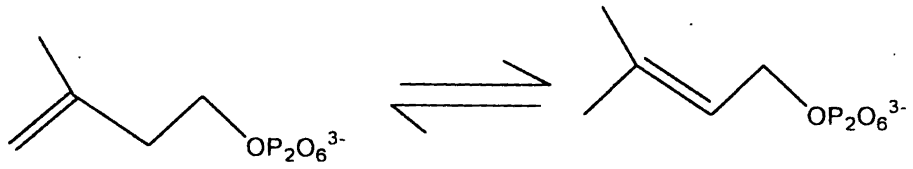


11 1.8-Cineol



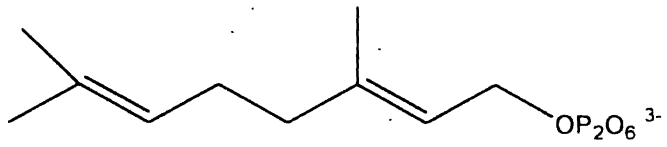
12. camphor

3IPP and 2IPP. 3IPP is isomerized by an enzyme to 2IPP this isomarization is an equilibrium and because of the cell has available both



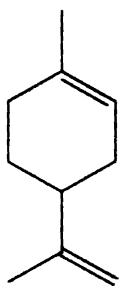
13. 3IPP

These two, five carbon units condensed with each other in another enzymetic reaction to C_{10} compound called Geranyl PP.

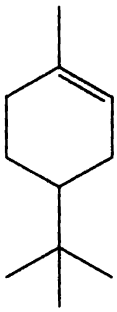


14. geranyll PP

Some important monoterpenoids are shown below.

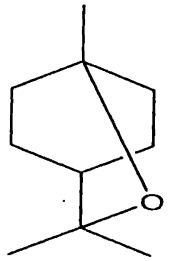


15. cimonene



16. α-terpinoel

are compound of citrus oils whereas 1,8-cineol occurs in wormwood and eucalyptus.

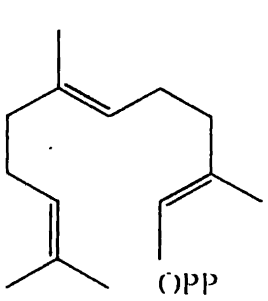


17. cineol

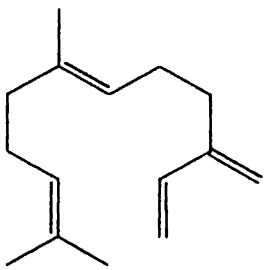
Sesquiterpenoids- C_{15} compounds

Addition of IPP to GPP as in above figure yields 2*E*, 6*E*-farnesyl pyrophoaphate(FPP), the parent of the sesquiterpenoids. Only a few sesquiterpenoids were characterized by 1920 but application of the Uesterberg dehydrogenation method led to a gradual acceleration of information and now over 200 skeletal types are known the most for any class of terpenoids. Sesquitepenoids are widespread but usually minor (5% w/w) compounds of plant oils but often endow crucial flavour characteristics (e.g. in oils of ginger, cloves. citronella and hops). Tracer studies have indicated that they are synthesized at intracellular sites denied to monoterpenoids. [1]

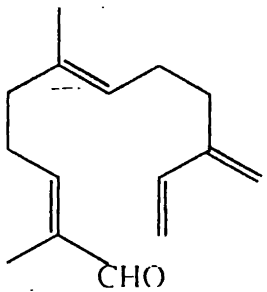
Farnesol(derived from **18**)is fairly rare in higher plants, and other acyclics of the class include trans-β-farnesene, a potent aphid-repellant present in hops and sweet potatoes, and β-sinesal a flavour component of orange. [1]



18. Derivative of farnesol



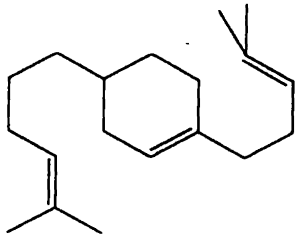
19. trans-β-farnesene



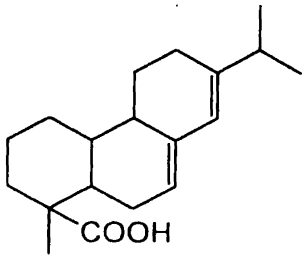
20. β-sinesal

Diterpenoids- C_{20} compounds

The diterpenoids are widely varied group of compounds based on four isoprene groups, most of which are of limited distribution in the plant kingdom. Because of their higher boiling points they are not considered to be essential oils. Instead, they are classically considered to be resins, the material that remains after steam distillation of a plant extract. [1]



