Investigation of Cytotoxicity and Anti-microbial property of the Methanolic extract of *Aphanamixis polystachya* Fruit

Submitted By
MD. Shafiul Alam
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Bachelor of Pharmacy

Under the Guidance of

Apurba Sarker Apu

Senior Lecturer

Department of Pharmacy, East West University

Dhaka, Bangladesh.

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East West University

In the name of ALLAH;

The most Gracious

The most Merciful

Declaration by the Research Candidate

I, MD. Shafiul Alam, hereby declare that the dissertation entitled **"Investigation of Cytotoxicity and Anti-microbial property of the Methanolic extract from *Aphanamixis polystachya* Fruit"**, submitted by me to the Department of Pharmacy, East West University , in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy (Honors) is a bonafide record of original research work carried out by me under the supervision and guidance of **Apurba Sarker Apu**, Senior Lecturer, Dept. of Pharmacy, East West University. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

Date: 29th may 2011

MD. Shafiul Alam

Signature of the Candidate

(MD. Shafiul Alam)

Thesis Certificate

This is to certify that the thesis entitled **"Investigation of Cytotoxicity and Anti-microbial property of the Methanolic extract of *Aphanamixis polystachya* Fruit"**, submitted by **MD. Shafiul Alam** to the Department of Pharmacy, East West University, and in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy (Honors) is a bonafide record of original work carried out by him under my direct supervision and guidance. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma or Fellowship.

Apurba

Mr. Apurba Sarker Apu

Senior Lecturer

Department of Pharmacy

East West University

Dhaka, Bangladesh.

Endorsement by Head of the Department

This is to certify that the dissertation entitled, **"Investigation of Cytotoxicity and Anti-microbial property of the Methanolic extract of *Aphanamixis polystachya* Fruit"** is a bonafide research work done by **MD. Shafiul Alam** under the guidance of **Apurba Sarker Apu,** Senior Lecturer, Department of Pharmacy, East West University, Dhaka.

27.06.2011

Dr. Sufia Islam

Chairperson and Associate Professor

Department of Pharmacy

East West University

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Dedication

This Research paper is dedicated to
My beloved parents,
Who are my biggest Inspirations…

ABSTRACT

The purpose of this study was to understand and investigate the cytotoxic and anti-microbial effect of the traditional medicinal plant *Aphanamixis polystachya*. Toxicologically it is classified under the family Meliaceae. In this paper the study was conveyed on the methanol extract of the fruit of *Aphanamixis polystachya*. Many researches were done on this plant species by many scholars at different time period but the intention of the study was to emphasize on the possibility of its cytotoxic property. To prove the cytotoxic property and detect its intensity, the Brine Shrimp Lethality Bio-assay and to measure the anti-microbial property Disk-diffusion method (Kirby-Bauer) was chosen. After collection, identification and drying the fruit sample, extraction was performed by using methanol solvent system. The extracted components were used to measure the cytotoxicity level over Brine Shrimp Nauplii and anti-microbial property was tested over *Candida albicans, Shigella dysentery* and *Staphylococcus aureus.*

Keywords: *Aphanamixis polystachya*, Meliaceae, Cytotoxicity, Brine Shrimp Lethality Bio-assay, Disk-diffusion method (Kirby-Bauer)

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Chapter One

INTRODUCTION

1.1 THE PLANT FAMILY: Meliaceae

1.1.1. Meliaceae: Prologue

Meliaceae, the mahogany family of flowering plants, of the order Sapindales, comprising about 51 genera and about 575 species of trees and (rarely) shrubs, native to tropical and subtropical regions. Most members of the family have large compound leaves, with the leaflets arranged in the form of a feather, and branched flower clusters. A large number and variety of plant species are listed under this family [1]. The China tree (*Melia azedarach*), also called chinaberry, bead tree, and Persian lilac, is an ornamental Asian tree with round yellow fruits, often cultivated in many tropical and warm temperate areas. Trees of the genus *Swietenia* and *Entandophragma,* commonly called mahogany, and of the genus *Cedrela* (especially the cigar-box cedar, *C. odorata*) are economically important timber trees. The neem, or nim, tree, also called the margosa tree (genus *Azadirachta*), grown throughout the Old World tropics, notably in India and Southeast Asia, is a source of timber and medicinal oils and resins [1].

Meliaceae are a large family of tropical woody species, comprised of 52 genera (approx. 575 species which were categorized into four sub-families: Melioideae (seven tribes, approx. 35 genera); Swietenioideae (three tribes, 13 genera); and the monogeneric Quivisianthoideae and Capuronianthoideae. These authors also considered the secondary xylem to be an important feature separating Swietenioideae and Melioideae [2].

Meliaceae, compared with other groups of similar size, contain a relatively wide range of floral, fruit and seed morphologies and plesiomorphic morphological characters occur side-by-side with derived ones and are frequently connected by intermediate states [2]. Molecular data based on the sequences of two plastid genes and

one nuclear gene confirmed the sub-familial status of both Melioideae and Swietenioideae. Furthermore, morphological and molecular data both indicate a close relationship between the genera *Cedrela* and *Toona*, which are sister taxa forming a monophyletic clade within Swietenioideae, justifying their positioning within the same tribe [2].

1.1.2 Meliaceae: List of Genera

Table 1.1: Genera of the family Meliaceae

Acanthotrichilia (Urb.) O.F.Cook & G.N.Collins = Trichilia P.Browne
Acrilia Griseb. = Trichilia P.Browne, Aglaia Lour.
Ailantopsis Gagnep. = Trichilia P.Browne
Aitonia Thunb. (SUH) = Nymania Lindb.
Alliaria Kuntze (SUH) = Dysoxylum Blume
Amoora Roxb. = Aglaia Lour.
Antelaea Gaertn. = Melia L., Anthocarapa Pierre, Aphanamixis Blume
Argophilum Blanco = Aglaia Lour., Astrotrichilia (Harms) T.D.Penn. & Styles
Aytonia L.f. (SUO) = Nymania Lindb., Azadirachta A.Juss.
Azedarach Mill. = Melia L.
Barbilus P.Browne = Trich.ilia P.Browne
Barola Adans. (SUS) = Trichilia P.Browne
Beddomea Hook.f. = Aglaia Lour.
Bingeria A.Chev. = Turraeanthus Baill., Cabralea A.Juss., Calodecaryia J.-F.Leroy
Calodryum Desv. = Turraea L.

Cambania Comm. ex M.Roem. = Dysoxylum Blume

Camunium Roxb. (SUH) = Aglaia Lour., Capuronianthus J.-F.Leroy, Carapa Aubl., Cedrela P.Browne

Cedrus Mill. (SUH) = Cedrela P.Browne

Charia C.E.C.Fisch. = Ekebergia Sparrm.

Chickassia Wight & Arn. = Chukrasia A.Juss, Chisocheton Blume, Chukrasia A.Juss.

Chuniodendron Hu = Aphanamixis Blume, Cipadessa Blume

Clemensia Merr. = Chisocheton Blume

Dasycoleum Turcz. = Chisocheton Blume

Didymocheton Blume = Dysoxylum Blume, Dysoxylum Blume, Ekebergia Sparrm.

Elcaja Forssk. = Trichilia P.Browne

Elutheria M.Roem. (SUH) = Swietenia Jacq.

Elutheria P.Browne = Guarea L.

Entandrophragma C.E.C.Fisch.

Epicharis Blume = Dysoxylum Blume

Euphora Griff. = Aglaia Lour.

Garretia Welw. = Khaya A.Juss.

Gilibertia J.F.Gmel. = Turraea L.

Ginnania M.Roem. (SUH) = Turraea L.

Grevellina Baill. = Turraea L., Guarea L.

Hartigshea A.Juss. = Dysoxylum Blume

Hearnia F.Muell. = Aglaia Lour., Heckeldora Pierre

Heimodendron Sillans = Entandrophragma C.E.C.Fisch.

Heynea Roxb. ex Sims = Trichilia P.Browne, Humbertioturraea J.-F.Leroy

Johnsonia Adans. (SUH) = Cedrela P.Browne, Khaya A.Juss.

Lamiofrutex Lauterb. = Vavaea Benth., Lansium Correa

Leioptyx Pierre ex De Wild. = Entandrophragma C.E.C.Fisch.

Lepiaglaia Pierre = Aglaia Lour.

Lepidotrichilia (Harms) J.-F.Leroy

Leplaea Vermoesen = Guarea L.

Litosiphon Pierre ex Harms = Lovoa Harms, Lovoa Harms

Macrochiton M.Roem. = Dysoxylum Blume

Mafureira Bertol. = Trichilia P.Browne

Mahagoni Adans. = Swietenia Jacq.

Mallea A.Juss. = Cipadessa Blume, Malleastrum (Baill.) J.-F.Leroy, Megaphyllaea Hemsl., Melia L.

Melio-Schinzia K.Schum. = Chisocheton Blume

Merostela Pierre = Aglaia Lour.

Milnea Roxb. = Aglaia Lour.

Monosoma Griff. = Xylocarpus J.Konig

Moschoxylum A.Juss. = Trichilia P.Browne, Munronia Wight

Napeodendron Ridl. = Walsura Roxb., Naregamia Wight & Arn.

Nelanaregam Adans. = Naregamia Wight & Arn.