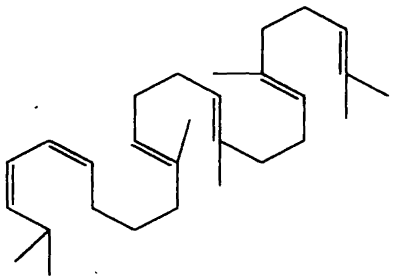
21. α-camphoraene



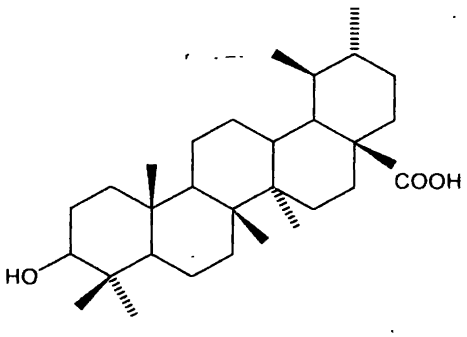
22. apietic acid

Triterpenoids- C_{30} compounds

The C_{30} compounds are based on six isoprene units and are biosynthetically derived from squalene. They are often high-melting colourless solids and are widely distributed amoung plant resin, cork, and cutin. There are several important groups of triterpenoids including common triterpenoids, steroids, saponins, and cardiac glycosides. Among these is 'azadirachtin', a powerful insect antifeidant, first isolated in 1985 from Neem oil. [1]



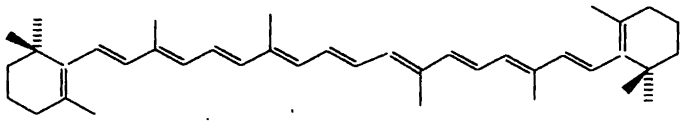
23. Squalene



24. Ursolic acid

Tetraterpenoids- C_{40} compounds

The most common tetraterpenoids are the 'carotenoids',a widely distributed group of C_{40} compounds. Whereas the structures of the di- and triterpenes can have a wide variety of fascinating structures, the carotenoids are generally derived from lycopene. Crystallization at one end gives gamma-carotene and at both ends provides β-carotene. These pigment; first isolated in 1831, is by far the most common of all of these pigments and virtually universal in the leaves of higher plants. [1]



25. β-Carotene

1.5.2. Steroids

Steroids are compounds possessing the skeleton of cyclopenta [α] phenanthrene or a skeleton derived there from by one or more bond scissions or ring expansions or contractions. Methyl groups are normally present at C_{10} and C_{13}. An alkyl side chain may also be present at C_{17}. Sterols are steroids carrying a hydroxyl group at C_3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain. [11]

1.5.3. Alkaloids

Alkaloids are a heterogeneous group of compounds containing Nitrogen as the heteroatom. Usually alkaloids are classified according to the nature of the nucleus present in the molecule. In many cases different alkaloids obtained from the same plant often have similar chemical structure and so some times the source of the alkaloids may indicate chemical similarity. Many alkaloids are used in modern medicine and the exploitation of alkaloidal plants in economically interesting and important. [1]

1.5.4. Xanthones

As a general rule, xanthones are formed by cyclization of benzophenones resulting from the addition of two carbon units (in fact of malonyl-CoA) onto a precursor in C_6-C_1, i.e., a benzoic acid arising from the shortening of a cinnamic acid.(see **5** and **6** for example). [12]

1.5.5. Saponins

Saponins are glycosides of triterpenoids, steroids, or steroidal alkaloids. As well as saponins are natural surfactants, widely distributed throughout the higher plants and animals. Mostly abundant in desert plants and marine animals (eg.Phylum Echinodermata). Due to the presence of both hydrophobic part(Sapogenin) and hydrophilic part(Sugar), saponins have soap like nature. [1]

Triterpenoid Spogenin

Varity of biological properties have been attributed to saponin including cytotoxicity, anti-cancer, immuno-modulating, anti-viral, antihepatotoxic and actions on cardiovascular system(reduce blood cholesterol). Many saponins show hemolytic activity, although this property can by no means be used as a test for saponins. The compounds other than saponins such as ecdysterone are hemolytic. Saponins containing plant material has been used as fish bait. Fish are dazed by the saponins but are still edible.[1]

1.6. Separation Methods

1.6.1. Chromatography

Introduction

The origins of the Chromatography are closely linked with the study of Natural Products. There is a constant need to separate both large and small quantities of mixtures efficiently, rapidly and inexpensively. [13]

Chromatographic processes can be classified according to the type of equilibration process involved, which is governed by the type of stationary phase. Various bases of equilibration are: (1) adsorption, (2) partition, (3) ion exchange, and (4) pore penetration (size exclusion). [14]

In the area of HPLC alone, applications are to be found in the pharmaceutical industry, biotechnological, biomedical and biochemical research, energy, food, cosmetics, environmental fields; drugs and vitamins with the increasing interest in the discovery of new lead compounds from micro organisms and both marine and terrestrial higher organisms; [13]

Adsorption (liquid-solid) Chromatography

The stationary phase is a solid on which the sample components are adsorbed. The mobile phase may be a liquid or gas. TLC is a special example of sorption chromatography in which the stationary phase is a plane, in the form of a solid support on an inert plate. [14]

Solvents	Adsorbents
water	alumina* , charcoal
acetonitrile	silica gel*
methanol	inorganic carbonates
ethanol	sucrose , starch
acetone	
ethyle acetate	
chloroform	
dichloromethane	
n-hexane	

Table 2. Decreasing adsorbent and solvent activity

(* activity can be diminished by treatment with water)

Partition Chromatography

The stationary phase on partition chromatography is a liquid supported on an inert solid. The mobile phase may be liquid or gas. [14]

Ion Exchange Chromatography

Ion exchange chromatography uses an ion exchange resin as the stationary phase. The mechanism of separation is based on ion exchange equilibria. [14]

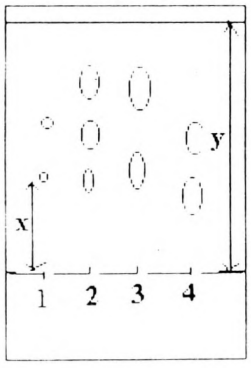
Size Exclusion Chromatography

In size exclusion chromatography, solvated molecules are separated according to their size by their ability to penetrate a sieve like structure. [14]

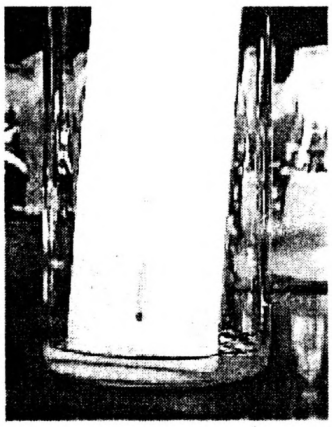
1.6.2. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a chromatographic technique that is useful for separating organic compounds. Because of the simplicity and rapidity of TLC, it is often used to monitor the progress of organic reactions and to check the purity of products. [15]

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown. The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light or by placing the plate in iodine vapor. The different components in the mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. [15]



$R_f = x/y$



26. A developed TLC showing the R_f value and Running the TLC plate in solvent

1.6.3. Preparative Thin-Layer Chromatography (PTLC)

Preparative scale TLC is a technique for the separation and recovery of milligram quantities of materials. In conventional preparative TLC, separation conditions developed on analytical plates (250 μm layer) are used with thicker adsorbent layer preparative plates (up to 2000 μm) for increased sample capacity.

A common problem with conventional preparative TLC procedures is loss of resolution compared to analytical TLC. Even with identical samples, resolution on a thicker adsorbent layer is often worse than that on a thin layer. [15]

Adsorbents

Various studies have been performed to investigate the effect of the 'Thickness of adsorbent' on the quality of separation but the most frequently employed thickness are 0.5-2.0 mm. The format of chromatography plate is generally 20*20cm or 20*40cm. Limitation to the thickness of the layer and on the size of the plates naturally reduces the amount of material that can be separated by PTLC. The maximum sample load for a silica layer 1.0mm thick is about 5mg/cm^2. Silica is the most common adsorbent and is employed for the separation of both lipophilic and hydrophilic substance mixtures. PTLC plates can either be self-made or purchased with the adsorbent already applied. The advantage of separating plates oneself is that any thickness(up to 5mm) or composition of plates can be accommodated. Thus, Silver nitrate, buffers etc. can be incorporated in the adsorbent. Application of the liquid sorbent can be performed with one of a number of commercially available spreaders. [13]

Sample Application

This is one of the most critical aspects of PTLC. Plates should be preferably being prewashed to minimize impurities that may be recovered when the compound of interest is removed from the sorbent. The sample is dissolved in a

small quantity of solvent before application of the plate. A volatile solvent hexane, CH_2Cl_2, EtOAc) is preferred since problems of band broadening occurs with less volatile solvents. The concentration of the sample should be about 5-10%. The sample is applied as a band, which must be as narrow as possible because the resolution depends on the width of the band. Application can be hand (pipette) but an automatic applicator is preferred. [13]

Choice of mobile phase and development of PTLC plate

The following binary mobile phases (in varying proportions) are very often applied to PTLC separations; n-hexane-ethyl acetate, n-hexane-acetone, and $CHCl_3$ -MeOH additions of acetic acid or diethyl amine in small amounts is useful for the separation of acidic and basic compounds, responding. [13]

Elution of PTLC plates generally takes place in glass tanks, which may hold several plates at one time. The tank is kept saturated with mobile phase by the presence of a sheet of filter paper dipping into the solvent. 'Multiple Development' can increase the separation efficiency. When a PTLC separation has been completed; the plate is dried and then re-introduced into the development tank. Depending the R_f of the band, this process can be repeated several times, albeit with a corresponding time penalty. [13]