Nemedra A.Juss. = Aglaia Lour., Neobeguea J.-F.Leroy

Nimmoia Wight (SUH) = Aglaia Lour.

Nurmonia Harms = Turraea L., Nymania Lindb.

Odontandra Willd. ex Roem. & Schult. = Trichilia P.Browne

Odontosiphon M.Roem. = Trichilia P.Browne

Oraoma Turcz. = Aglaia Lour, Owenia F.Muell.

Payeria Baill. = Turraea L.

Persoonia Willd. (SUH) = Carapa Aubl.

Philastrea Pierre = Munronia Wight

Pholacilia Griseb. = Trichilia P.Browne

Picroderma Thorel ex Gagnep. = Trichilia P.Browne

Plagiotaxis Wall. ex Kuntze = Chukrasia A.Juss.

Plumea Lunan = Guarea L.

Portesia Cav. = Trichilia P.Browne

Prasoxylon M.Roem. = Dysoxylum Blume, Pseudobersama Verdc., Pseudocarapa Hemsl., Pseudocedrela Harms, Pterorhachis Harms

Pterosiphon Turcz. = Cedrela P.Browne

Quivisia Cav. = Turraea L., Quivisianthe Baill.

Racapa M.Roem. = Carapa Aubl., Reinwardtiodendron Koord.

Rhetinosperma Radlk. = Chisocheton Blume

Rochetia Delile = Trichilia P.Browne

Roia Scop. = Swietenia Jacq., Ruagea H.Karst.

Rutea M.Roem. = Turraea L.

Samyda L. (SUH) = Guarea L., Sandoricum Cav.

Schizochiton Spreng. = Chisocheton Blume, Schmardaea H.Karst.

Scyphostigma M.Roem. = Turraea L.

Selbya M.Roem. = Aglaia Lour, Soymida A.Juss., Sphaerosacme Wall. Ex M.Roem.

Suitenia Stokes (SUO) = Swietenia Jacq.

Surenus Kuntze = Toona (Endl.) M.Roem.

Surwala M.Roem. = Walsura Roxb., Swietenia Jacq.

Sycocarpus Britton = Guarea L.

Symphytosiphon Harms = Trichilia P.Browne, Synoum A.Juss., Toona (Endl.) M.Roem.

Torpesia (Endl.) M.Roem. = Trichilia P.Browne

Touloucouna M.Roem. = Carapa Aubl., Trichilia P.Browne, Turraea L., Turraeanthus Baill.

Urbanoguarea Harms = Guarea L., Vavaea Benth., Walsura Roxb.

Wulfhorstia C.E.C.Fisch. = Entandrophragma C.E.C.Fisch., Xylocarpus J.Konig

Zederachia Heist. ex Fabr. = Melia L.

Zurloa Ten. = Carapa Aubl.

1.1.3 Characteristics of the family: Meliaceae

Habit and leaf form: Trees, or shrubs, or herbs (rarely, e.g. *Naregamia*); laticiferous (rarely, with milky juice exuding from the bark), or with colored juice; bearing essential oils. They are characterized by alternate, usually pinnate leaves without stipules, and by syncarpous, apparently bisexual (but actually mostly cryptically unisexual) flowers borne in panicles, cymes, spikes, or clusters. Most species are evergreen, but some are deciduous, either in the dry season or in winter [3].

Leaves: Leaves are alternate (nearly always), or opposite (rarely, decussate); nearly always spiral; petiolate; non-sheathing; not gland-dotted; compound (usually) or simple; pinnate (mostly, pari- or imparipinnate) or unifoliolate (rarely), or ternate (rarely) or bifoliolate (very rarely) or bipinnate (very rarely). Mucilaginous epidermis present or absent [3].

Stem: Cork cambium present, initially superficial. Secondary thickening develops from a conventional cambial ring [3].

Reproductive System: Reproductive type is pollination. Plants hermaphrodite (usually) or monoecious or dioecious or polygamomonoecious. Gynoecium of male flowers pistillodial or vestigial (present as well developed rudiments, perhaps important in pollination). Pollination usually) entomophilous; via hymenoptera or via lepidoptera; mechanism conspicuously specialized (passive pollen presenters, in at least three genera), or unspecialized [3].

Inflorescence, floral, fruit and seed morphology: Flowers solitary, or aggregated in 'inflorescences'; in cymes, in racemes, in panicles, and in spikes. Flowers minute to large; calyptrate or not calyptrate; regular; cyclic; tetracyclic to polycyclic. Floral receptacle with neither androphore nor gynophore [3].

Fruit: Fruits are fleshy or non-fleshy; dehiscent or indehiscent; a capsule or a berry or a drupe or a nut (rarely); without fleshy investment [3].

Seedling: Germination phanerocotylar or cryptocotylar [3].

1.1.4 Some economically important species belong to this family

Table 1.2: Some members of the family Meliaceae

Neem tree: *Azadirachta indica* (India)
Crabwood Tree: *Carapa procera* (South America and Africa)
Cedrela: *Cedrela odorata* (Central and South America; timber also known as Spanish-cedar)

Sapele: *Entandrophragma cylindricum* (tropical Africa)

Utile or Sipo: *Entandrophragma utile* (tropical Africa)

Bossé: *Guarea cedrata* (Africa)

Bossé: *Guarea thompsonii* (Africa)

Ivory Coast Mahogany: *Khaya ivorensis* (tropical Africa)

Senegal Mahogany: *Khaya senegalensis* (tropical Africa)

Chinaberry or Bead Tree: *Melia azedarach* (North America, Queensland, India and southern China)

Mahogany *Swietenia* species (tropical Americas)

Australian Redcedar: *Toona australis* (Australia), often included in *Toona ciliata* (seq.)

Toon, surian: *Toona ciliata* (India, southeast Asia and eastern Australia)

1.2 INFORMATION ABOUT THE PLANT INVSTIGATED

1.2.1 *Aphanamixix polystachya*: Taxonomic hierarchy

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Meliaceae

Genus: *Aphanamixis*

Species: *Aphanamixis polystachya*

Taxonomy:

Current Name: *Aphanamixis polystachya*

Authority: (Wallich) R.N. Parker

1.2.2 *Aphanamixis polystachya*: Common name

Table 1.3: Common names of *Aphanamixis polystachya*

Bengali	Tiktaraj, Pithraj
Hindi	Harin-hara, Harinkhana
Manipuri	Heirangkhoi
Marathi	Raktharohida
Tamil	Malampuluvan, Sem, Semmaram
Malayalam	Chemmaram, Sem
Telugu	Chevamanu, Rohitaka
Kannada	Mukhyamuttage, Mullumuttaga, Mulluhitthalu, Roheethaka
Kuki	Sahala
Khasi	Dieng rata
Rongmei	Agan
Assamese	Hakhori bakhori
Sanskrit	Anavallabha, Ksharayogya, Lakshmi, Lakshmivana, Lohita

Botanical Name: *Aphanamixis polystachya*

Family: Maliaceae

Synonyms: *Aglaia polystachya*, *Amoora rohituka*, *Andersonia rohituka*

1.2.3 *Aphanamixis polystachya*: Description

Family: Meliaceae

Seed: Dicotyledon

Field Characters: Large canopy tree (up to 25 m high, rarely to 35 m); Bole cylindrical or markedly fluted (slightly up to 100 cm diam.); often crooked or straight (bole up to c. 15 m long); buttresses present (1-4 m high); spines absent; aerial roots absent; stilt roots stilt roots absent; Bark brownish red or pale brown, rough, scaly or flaky; Subrhytidome (under-bark) red (bright red); less than 25 mm thick (5-6 mm thick), 5.0-8.0; bark blaze consisting of one layer; strongly aromatic; pleasant; outer blaze red, markings absent, fibrous; inner blaze red, markings absent, fibrous; bark exudates (sap) present, white/milky, not readily flowing (spotty), color not changing on exposure to air, sticky; terminal buds not enclosed by leaves [4].

Indumentum: Complex hairs absent; stinging hairs absent; mature twig indumentum (hairs) absent [4].

Leaves: Leaves spaced along branches, spiral (leaves occurring singly at a node and arranged spirally up the branchlet), compound (a leaf made up from two or more leaflets); petiole present, not winged, attached to base of leaf blade, swollen (at base and inhabited by ants); leaves pinnate (unbranched with more than three leaflets);

petiolule not swollen; asymmetric, terminal developing leaflet buds straight; venation pinnate, secondary veins open, prominent, intramarginal veins absent; leaves lower surface green, upper surface green, indumentum (hairs) absent; absent; domatia absent; stipules absent [4].

Flowers: Inflorescence axillary (sweetly aromatic), flowers on an unbranched axis, cones absent; flowers unisexual or bisexual, unisexual with male and female flowers on different plants, stalked (shortly), flowers with many planes of symmetry, 3.0-5.0 (-7.0) mm long, diameter small (up to10 mm diam.) (4-9 mm diam.); perianth present, with distinct sepals and petals whorls, inner perianth pale yellow or cream-coloured (sometimes tinged with red); 3, some or partly joined; stamens 3-8, present, joined (to form a staminal tube), at base joined to the perianth; ovary superior, carpels joined (when more than one) [4].