There are many variables in PTLC but as a general guideline, 10-100mg of sample can be separated on a 1mm thick 20*20cm silica gel or Auminium oxide layer. Doubling the thickness allows the application of 50% more sample. [13]

Isolation of Sample

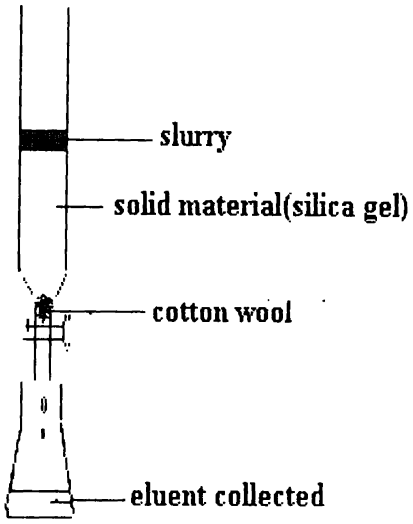
Most PTLC adsorbents contain a fluorescent indicator, which aids localization of the separated bands, as long as the separated compounds absorb UV light. A problem with some indicators; however, is that they may react with acids-occasionally even with acetic acid. [13]

For non-UV absorbing compounds, there are several alternatives;

- Spraying the plate with water (e.g., Saponins)
- Covering the plates with a sheet of glass and spraying one edge with a spay reagent
- Addition of a reference substance.

The bands, having been localized; are scraped off the plate with a spatula or with a tubular scraper connected to a vacuum collector. This later methods is not very practicable for sensitive substances because the adsorbent containing the purified product is in a risk of autoxidation. Whatever the collection method; the substance has to be extracted from the adsorbent with the least polar solvent possible (eg. 10%MeOH-$CHCl_3$). [13]

1.6.4. Column Chromatography



27. A typical chromatographic column

Column packing and Sample introducing

A column prepared by carefully packing the solid material in a column, usually by adding it to the column filled with solvent or by pouring silica gel of it into the column and allowing this to settle. The column can be mechanically vibrated or

the solid material tamped with a long plunger during packing. Care must be taken to keep out air bubbles or channeling will result in the column, rendering is less effective. (see **27**.) [13,14]

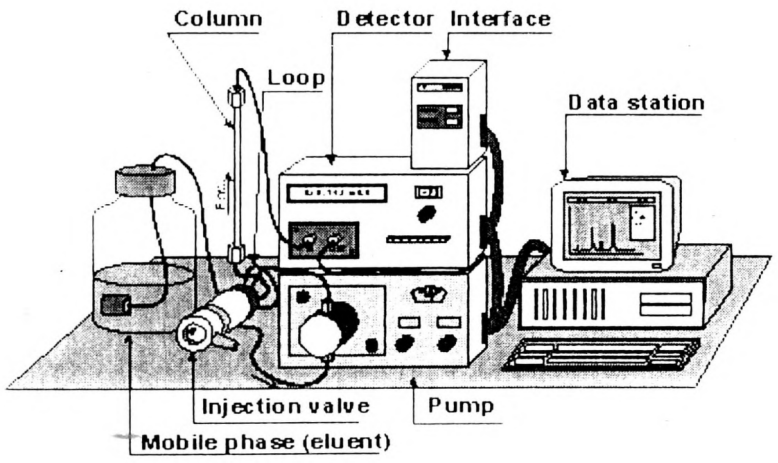
The sample is placed in a small volume on top of the column. A solvent should be used in which the sample is readily soluble, so that the volume can be kept small. The solvent flow must be slow enough to allow equilibrium between the two phases and prevent the 'tailing' of the chromatographic peaks or bands. [14]

Sample Preparation (Slurry)

The concentrated extract is evaporated with suitable amount of gravity silica gel. Then the liquid free (MeOH, H_2O, etc.) slurry may be obtained.

1.6.5. High Performance Liquid Chromatography (HPLC)

HPLC is unquestionably the most widely used of all of the analytical separation techniques. The reasons for the uses of HPLC are its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separating non-volatile species. [14]



28. HPLC system

Stationary Phases (Adsorbents)

HPLC separations are based on the surface interactions, and depend on the types of the adsorption sites. [16]

1.6.6. Reversed-Phase HPLC

The chromatographic technique, which utilizes non-polar adsorbent surface and a polar eluent historically, has been named "Reversed-Phase HPLC". RP-HPLC quickly grown in its number of applications, and now it is the most widely used HPLC mode. This success could be explained by its ability to separate a large variety of organic compounds and related components especially. [15]

1.7. Antifungal Activity

Fungi cause wide range of diseases both plant and animals, treatment with immune suppressive drugs and the spread of AIDS have meant that disease caused by weakness in immunity system, of human are becoming more and more prevalent. Associated with these problems is an increase predisposition to fungal attack. As there are few really effective antifungal preparations currently available for the treatment of systematic mycosis and the efficiency of existing drugs is rather limited. It is important to find new source of antifungal agents. [18]

2.0. OBJECTIVES

- Study the chromatographic techniques such as TLC, PTLC, Sephadex LH-20, Reversed phase C_{18} , and HPLC to separate secondary metabolites from plant extract .
- Determination of anti-fungal activity of crude extracts and pure compounds.
- Study the Spectroscopic techniques such as NMR, UV, IR, and Mass to determine the structures of natural products.

3.0. MATERIALS AND METHODS

Stem bark of ***Artocarpus nobilis*** was collected from Pilimatalawa, Central province of Sri Lanka in September 2002. A voucher specimen is deposited at the Institute of Fundamental Studies, Kandy.

Extraction, Isolation and Antifungal Bioassay

Dried, ground stem bark of *Artocarpus nobilis* (400g) was extracted with cold MeOH in 12 times within 3 hours interval using laboratory shaker. The MeOH extract was evaporated to dryness to give 'MeOH extract' (21.74g). Then the MeOH extract was partitioned with n-butanol and water. Evaporation of n-butanol extract gave dark brown solid (15.69g). Slurry was made with 15g of butanol extract and 30g of gravity silica gel (0.063-0.200mm size). Column was packed with silica gel and hexane as column bed, and was eluted with hexane, EtOAc, MeOH; and gave 6 fractions. (where the fractions were combined with their TLC pattern). All these fractions were subjected to TLC bioassay and observed that the fractions which are labeled as ANSM and ANSN were highly active against *C. cladosporioides*. (see Fig. 30) In order to isolate antifungal constitute in pure form, attempted to separate combined fractions by gravity silica gel column, Sephadex LH-20 column none of these techniques was failed to isolate any pure compound due to their close polarity. Finally, the pure compounds were isolated from reversed phase HPLC (solvent system was 40% water with MeOH, λ_{max}=254nm).

The n-butanol extract did not show a positive result for 'Dragendorff reagent'.

Bioassay for Antifungal activity[17]

TLC bioassay

For antifungal TLC bioassay we used typical fungus named as *Cladosporium cladosporoids*. This was inoculated to Potato Dextrose Agar (PDA) media from isolated mycelia of C.cladosporoides and maintained at 25^0 C. At leased ten days needed to obtain flat dirty green-colored mycelia.

Preparation of PDA media

Peeled off Potatoes (50g) were cut into small pieces and boiled with distilled water (200ml), until piece of potatoes were soft. Water extract was decanted into measuring cylinder and distilled water was added up to 250ml. Dextrose 5g and agar 15g were also added to a solution in a conical flask. The solution was autoclaved about/more than 5 minutes to sterilize the media.

Preparation of Czepeck-Dox nutrient solution for *C. cladosporioides.*

Materials:

KNO_3	2g
KH_2PO_4	1g
$MgSO_4$	0.5g
KCl	0.5g
$FeSO_4$	0.01g
Sugar	30g
Water	1*L*

1/ medium divided into 2 parts and use 500ml conical flasks autoclaved (121° C, 20 Minutes)

Preparation of suspension of conidia:

Culture plates of 1/ *cladosporium* (previously grown) were taken and 10ml of sterilized Czepeck-Dox medium was put into each culture plate, under aseptic condition and help of spatula the mycelium were scraped. Mycellical suspension was filtered out through glass wool to remove hyphal fragments into

spraying apparatus. Mycellium was remained in glass wool and spores were carried Czepeck-Dox medium (spore suspension) taken into separation apparatus. Spore suspension, was sprayed into TLC plates, and was kept in moisture chamber at room temperature.

Reagents for detection of Alkoloids

Dragendorff's reagent:

Bismath carbonate (2.60g) and Sodium iodide (7.00g) in glacial acetic acid (25ml) were boiled for few minutes. After about 12 hours the insoluble materials was filtered through a sintered glass funnel. The clear reddish brown solution (20ml) was mixed with ethyl acetate (8ml) and stored in a brown bottle.

Spraying solution:

Stocked solution (10ml) was mixed with glacial acetic acid (25ml) and ethyl acetate (60ml), 50% of H_2SO_4 was sprayed on TLC after spraying Dragendorff's reagent.

Preparation of anisaldehyde spray reagent for sugar, steroids, and terpenoids

Heat 100-150°C sprays a solution of 0.5mg anisaldehyde in 500ml glacial acetic acid and 1ml of 97% conc. H_2SO_4 acid were mixed together to form a solution. 5ml was needed to maximal visualization of spots. The background was brishtended by water paper lichen constituents phenols, terpenes, sugar, steroids,turn violet blue, red gray or green.