Fruits: Infrutescence arranged on unbranched axis, fruit 20.0-40.0 mm long, yellow when young or pale red, not spiny, slightly fleshy, simple, dehiscent, capsule; seeds 1-3, much more than 10 mm long (17-20 mm long), not winged, narrow (longer than wide), seed 1-10 mm diam. (c. 6 mm diam.) [4].

Ecology: In undisturbed mixed dipterocarp and coastal forests up to 700 m altitude. Usually on hillsides and ridges with sandy to clay soils. Also on limestone. In secondary forests usually present as a pre-disturbance remnant [5].

Uses: The wood is used for construction purposes. The bark is used medicinally against rheumatism. Mashed leaves in water solution are effective antifeedants, able to protect crops against insect herbivores. Oil for making soap is extracted from the seeds [5].

Distribution: Tropical Asia and Pacific areas [5].

Medicinal Uses: Bark is used in spleen, liver diseases, tumor and abdominal complaints. Seed-oil is used in rheumatism [5].

Figure 1: *Aphanamixis polystachya* tree

Figure 2: *Aphanamixis polystachya* fruit in tree

Figure 3: *Aphanamixis polystachya* stem and bark

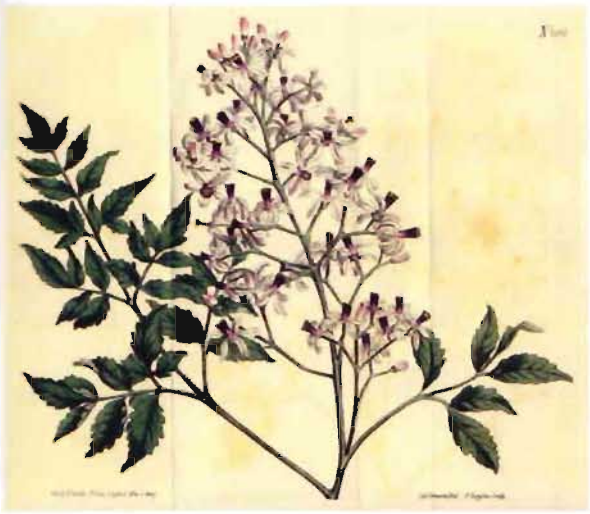


Figure 4: *Aphanamixis polystachya* flower

Figure 5: *Aphanamixis polystachya* fruit

Figure 6: Aphanamixis polystachya dried leaf, flower and fruit

1.3 BRINE SHRIMP LEATHALITY BIOASSAY: AN INTRODUCTION

1.3.1 Brine Shrimp Lethality Bioassay: History

Over the last decade, interest in drugs of plant origin has been growing steadily. The study of bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple, and rapid screening procedure [6]. There are, of course, many procedures for bioassay, but unless collaborative programs with biologists or pharmacologists are in place, the typical chemical laboratory is not suitably equipped to perform the usual bioassays with whole animals or isolated tissues and organs, as well aseptic techniques [6].

When screening for biologically active plant constituents, the selection of the plant species to be studied is obviously a crucial factor for the ultimate success of the investigation. Plants used in traditional medicine are more likely to yield pharmacologically active compounds [6]. The *in vivo* lethality in a simple zoological organism, such as the brine shrimp lethality test (BST), developed for Meyer *et al.*, might be used as a simple tool to guide screening and fractionation of physiologically active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive [6].

Figure 7: Brine Shrimp

1.3.2 Brine Shrimp Lethality Bioassay: Facts

Bioactive compounds are always toxic in high doses. Thus in vivo lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive molecules. The eggs of brine shrimp, Artemia salina can be a useful tool here [7]. They costs low and remain viable for years in dry state. Upon placed in sea water, they hatch in 48 hours and provide a large number of nauplii for experimental use [7].

Brine shrimp nauplii have been used and proved useful in assaying natural products extracts, fractions and pure compounds. Sample compounds are applied in different concentrations in small vials containing brine and a specific number of nauplii. Survivors are counted 24 hours later[7]. The result can be used to determine LC50 of the applied compound and measure the comparative cytotoxicity against a standard cytotoxic compound [7].

This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise here is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, perturbation on a physiologic system [6]. However, it has been demonstrated that BST correlates reasonably well with cytotoxic and other biological properties. Brine shrimp have been previously utilized in various bioassay systems. There have been many reports on the use of this animal for environmental studies, screening for natural toxins and as a general screening for bioactive substances in plant extracts [6].

1.4 ANTIMICROBIAL INVESTIGATION: AN INTRODUCTION

1.4.1 History

The publication on penicillin by Alexander Fleming in 1928 is a milestone in the history of medicine. As more antimicrobial compounds were discovered, it was predicted that infectious diseases would be eliminated through the use of these antimicrobials. Unfortunately, the development of bacterial resistance to these antimicrobials quickly diminished this optimism and resulted in the need for physicians to request the microbiology lab to test a patient's pathogen against various concentrations of a given antimicrobial to determine susceptibility or resistance to that drug [8]. The original method of determining susceptibility to antimicrobials was based on broth dilution methods, which although still the gold standard today, is time consuming to perform. This prompted the development of a disk diffusion procedure for the determination of susceptibility of bacteria to antimicrobials [8].

1.4.2 Purpose

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a physician in selecting treatment options for his or her patients [8]. The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism [8].

1.4.3 Facts

Antimicrobials are one of our most important weapons in fighting bacterial infections. However, over the past few decades, the health benefits offered by the

antimicrobials are diminishing due to increased resistance by the microorganisms [9]. It is essential to investigate newer drugs with lesser resistance. Antimicrobials can be synthetic or can be isolated from the microorganism itself. But the implication of plant extract for the curing of microbial infection has been since the dark ages [9]. And plant extracts and their isolated compounds are still valuable antimicrobials which are mostlyact as lead compounds for the synthesis of an array of antimicrobials [9].

The use of herbs and medicinal plants were carried out by every culture on earth, through written or oral tradition. They relied on vast variety of natural chemicals found for their therapeutic properties. The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms [9].

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc [9]. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition" [9].

1.4.4 Antimicrobial Screening

The disk-diffusion method (Kirby-Bauer) is more suitable for routine testing in a clinical laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics. An agar plate is uniformly inoculated with the test organism and a paper disk impregnated with a fixed concentration of an antibiotic is placed on the agar surface [10]. Growth of the organism and diffusion of the antibiotic commence simultaneously resulting in a circular zone of inhibition in which the amount of antibiotic

exceeds inhibitory concentrations. The diameter of the inhibition zone is a function of the amount of drug in the disk and susceptibility of the microorganism [10].

This test must be rigorously standardized since zone size is also dependent on inoculum size, medium composition, temperature of incubation, excess moisture and thickness of the agar. If these conditions are uniform, reproducible tests can be obtained and zone diameter is only a function of the susceptibility of the test organism [10].

Zone diameter can be correlated with susceptibility as measured by the dilution method. Further correlations using zone diameter allow the designation of an organism as "susceptible", "intermediate", or "resistant" to concentrations of an antibiotic which can be attained in the blood or other body fluids of patients requiring chemotherapy [10].

1.5 CYPROFLOXACINE: DESCRIPTION

1.5.1 Overview

Cyclosporine is a drug that decreases the pain and swelling of arthritis. It also may slow down progression of arthritis over time. It originally was used to prevent rejection in individuals after a kidney transplant [11].

It is the antimicrobial agent which was used in this research as control antimicrobial substance for the antimicrobial susceptibility test.

1.5.2 Chemical origin

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluroquinolone drug class; it is a second generation fluroquinolone. It kills bacteria by interfering with

the enzymes that cause DNA to rewind after being copied, which stops synthesis of DNA and of protein [12]. This is the most frequently used fluoroquinolone in the United States [12].

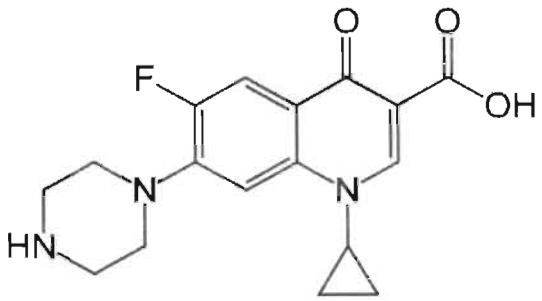


Figure 8: Chemical structure of ciprofloxacin.

Ciprofloxacin is effective against many systemic infections, with the exception of serious infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), the enterococci, and pneumococci. Ciprofloxacinis also particularly useful in treating infections caused by many Enterobacteriaceae and other gram-negative bacilli. It is the most potent of the fluoroquinolones for *Pseudomonas aeruginosa* infections and, therefore, is used in the treatment of pseudomonal infections associated with cystic fibrosis [12]. The drug is also used as an alternative to more toxic drugs, such as the aminoglycosides. It may act synergistically with β-lactams and is also of benefit in treating resistant tuberculosis [12].

1.6 SOLVENT SYSTEM: METHANOL

1.6.1 Introduction

Methanol is the simplest alcohol, containing one carbon atom. It is a colorless, tasteless liquid with a very faint odor [13].