Column Chromatography separation of n-BuOH extract of MeOH extract of stem bark of *Artocarpus nobilis*

The n-BuOH extract was chromatographed over silica gel with n-hexane, EtOAc, MeOH

"Column 1"Gravity column for 'BuOH' extract

Solvent system	Volume (ml
100% hexane	100
25%EtOAc+hexan	200
50%EtOAc+hexan	100
75%EtOAc+hexan	100
100%EtOAc	300
1%MeOH+EtOAc	100
2%MeOH+EtOAc	100
3%MeOH+EtOAc	100
4%MeOH+EtOAc	100
5%MeOH+EtOAc	100
7.5%MeOH+EtOAc	100
10%MeOH+EtOAc	100
12%MeOH+EtOAc	100
15%MeOH+EtOAc	100
20%MeOH+EtOAc	100
30%MeOH+EtOAc	100
40%MeOH+EtOAc	100
50%MeOH+EtOAc	100
60%MeOH+EtOA	100
80%MeOH+EtOAc	100

Fraction #	Label	Weight of the extract (g)
1-3	ANSL	3.0724
4-10	ANSM	7.7970
11-14	ANSN	0.3403
15-32	ANSO	0.6350
33-48	ANSP	0.8658
49-59	ANSQ	1.2694

Table 3. Solvent system and fractionations obtained from column 1

"Column 2" Gravity column for ANMN

ANSM, ANSN (ANSM+ANSN=ANMN) were combined and the combined fraction was taken to do a gravity column.

Solvent system	Volume (ml)
100%hexane	100
10%EtOAc+hexan	100
20%EtOAc+hexan	100
30%EtOAc+hexan	100
40%EtOAc+hexan	100
50%EtOAc+hexan	100
60%EtOAc+hexan	100
70%EtOAc+hexan	100
80%EtOAc+hexan	100
10%MeOH+EtOAc	100
20%MeOH+EtOAc	100

Fraction	Label	Weight of the extract (g)
1-16	ANMN1	4.1949
17-23	ANMN2	0.4055
24-29	ANMN3	2.4954
30-32	ANMN4	0.5694
33-54	ANMN5	0.7690

Table 4. Solvent system and fractionations obtained from column 2

"Column 3" Gravity column for ANMN6

ANMN3, ANMN4 (ANMN3+ANMN4=ANMN6) were combined and the combined fraction was taken to do a Gravity column. The above gravity column was not giving any good separation. The solvent system was hexane, CH_2Cl_2, and MeOH. And then the column was washed with 100% MeOH and the 'MeOH wash with collected fractions' was taken to do a "column 4"

"Column 4" Sephadex LH-20 column for ANMN6

The above sample (ANMN6) was taken to do a sephadex LH-20 column. Solvent system for the sephadex LH-20 column was only 100% MeOH.

Fraction #	Label	Weight of the extract (g
1-10	ANMN61	0.0634
11-30	ANMN62	0.2127
31-63	ANMN63	1.9953
64-86	ANMN64	0.3188
87-90	ANMN65	0.0595

Table 5. Fractionations obtained from column 4

"Column 5" Reversed phase C_{18} Column for ANSW

ANMN64, ANMN65 (ANMN64+ ANMN65=ANSW) were combined and the combined fraction ANSW was taken to do a Reversed phase Column (R_p-18 column). Solvent system was 50% H_2O-MeOH.

Fraction #	Label	Weight of the extract (g)
1-2	ANSW1	0.2376
3-6	ANSW2	0.0817
7-9	ANSW3	0.0617
10-19	ANSW4	0.0964
20-27	ANSW5	0.0161
MeOH Washed	ANSW6	0.1100

Table 6. Fractionations obtained from column 5

"Column 6" HPLC for ANSW1

Fraction	Weight of the extract (g)
HPLCA1	0.0995
HPLCA2	0.0405
HPLCA3	0.0232
HPLCA4	0.0200

Table 7. Fractionations obtained from column 6

"Column 7" HPLC for ANSW2+ANSW3

Fraction #	Weight of the extract (g)
HPLCB1	0.0243
HPLCB2	0.0090
HPLCB3	0.0116
HPLCB4	0.0010