1.6.2 Chemical Structure

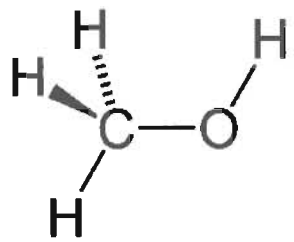


Figure 9: Methanol

1.6.3 Physical and Chemical Properties of Methanol

Table 1.4: Physical and Chemical Properties of Methanol

Molecular weight	32.04
Boiling point	64.7°C
Vapor pressure	97 Torr at 20°C
Freezing point	-97.68°C
Refractive index	1.3284 at 20°C
Density	0.7913 g/mL (6.603 lb/gal) at 20°C
	0.7866 g/mL (6.564 lb/gal) at 25°C
Dielectric constant	32.70 at 25°C
Dipole moment	2.87 D at 20°C
Polarity index (P')	5.1
Viscosity	0.59 cP at 20°C
Surface tension	22.55 dyn/cm at 20°C
Solubility in water	Miscible in all proportions
DOT Hazard Class	3, Flammable Liquid (Domestic) or Flammable Liquid and Poison (International)
Storage	Store in an area designed for flammable storage, or in an approved metal cabinet, away from direct sunlight, heat and sources of ignition.

1.6.4 Methanol: Uses [14]

Table 1.5: Uses of Methanol

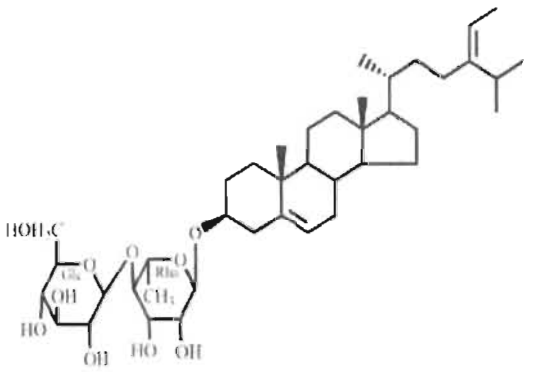
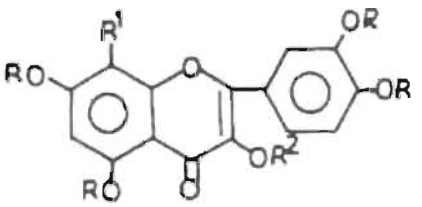
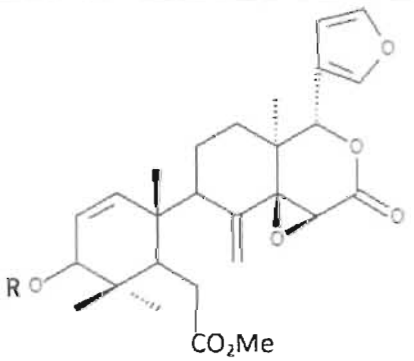
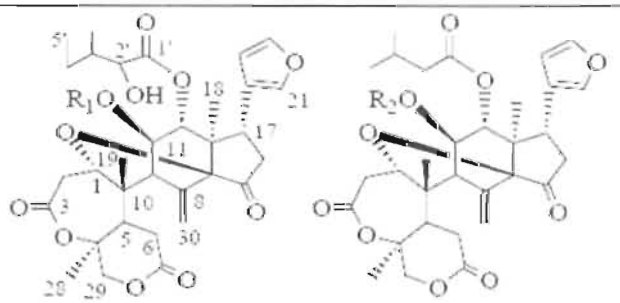
Precipitation of polystyrene and chloroprene resins
Washing and drying of powdered coal fractions
Paint stripping
Metal surface washing
Cleaning of ion exchange resins
Moisture and resin removal from lumber
Extraction agent in the oil, chemical and food industries
Fondue fuel
Fuel for picnic stoves and soldering torches
De-icer and windshield washer fluid for automobile
Antifreeze for pipeline dehydration.
Biodiesel Production
Waste Water Treatment
Fuel Cell Applications
The primary uses for methanol are the production of chemical products and use as a fuel

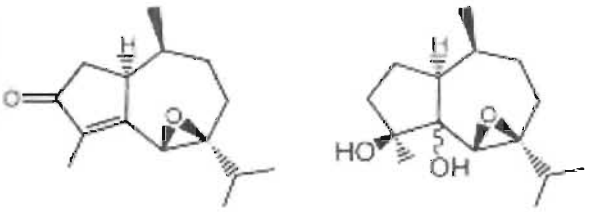
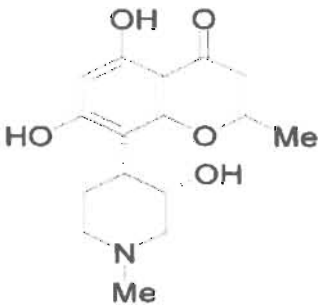
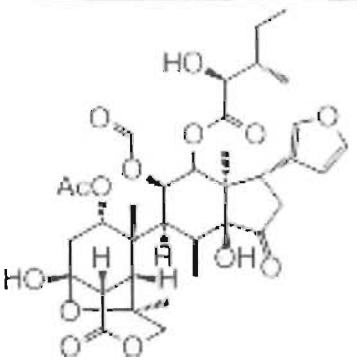
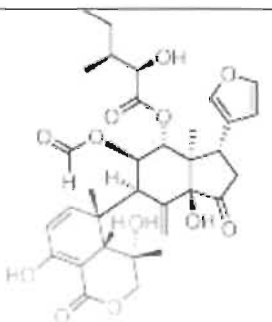
Chapter Two

LITERATURE REVIEW

2.1 *Aphanamixis polystachya*: LIST OF ISOLATED COMPOUNDS

Table 2.1: List of isolated compounds

Structures of isolated compounds	Name of the compound
	Stigmata-5,24(28)-dien-3-*O*-[β-D-glucopyranosyl-α-L-rhamnopyranoside].
	8-*C*-methyl-quercetin-3-*O*-β-D-xylopyranoside; R = H, $R^1 = CH_3$, R^2=Xylose.
	Amoorinin; R = H
	R_1 = H, Rohituka-15; R_1 = HCO, Dregeana-1; R_2 = H, Rohituka-12; R_2 = HCO, Polystachin.

	6β,7β-Epoxyguai-4-en-3-one and 6β,7β-Epoxy-4β,5-dihydroxyguaiane
	Rohitukine.
	Aphanamolide
	Aphapolynin

2.2 *Aphanamixis polystachya*: PHYTOCHEMICAL STUDIES

Phytochemical examination of the seeds of *Aphanamixis polystachya* resulted in the isolation and identification of a new saponin, Stigmata-5,24(28)-dien-3-*O*-[β-D-glucopyranosyl-α-L-rhamnopyranoside][15].

It also has an astringent action and has been used in the treatment of spleen and liver diseases, tumors, and abdominal complaints. Isolation and characterization of a new flavone glycoside whose structure as 8-*C*-methyl-5,7,3′,4′-tetrahydroxy-flavone-3-*O*-β-D-xylopyranoside have been reported[16].

The structure of aphananin isolated from the fruits of *Aphanamixis polystachya* was established as 21,23*S*-epoxytirucall-7-ene-3β,21β,24,25-tetrol 3β-monoacetate from spectral analyses and chemical transformations [17].

The study of the plant has also disclosed the presence of a class of limonoid, andirobin. Here, the isolation and characterization of a new limonoid, Amoorinin on the basis of spectral and chemical methods [18].

A new limonoid named Rohituka-15, with a known limonoid Dregeana-1 , was isolated from the seed of *Aphanamixis polystachya*. The ^{13}C NMR data assignment of dregeana-1 and the structural elucidation of the new compound were based on spectral analysis including ^{1}H-^{1}H COSY, HMQC and HMBC experiments [19].

The petroleum ether extract of the stem bark of *Amoora rohituka* afforded two novel guaiane-derived sesquiterpenoids, 6β,7β-Epoxyguai-4-en-3-one and 6β,7β-epoxy-4β,5-dihydroxyguaiane. The structures were determined by extensive NMR and MS analyses and by comparison of their spectral data with related compounds. The relative stereochemistries of the asymmetric centers in were determined by selective 1D-NOESY experiments [20].

A new lignan, polystachyol, two lignan glycosides, lyoniside and nudiposide and a sterol, ergosta-4,6,8(14),22-tetraen-3-one, with stigmasterol, and oleic and linoleic acids, have been isolated from an MeOH extract of the dried bark of *Aphanamixis polystachya*. The structures of the isolated compounds were elucidated by analysis of 1D and 2D NMR and mass spectroscopic data [21].

Rohitukine, a chromane alkaloid, is a precursor of flavopiridol, a promising anti-cancer compound. Flavopiridol is a potent inhibitor of several cyclin-dependent kinases (CDKs) [22].

In a study, two novel limonoids, Aphanamolides A and B, along with a structurally related known limonoid, were isolated from the EtOH extract of the seeds of *Aphanamixis polystachya*. Aphanamolide A featured an unprecedented carbon skeleton via the formation of a C-3-C-6 bond. Aphanamolide A was isolated as white amorphous powders. The molecular formula was determined to be $C_{35}H_{44}O_{14}$. Compounds showed cytotoxic activity against two tumor cell lines [23].

Two new highly oxidized A, B-*seco* limonoids, aphapolynins A and B, were isolated from the fruits of *Aphanamixis polystachya*. Their structures were elucidated by spectroscopic analysis; in particular, the absolute configuration of aphapolynin A was determined by a single-crystal X-ray study using a mirror Cu Kα radiation. Aphapolynin A exhibit moderate cytotoxicities when tested against a panel of tumor cell lines [24].

Table 2.2: Summary of the Phytochemical Studies on *Aphanamixis polystachya* [15-24]

Time / Chronicles	Names of the Researchers	Part of the plant	Findings
1981	Bhatt, S. K., Saxena, V. K. & Nigam, S. S.	Seed	Saponin [15]
1985	Jain, A. & Srivastava, S. K.	Root	Flavone glycoside [16]
1985	Kundu, A.B., Ray, S., Chatterjee, A.	Fruits	Aphananin [17]
1987	Agnihotri, V. K., Srivastava, S. D. & Srivastava, S. K.	Stem bark	Amoorinin [18]
2002	Zhang, H., Chen, F., Wang, X., Wu, D. & Chen, Q.	Seed	Rohituka-15 [19]
2003	Chowdhury, R., Hasan, C. M. & Rashid, M. A.	Stem bark	Guaiane sesquiterpenes [20]
2006	Sadhu, S.K., Phattanawasin, P., Choudhuri, M.S.K., Ohtsuki, T., Ishibashi, M.	Stem bark	Lignan [21]
2010	Mohanakumara, P., Sreejayan, N. & Priti, V.	Whole plant	Rohitukine [22]
2010	Yang, S.P., Chen, H.D., Liao, S.G., Xie, B.J., Miao, Z.H., Yue, J.M.	Seed	Aphanamolides A and B [23]
2011	Zhang, Y., Wang, J.S., Wang, X.B., Wei, D.D., Luo, J.G., Luo, J., Yang, M.H., Kong, L.Y.	Fruits	Aphapolynins A and B [24]

2.3 *Aphanamixis polystachya*: PHARMACOLOGICAL STUDIES

Aphanamixis polystachya seed extracts were evaluated for their repellency, feeding deterrency, contact toxicity and oviposition deterrency to rice weevils. The extracts had strong repellent and feeding deterrent effects on rice weevils. The extracts were moderately toxic to rice weevils. An ethanol extract was the most toxic of four extracts tested and showed the lowest LD_{50} and LT_{50} values. The ground leaves, bark and seeds at a 2.5% ratio provided good protection for rice grains by reducing the F1 progeny emergence and the grain infestation rates [25].

Seed extracts of *Aphanamixis polystachya* were evaluated for their repellent, anti-feedant and contact toxicity to adults of *Tribolium castaneum*. The crude seed extracts were strong repellents and moderate feeding deterrents to *T. castaneum*. All extracts were toxic to beetles [26]. The ethanol extract was the most toxic of four extracts tested and showed the lowest LD_{50} value. Ground leaves, barks and seeds were also tested for oviposition deterrency to *T. castaneum*. The ground leaves, bark and seeds in a 2.5% mixture provided some protection for wheat flour by reducing F1 progeny [26].

The oil and 20% crude alkaloid solution from *A. polystachya* seeds, both at 20 and 40 µl/disc, were tested for antimicrobial activities against human bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Bacillus cereus* and *B. subtilis*) and plant pathogenic fungi (*Alternaria alternata, Cochliobolus lunatus, Colletotrichum corchori, Fusarium equiseti, Macrophomina phaseolina, Drechslera oryzae* and *Botryodiplodia theobromae*). [27] Among the bacteria, *B. cereus, E. coli* and *Staphylococcus aureus* showed mild sensitivity (13-14 mm) to the seed oil while the rest did not exhibit any degree of sensitivity. *S. aureus* and *E. coli*

were mildly sensitive (15-16 mm) to the alkaloid solution. The oil and alkaloid solution showed antifungal activities in different degrees against all fungi tested. The highest growth inhibition exhibited by the seed oil (40.28 and 50.24% at 20 and 40 µl, respectively) was recorded for *D. oryzae*. The alkaloid showed the highest inhibition 71.47 and 78.87% at 20 and µl, respectively) on *M. phaseolina* [27].

Sub-fractions of an acetone extract of *A. polystachya* seeds were evaluated for their feeding deterrent effects on adult *T. castaneum*. The results showed that a sub-fraction was highly deterrent to *T. castaneum* feeding. This fraction was isolated and analyzed by gas chromatography-mass spectrometry. Four compounds were identified: glycerol, 2-methoxy-2-hydroxy propanoic acid, 3-methyl-2-hydroxy pentanoic acid and 2,3,4-trihydroxy butanal. 2-methoxy-2-hydroxy propanoic acid might be the active compound against *T. castaneum* [28].

Laboratory experiments were carried out to investigate the efficacy of seed extracts of *Aphanamixis polystachya*, against the red flour beetle, *Tribolium castaneum*. Results of three different bioassays showed that crude extracts of seeds have strong repellent effects and moderate feeding deterrent and insecticidal effects on adult *Tribolium castaneum* [29].

Ground leaves, bark, seeds, and four seed extracts of *Aphanamixis polystachya*, were evaluated for their repellency, contact toxicity, and food protectant efficacy against adult pulse beetle (*Callosobruchus chinensis* L.). The seed extracts showed poor repellent effects, but high contact toxicity to adults at 72 hr after application. The ground leaves, bark, and seeds provided good protection for mung beans against pulse beetles, and the seed powder greatly reduced the F1 progeny and seed damage rates [30].

A crude ethanolic extract of the leaf of this plant shows a beneficial effect on toxic liver injury. Its anti-hepatotoxic activity was evaluated on carbon tetrachloride (CCl_4)-induced liver injury in a rat model. The assessment of hepatoprotective activity was evaluated by measuring the activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, acid phosphatase and lactate dehydrogenase, serum total bilirubin and albumin and histology of the liver [31].

The petroleum ether, dichloromethane and methanol extracts of *Aphanamixix polystachya* demonstrated good laxative potential at 400, 250 and 400 mg/kg respectively and the data obtained after 1 h of drug administration were statistically significant. The petroleum ether and methanol extracts also showed significant gastrointestinal hypermotility following barium sulphate milk in mice. The data showed dose dependency and were well correlated with the findings of laxative screening. The crude extracts of have laxative principle comparable to those of a stimulant laxative, sennoside B [32].

Amooranin is a triterpene acid isolated from the stem bark of *Aphanamixix polystachya*. The stem bark is one of the components of a medicinal preparation used in the Ayurvedic system of medicine for the treatment of human malignancies. The mechanism of cell death was investigated associated with AMR cytotoxicity in human mammary carcinoma MCF-7, multidrug resistant breast carcinoma MCF-7/TH and breast epithelial MCF-10A cell lines. The induction of apoptosis in AMR treated cells was accompanied by the elevation of total caspase and caspase-8 activities [33].

The effect of radiation on tumor tissue can be optimized by adding radiosensitizing agents, in order to achieve a greater degree of tumor damage than expected from the use of either treatment alone. The ethanolic extract of *Aphanamixis polystachya* (APE) was tested in Swiss albino mice transplanted with Ehrlich ascites

carcinoma (EAC) and exposed to various doses of gamma-radiation [34]. The best effect of APE and radiation was observed for 6 Gy gamma-radiations. The APE treatment before irradiation elevated lipid peroxidation followed by a reduction in the glutathione contents. Treatment of tumor bearing mice with APE before irradiation further reduced the activities of various antioxidant enzymes like glutathione peroxidase, glutathione-s-transferase, superoxide dismutase and catalase at different post last drug administration (PLDA) times [34].

Normal tissue radiosensitivity is the major limiting factor in radiotherapy of cancer. The use of phytochemicals may reduce the adverse effects of radiation in normal tissue. The effect of ethyl acetate fraction of *Aphanamixis polystachya* was investigated on the radiation-induced chromosome damage in the bone marrow cells of Swiss albino mice exposed to various doses of gamma-radiation [35]. Irradiation of mice to different doses of gamma radiation caused a dose dependent elevation in the frequency of aberrant cells and chromosome aberrations like chromatid breaks, chromosome breaks, dicentrics, acentric fragments and total aberrations at all the post-irradiation times studied [35].

Preliminary phytochemical investigation revealed the presence of carbohydrates, saponins and triterpenoids in AqE of *Aphanamixis polystachya*. LD50 cut-off dose was found to be 2500 mg/kg b.w. for the extracts of *Aphanamixis polystachya*. Hence, therapeutic dose was taken as 250 mg/kg b.w. for all four extracts [36]. Treatment with AqE of *Aphanamixis polystachya* significantly showed the anti-ulcer activity as compared to control and other extracts. The histopathological study of the stomach also supported the above results. The results were comparable to that of standard drug (Omeprazole). From the literature survey and the work carried out, it may be confirmed that bark of *Aphanamixis polystachya* does possesses anti-ulcer property. Phytochemical

investigation suggests the presence of saponins which may be responsible for the anti-ulcer activity [36].

The investigation of the possible CNS depressant and analgesic action of the methanol extract of *Aphanamixis polystachya* leaf was done. Its CNS depressant activity was evaluated by using thiopental sodium-induced sleeping time, hole cross and open field tests. The analgesic activity was also investigated for its central and peripheral pharmacological actions using hot plate and tail immersion test and acetic acid-induced writhing test in mice respectively [37]. The extract significantly maximized the duration of sleeping time when administered with thiopental sodium. The extract increased in pain threshold both in hotplate and tail immersion methods in a dose dependent manner. These results suggest that the extract possesses strong CNS depressant and analgesic activity in mice [37].