Table 8. Fractionations obtained from column 7

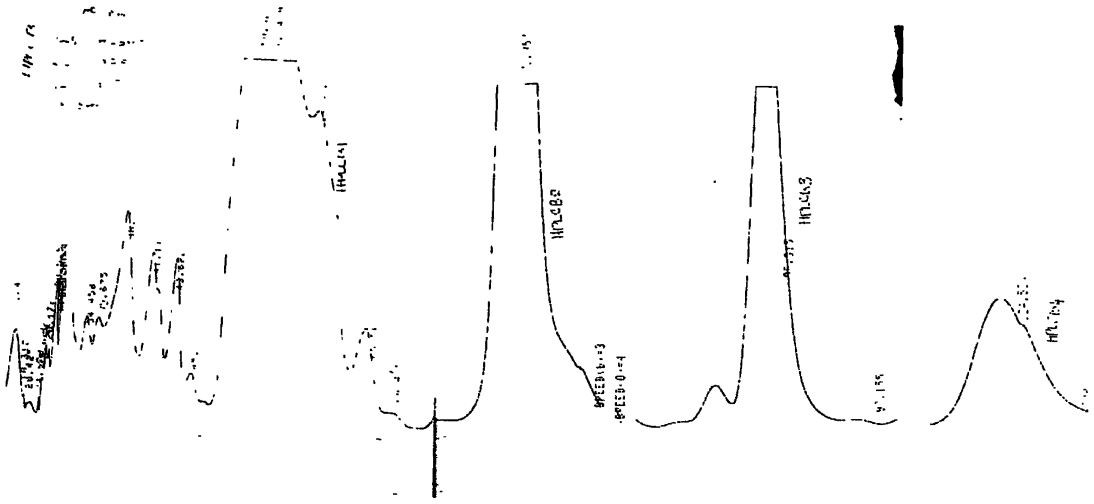
According to the (HPLC) analytical chromatogram HPLCA4 and HPLCB3 were combined and were purified to get a pure sample.

4.0. RESULTS AND DISCUSSION

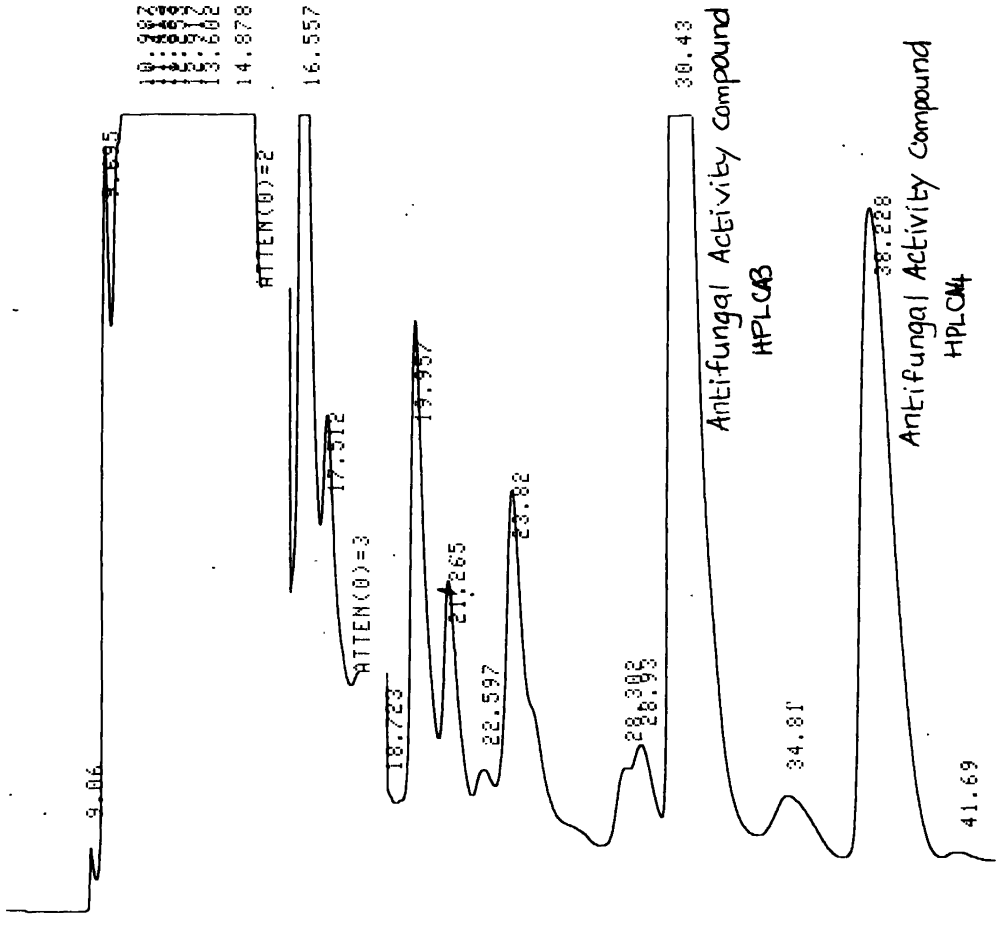
Activity guided fractionation of extracts by column chromatography