Free radical stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging and ischemia, reperfusion injury of many tissues, gastritis, tumor promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders. Due to its natural origin and potent free-radical scavenging ability *Aphanamixis polystachya* could be used as a potential preventive intervention for free radical-mediated diseases [38].

The dried stem bark of *Aphanamixis polystachya* was extracted with alcoholic, hydroalcoholic and aqueous solvent for 36 hours in soxhlet apparatus and solvents from extracts was evaporated under vacuum. All three extracts were further fractionized in to the petroleum ether, ethyl acetate and n-butanol fractions and studied for *in vitro* antimicrobial activity by Agar cup method using different bacterial strains in nutrient

agar media [39]. For screening of antimicrobial activity, these extracts of dried stem bark of *Aphanamixis polystachya* was used at the dose of 500 mcg/cup against the kanamycin, which was used as standard antimicrobial agent at the dose of 30mcg/cup. The zone of inhibitions indicates that the extracts of dried stem bark of *Aphanamixis polystachya* showed significant antimicrobial activity as comparison to kanamycin [39].

An invention discloses that 5-lipoxygenase inhibitory product prepared from botanical sources. More specifically, the invention describes 5-lipoxygenase inhibitory extracts or bio-enriched extracts or fractions of *Aphanamixis polystachya*, methods of making 5-lipoxygenase inhibitory extract, and methods of treating and preventing disease conditions mediated by 5-lipoxygenase by using the said extract [40]. The invention further discloses pharmaceutical or dietary compositions containing therapeutically effective amounts of the extracts of *Aphanamixis polystachya* in combination with other known anti-inflammatory agents useful for oral, parenteral and topical administration [40].

Table 2.3: Summary of the Pharmacological Studies on *Aphanamixis polystachya* [25-40]

Time Chronicles	Names of the Researchers	Plant part	Assertions
1994	Howse, P. E.	Seed	Toxic & repellent properties [25]
1995	Talukder, F. A.; Howse, P. E.	Seed	Repellent, Antifeedant and Contact toxicity [26]
2000	Bhuyan, M. A. K.; Begum, J.; Chowdhury, J. U.; Ahmed, K.; Anwar, M. N.	Seed	Antimicrobial activity [27]
2000	Talukder, F. A.; Howse, P. E.	Seed	Feeding deterrent [28]
2000	Talukder, F. A.; Howse, P. E.	Seed	Deterrent and insecticidal effects [29]
2000	Talukder, F. A.; Howse, P. E.	Whole plant	Repellent, Toxic, and Food protectant effects [30]
2002	Gole, M. K. & Dasgupta, S.	Leaf	Hepatoprotective activity [31]
2003	Chowdhury, R. & Rashid, R.	Stem bark	Laxative properties [32]
2003	Rabi, T., Ramachandran, C., Fonseca, H. B. & Nair, R. P.	Stem bark	Anticancer activity on human breast carcinoma [33]
2005	Jagetia G. C. & Venkatesha V. A.	Whole plant	Anticancer activity on ehrlich ascites carcinoma [34]
2006	Jagetia G. C. & Venkatesha V. A.	Stem bark	Reduction of radiation-induced chromosome injury [35]
2007	Shinkar, A.S.	Whole plant	Antiulcer activity [36]
2009	Hossain, M., Biva, I. J., Jahangir, R. & Vhuiyan, I.	Leaf	CNS depressant and analgesic activity [37]
2009	Krishnaraju, A. V., Rao, C. V., Rao, V. N., Reddy, K. N. & Trimurtulu, G.	Bark	*In vitro* and *in vivo* antioxidant activity [38]
2010	Yadav, R., Chauhan, N. S., Chouhan, A. S., Soni, V. K. & Omray, L.	Stem bark	Antimicrobial activity [39]
2010	Gokaraju, G.R., Gokaraju, R.R., Golakoti, T., Chirravuri, V.R., Raju, V.K., Bhupathiraju, A.K.	Whole plant	5-lipoxygenase inhibitory product [40]

Chapter Three

MATERIALS AND METHOD

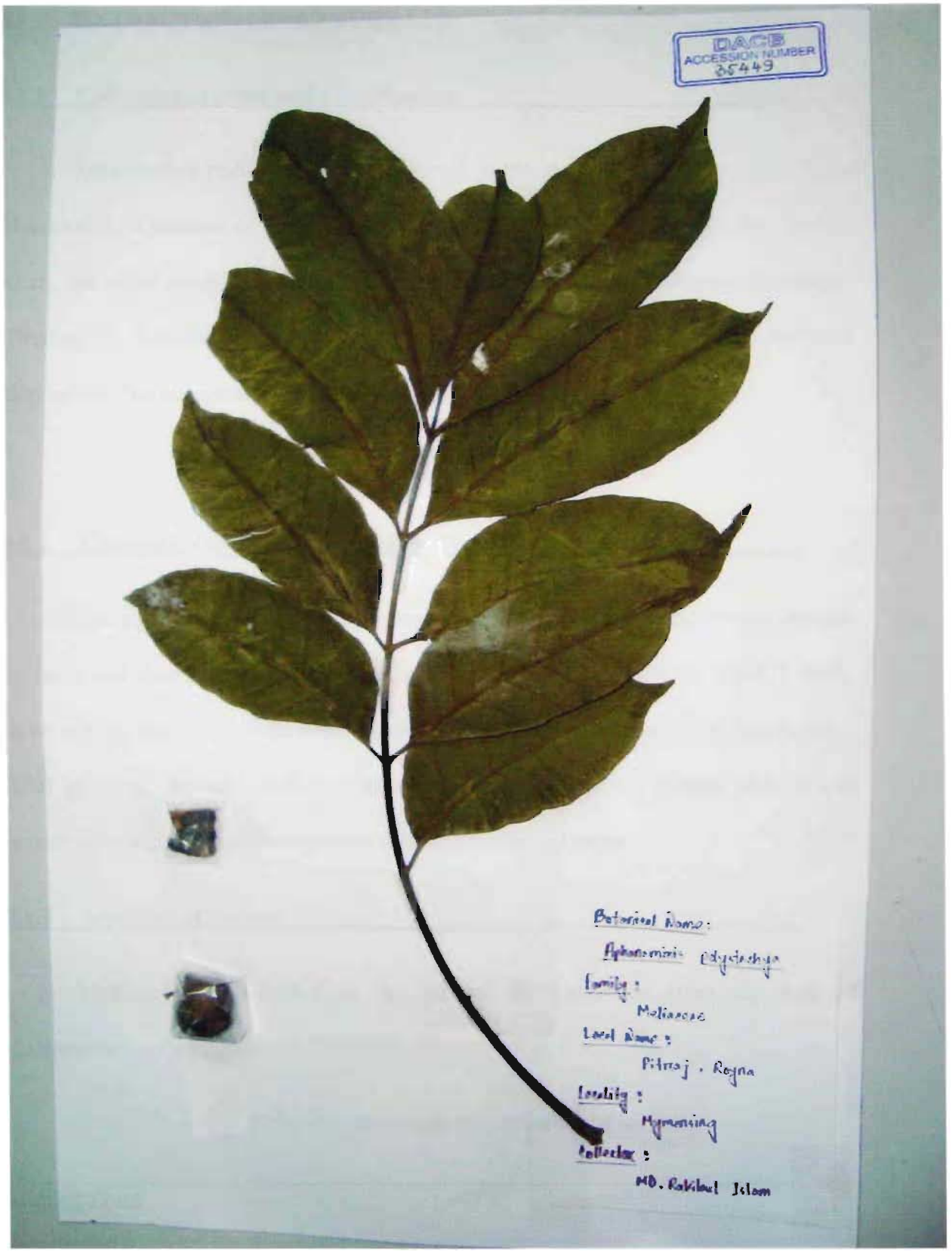


Figure 10: Identification sheet of *Aphanamixis polystachya*

3.1 EXTRACTION PROCEDURE

3.1.1 Collection of plant and Identification

Aphanamixis polystachya was collected in the month of February, 2011 from Mymensing, a District of Bangladesh. Collected plant was identified by Dr. Bushra Khan, the chief scientist and taxonomist of Bangladesh National Herbarium, Mirpur (Dhaka). A duplicate specimen has been deposited in the Bangladesh National Herbarium. The accessesion number of the specimen is 35449.

3.1.2 Crushing, drying and grinding the fruit

The collected fruit sample weighted about 400 gm. At first those were crushed by hand and then by mortar-pastel and were dried under sun-light for about 1 week. After drying, the dried fruits were grinded into grinding machine to get fine powder. After grinding, the total fruit powder weighted 144.1 gm. All grinded powder was preserved in a glass container covered with aluminum foil paper.

3.1.3 Selection of Solvent

Methanol was selected as the solvent for extraction from the fruit of *Aphanamixis polystachya.*

Table 3.1: Important properties of the solvent

Melting Point	-97^0C
Boiling Point	64.6^0C
Relative Density	0.8

3.1.4 Methanolic extraction process of *Aphanamixis polystachya*

The extraction from the fruits of *Aphanamixis polystachya* was done by hot extraction method, by a mechanism called Soxhlet apparatus. Franz Ritter von Soxhlet a German agricultural chemist invented the Soxhlet apparatus in 1879.