The dried stem bark of ***Artocarpus nobilis*** was extracted with cold MeOH. The extract was evoparated to dryness in reduced pressure using rotary evaporator.The MeOH extract was partitioned with n-butanol and water. The butanol crude extract was subjected to antifungal activity test against ***Cladosporium cladosporioides*** by TLC bio-autography method. The n-butanol extract was shown positive response while the above n-butanol ectract did not show a positive result for dragendorff reagent. The n-butanol extract was used to make slurry. The slurry was placed on the suitable (diameter and length) column of gravity silica gel packed with n-hexane, since the n-hexane is one of the low polar solvent which is available in the laboratory. Combination of organic solvents (n-hexane, EtOAc, and MeOH) in increasing polarity pass through the column and fractions were collected in to the test tube. Aliquots of each fraction eluted were spotted on a small TLC plate. Using capillary tube. After air drying the TLC plate, it was developed in a suitable solvent system (eg. 10%MeOH in $CHCl_3$, $CHCl_3$: MeOH: H_2O ; 7:3:1). Thereafter, anisaldehyde reagent was sprayed on to TLC plate and heated on a hot plate about one minute. The fractions giving same TLC patterns were combined and evaporated to dryness. Then dry weights of combined fractions were taken.

These fractions were also tested for a selected bioassy (eg. antifungal). After selection of the bioactive fractions, it was further chromatographed following bioassay until the pure compounds obtained. Since normal gravity silica gel column chromatography, gel chromatography (sephadex LH-20) and reversed phase-C_{18} were failed to isolate pure compounds. At the last, HPLC and TLC were used to isolate pure 'antifungal active' compounds which are labeled as HPLCA3 and HPLCA4 and a pure 'non-antifungal active' compound which is labeled HPLCB3. Their NMR, IR, Mass, UV, Physical constants and structure determination studies are in progress.



HPLC chromatogram for the sample HPLCB



HPLC chromatogram for the sample HPLCA

29. Some HPLC chromatogram for samples HPLCB and HPLCA.

for ANMN, 10%MeOH-$CHCl_3$ for ANMN6, 15%MeOH-$CHCl_3$ for HPLC, 10%MeOH-$CHCl_3$

30. Antifungal activity TLCs

The n-butanol extract of methanol extract of stem bark of *Artocarpus nobilis* and the fractions of column-1 was tested against *Cladosporium cladosporioides* by TLC bioassay method. There are some antifungal areas in the fractions of Column-1. Two inhibition areas were observed in the fractions ANSM and ANSN. According to the TLC bioassay, inhibition areas on both fractions ANSM and ANSN appeared at the same level. Therefore both fractions were combined together and chromatographed to isolate the antifungal compounds. All the resulted fractions were subjected to TLC bioassay(see **30**).

5.0.CONCLUSION

Chromatographic techniques such as normal silica gel, reversed phase silica gel C_{18}, Sephadex LH-20, and HPLC followed by activity guided fractionations of n-butanol extract of the methanol extract of stem bark of the ***Artocarpus nobilis*** yield three pure compounds. Out of three, two compounds showed strong antifungal activity against *Cladosporium cladosporioides* on TLC bioassay.

REFERENCE

1. Peter B. Kaufman, et al, (1999) Natural products from plants, University of Lausanne press, pp. 13-32.

2. Narong C. C., and Hanc V.Hende, (1993), Medicinal and Aromatic Plants in Asia Breeding and improvement, pp. 6-8.

3. Pandey B.P., (1997) Taxonomy of angiosperms for university students, pp. 209-210.

4. Dassanayake M.D., (1981), A revised handbook of the FLORA OF CEYLON, vol-III, Amerinel Press, pp.213-292.

5. Robert F.Raffauf, (1996), Plant Alkaloids, Food products press, pp.143-144.

6. Sultanbawa M.U.S., (1981), Chemical Investigation of Endemic Plants of Sri Lanka, pp.183-196.

7. Jayaweera D.M.A., (1982), Medicinal Plants (Indigenous and Exotic) used in Ceylon, part IV, The national Science council of Sri Lanka Press, pp. 82-95.

8. http://www.brunel.ac.uk/depts/bl/project/molgen/biotech /grometab/primetab.htm.

9. http://www.aspb.org/publications/biotext/intros/ch24.cfm

10. http://www.chemie.fu-berlin.de/chemistry/

11. http://www.chem.qmul.ac.uk/iupac/steroid/3S01.html#3S10

12. Jean Bruneton, (1999), Pharmacognosy-Phytochemistry Medicinal Plants', pp 303-304.

13. Hostettmann K., *et al*, (1998), Preparative Chromatography Techniques- Application in natural Products Isolation, University of Lausanne Press, pp.15-17.

14. Watson.T.E., (1985), Chromatographic methods, pp. 506-629.

15. http://www.chem.vt.edu/

16. http://hplc.chem.shu.edu/

17. Takeya.T, *et al*, (1995), Chem.Parm.Bull 43, pp.1970-1976.

18 http://kerouac.pharm.edu/

19. World health organization report no-622 on the promotion and development of Traditional Medicine", Geneva.

20. Udaya Rajapaksha (1998), Traditional food plants in Sri Lanka, HKARTI Press, pp.305-307.

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