The grinded powder of the fruit was taken in a cloth bag, which served as a porous medium. The bag held the sample and also as a medium for the solvent (methanol) to get in contact with the powder. In the lower round bottom flask the solvent was heated to its boiling point, the vapor passes through the bypass stream line, become condensed and liquefy over the cloth bag where the sample was held. Extraction continues while the solvent kept evaporating from the flask and condensing over sample. After a specific level when the smaller side-arm fills to overflowing, it initiates a siphoning action. The solvent, containing the dissolved component, was siphoned into the boiler below. The residual solvent then drains out of the cloth bag, as fresh solvent drops continue to fall into it and the cycle repeats. After that, by using the rotator evaporator the solvent was removed and the remaining crude extract was collected.

3.1.5 Apparatus & reagent used for plant extraction

Table 3.2: Apparatus & reagent used for plant extraction

Plant sample
Blender machine (for obtain the powder of plant)

Acetone (Merck, Germany)

Glass container (storing of plant powder)

Aluminum foil (covered the beaker and conical flask)

Electric Balance; SHIMADZU AY220 & SCALTEC SPB31

Conical flask; 1000ml

Methanol, ethyl acetate and n-hexane (Merck, Germany)

Filter paper (Double Rings 102 – 11cm, HANGZHOU XINHUA PAPER Industry Co. Ltd., China)

Volumetric Flask; 250ml, 500ml and 1000ml

Round Bottle Flask; BOROSIL 1000ml

Rotary evaporator (IKA RRV05 Basic, Biometra, Germany)

Di-ethyl-ether (Merck, Germany)

Beaker; 25ml

Figure 11: Soxhlet Apparatus and Rotatory Evaporator

3.2 BRINE SHRIMP LETHALITY TEST OF *Aphanamixis polystachya*

3.2.1 Brine Shrimp (*Artemia salina* Leach) hatching procedure

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38 gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000ml by distilled water in a 1000ml beaker for *Artemia salina* hatching. 1-2 drops of NaOH solution of 1N was added with a dropper to obtain the pH 8.4 as sea water. Then 0.25gm of dry preserved eggs of *Artemia salina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. After 24 hours incubation at room temperature, newly hatched free-swimming red-colored nauplii

were harvested from the bottom outlet and place into another beaker with a dropper where previously made sea water was present. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. Those freshly hatched free-swimming nauplii were used for the bioassay.

3.2.2 Arranging the Bioassay procedure

30 test tubes were cleaned with acetone and then every test tube was filled with 10ml of water and then the water level was tagged with masking tape. Then the water removed from all the test tube and kept for the assay. A serial number was also written on the masking tape to identify the drugs concentration. 20 test tubes were for positive and negative control test and 10 test tubes for the cytotoxic test with methanol extract of *Aphanamixis polystachya*. After preparing the test tubes 10 nauplii were taken into each test tube with a dropper from the freshly hatched free-swimming red nauplii. The sea water level in the test tube was below 10 ml for the further adjustment with crude drugs.

3.2.3 Running the Positive control test

10 test tubes were taken containing 10 nauplii each. For positive control $KMnO_4$ was used as the standard drug. 50 mg KMnO4 was dissolved in 10 ml of sea water. So, 50000 µg of $KMnO_4$ was dissolved in 10000 µl of sea water which made the concentration of $KMnO_4$ in sea-water was 5µg/µl. Then 2 µl of $KMnO_4$ containing sea-water was taken into the first test tube with a micro pipette for positive control and then the test tube was adjusted with normal sea-water up to 10ml, so the final concentration of the first test tube was 1µg/ml. This solution was given to the other test tubes for positive control test in the same manner but the final concentration varied as different

amount of solution to each test tubes. After 24 hours rest the test tubes were checked for lethality assay.

Table 3.3: Preparation of test solution for positive control

Serial number	Amount of KMnO4 in the solution	Final volume	Final concentration
1	4 µg	10 ml	2 µg/ml
2	8 µg	10 ml	4 µg/ml
3	12 µg	10 ml	6 µg/ml
4	16 µg	10 ml	8 µg/ml
5	20 µg	10 ml	10 µg/ml
6	24 µg	10 ml	12 µg/ml
7	28 µg	10 ml	14 µg/ml
8	32 µg	10 ml	16 µg/ml
9	36 µg	10 ml	18 µg/ml
10	40 µg	10 ml	20 µg/ml

3.2.4 Addition of *Aphanamixis polystachya* methanolic fruit extract

200 mg of methanolic solvent fruit extract was taken into a 25ml beaker and was dissolved in 10 ml of Dimethyl Sulfoxide (DMSO) and two drops of TWEEN 80. 10 test tubes were taken each containing 10 nauplii. In the first test tube, 10 µl of methanol fruit extract was added and then the solution volume was adjusted up to 10 ml

by adding freshly prepared sea water. Micro tips and micro pipette were used to transfer the crude extracts into each test tube for accurate measurement. After that the test tubes were filled up to 10ml with the previously made fresh sea water. Then these test tubes were kept for 24 hours to get the result of lethality bioassay.

Table 3.4: Preparation for *Aphanamixis polystachya* extracts for brine shrimp test

Amount of Methanolic extract	**Final volume**	**Final Concentration (µg/ml)**
50 µg	10 ml	5
75 µg	10 ml	7.5
125 µg	10 ml	12.5
150 µg	10 ml	15
200 µg	10 ml	20
250 µg	10 ml	25
300 µg	10 ml	30
350 µg	10 ml	35

3.2.5 Running the Negative control test

To run the negative test procedure, 10 test tubes were taken each containing 10 nauplii. Then 2 drops of TWEEN 80 were added with 10 ml of DMSO which was the parent solution for negative control test. In each test tube that solution was added according to the volume calculated as the *Aphanamixis polystachya* methanol fruit

extracts. Then these test tubes were kept for 24 hours to get the result of brine shrimp lethality test.

3.2.6 Apparatus & Reagent used for Brine Shrimp Lethality Test

Table 3.5: Apparatus & Reagent used for Brine Shrimp Lethality Test

Pure NaCl (Merck, Germany)
Measuring cylinder
Brine shrimp eggs
1000 ml beaker
Table lamp
Aquarium air pump (SB2488, Sovo)
Crude extracts
Test tubes and racks
Electric Balance; SHIMADZU AY220 & SCALTEC SPB31
Filter paper (Double Rings 102 – 11cm, HANGZHOU XINHUA PAPER Industry Co. Ltd., China)
DMSO (Merck, Germany)
TWEEN80 (Merck, Germany)
$KMnO_4$ (Merck, Germany)

3.3 ANTI-MICROBIAL SUSCEPTIBILITY TEST OF *Aphanamixis polystachya*: DISC-DIFFUSION METHOD (KIRBY-BAUER)

3.3.1 Petri dish Sterilization

To perform the antimicrobial susceptibility test, first we need to sterilize Petri dishes for pure culture of microbes. 3 Petri dishes were wrapped with paper and placed inside an autoclave machine (HIRAYAMA, Japan) for sterilization at 121°C for 15 minutes. After that, the dishes were washed with detergent properly. Then they were allowed to dry and then placed these Petri dishes into hot air oven (FN-500, Niive) for 20 minutes. Finally the Petri dishes were placed into the laminar flow cabinet for prevention of further contamination from the environment.

Figure 12: Autoclave machine

3.3.2 Agar Preparation

The standard rule of preparing agar is to take 28 gram Nutrient Agar and to dissolve it in 1000 ml of water. So to prepare 300 ml of agar, 8.4 gram Nutrient Agar was weighted and dissolved in 300 ml distilled water. This preparation was kept in a

400 ml glass container. And then the glass container was kept in the autoclave machine for 15 minutes at 121°C temperatures. After that the agar solution was placed in the laminar air flow cabinet.

3.3.3 Inoculation of micro-organisms in Agar medium

The prepared Agar solution was poured into each of the 3 Petri dishes in a way so that each Petri dish gets 12-15 ml agar medium. Agar medium was dispensed into each Petri dish to get 3-4 mm depth of agar media each. After pouring the agar medium, all Petri dishes were kept in room temperature so that the medium can properly solidify. *Candida albicans, Staphylococcus aureus* and *Shigella dysentery* were used as test micro-organisms and the concentration of the suspension of test micro-organism was 1480 CFU/ml for *Candida albicans,* 320 CFU/ml for *Staphylococcus aureus* and 1000 CFU/ml for *Shigella dysentery.* Petri dishes were labelled with the name of the microorganisms.

0.5 ml of bacterial suspension was taken from the each vial with micropipette and placed on the surface of the agar media on each Petri dish according to their labelled name. After that the suspension was spread with glass rod spreader gently on the agar media. Then the ciprofloxacin disc and other two discs (one discs having 500μg and another having 1000 μg of crude drugs) containing crude extracts were placed into the agar media. All these work were done in the laminar air flow cabinet.

3.3.4 Incubation of the micro-organisms

All the prepared agar plates with respective microorganisms were placed inside a bacteriological incubator at 36°C temperatures for 24 hours for obtaining the result of anti-susceptibility test of *Aphanamixis polystachya.*

Figure 13: Incubator

Figure 14: Micropipette

Figure 15: Spreader

Figure 16: Laminar Air Flow

Figure 17: Air Dryer (Left) & Incubator (Right)

3.3.5 Apparatus & reagents used for Antimicrobial Susceptibility Test

Table 3.6: Apparatus & reagents used for Antimicrobial Susceptibility Test

Micro-organisms suspensions
Laminar air flow cabinet (ESCO, Singapore)
Petri dishes
Sterile forceps
Vials
Autoclave (HIRAYAMA, Japan)
Hot air oven (FN-500, Niive)
Nutrient Agar (TECHNO PHARMCHEM, India)
Filter paper (Double Rings 102 – 11cm, HANGZHOU XINHUA PAPER Industry Co. Ltd., China)
Filter paper discs
Inoculating loop
Crude extracts of experimental plant
Micropipette (Eppendrof, Germany)
Micropipette tips (Eppendrof, Germany)
Spirit burner
Incubator
Measuring Cylinder (10ml & 100ml)
Distilled water

Chapter Four

RESULT AND DISCUSSION

4.1 RESULT: BRINE SHRIMP LETHALITY TEST

The lethality of methanol extracts of *Aphanamixis polystachya* fruit to brine shrimp was investigated in this study by this following procedure:

After 24 hours, the test tubes were inspected using a magnifying glass and the number of survivors was counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed by using Microsoft Excel. The effectiveness or the concentration-mortality relationship of plant product was expressed as a median Lethal Concentration (LC_{50}) value. This represents the concentration of the crude extracts that produces death in half of the test subjects after a certain exposure period.

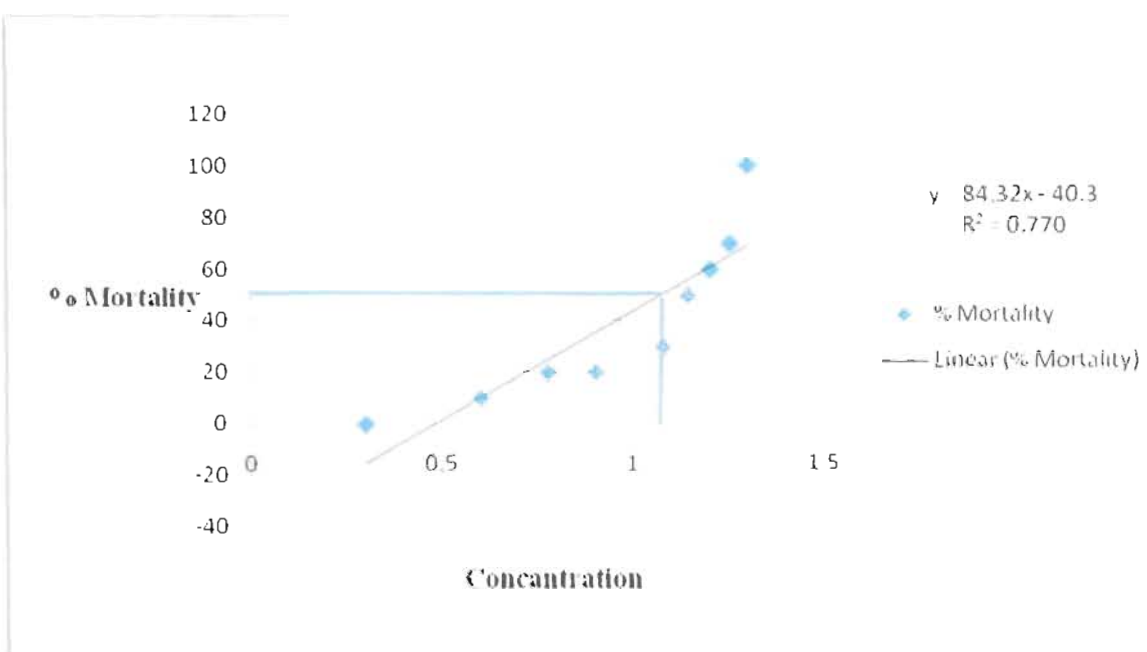
Table 4.1 and 4.2 gives the results of the brine shrimp lethality after 24 hours exposure to the positive control $KMnO_4$ and methanol extraction of *Aphanamixis polystachya* fruit respectively. The positive control, compared with the negative control was lethal, giving significant mortality to the shrimp as there was no mortality found in the negative control.

The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the shrimps killed against the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis.

Table 4.1: Effect of $KMnO_4$ on Brine Shrimp nauplii.

Concentration (µg/ml)	Total population	Survivors	Death	% Mortality	LC_{50} (µg/ml)
2	10	10	0	0	
4	10	9	1	10	
6	10	8	2	20	
8	10	8	2	20	
12	10	7	3	30	
14	10	5	5	50	13
16	10	4	6	60	
18	10	3	7	70	
20	10	0	10	100	

Figure 18: Effect of $KMnO_4$

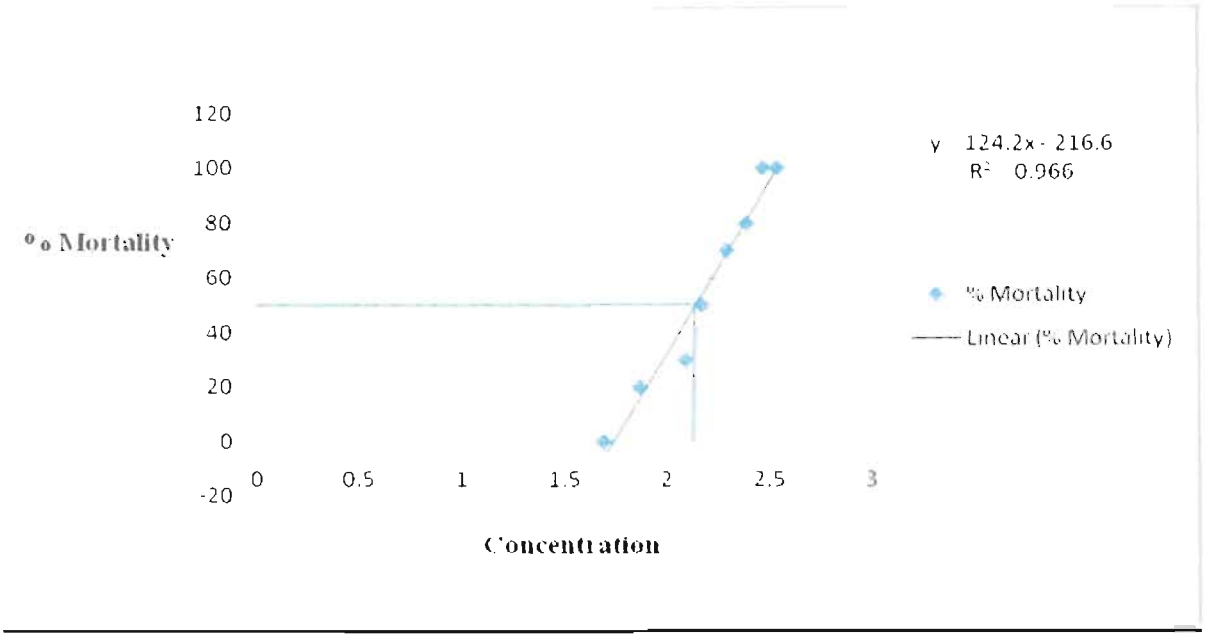


Here, LC_{50} =13 µg/ml.

Table 4.2: Effect of methanolic extracts of *Aphanamixis polystachya* on brine shrimp nauplii.

Concentration (µg/ml)	**Total population**	**Survivors**	**Death**	**% Mortality**	**LC_{50} (µg/ml)**
50	10	10	0	0	
75	10	8	2	20	
125	10	7	3	30	
150	10	5	5	50	129
200	10	3	7	70	
250	10	2	8	80	
300	10	0	10	100	
350	10	0	10	100	

Figure 19: Effect of Methanolic extract from *Aphanamixis polystachya*



Here, LC_{50} was 129 µg/ml

Table 4.3: Results of the test samples *Aphanamixis polystachya.*

Sample	LC_{50} (µg/ml)	Regression equation	R^2
$KMnO_4$ (Std.)	13	y = 84.329x – 40.3	0.7704
Methanol extract	196	y = 124.27x – 216.64	0.966

The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration to highly significant with the highest concentration (400µg/ml). Maximum mortalities took place at a concentration of 400µg/ml, whereas least mortalities were at lowest concentration. In other words, mortality increased gradually with the increase in concentration of the test samples.

4.2 RESULT: ANTI-MICROBIAL SUSCEPTIBILITY TEST

The methanolic extracts of the plant exhibited very low activity against the tested micro organisms compared to the Ciprofloxacin (std.) disc. The average zone of inhibition produced by Ciprofloxacin was 42.33 mm, where the average zone of inhibition for methanolic extract was 2.3 mm and 6 mm at a concentration of 500 µg/disc and 1000 µg/disc respectively.

Table 4.4: Antimicrobial activity of methanolic crude extract

Microorganisms	Zone of inhibition (mm)		
	500µg/disc	1000µg/disc	Ciprofoxacin (Std.)
Staphylococcus aureus (gram +ve bacteria)	7	10	42

Microorganisms	Zone of inhibition (mm)		
	500µg/disc	1000µg/disc	Ciporfloxacin (std)
Shigella dysentery (gram -ve bacteria)	0	8	38
Candida albicans (fungi)	0	0	47

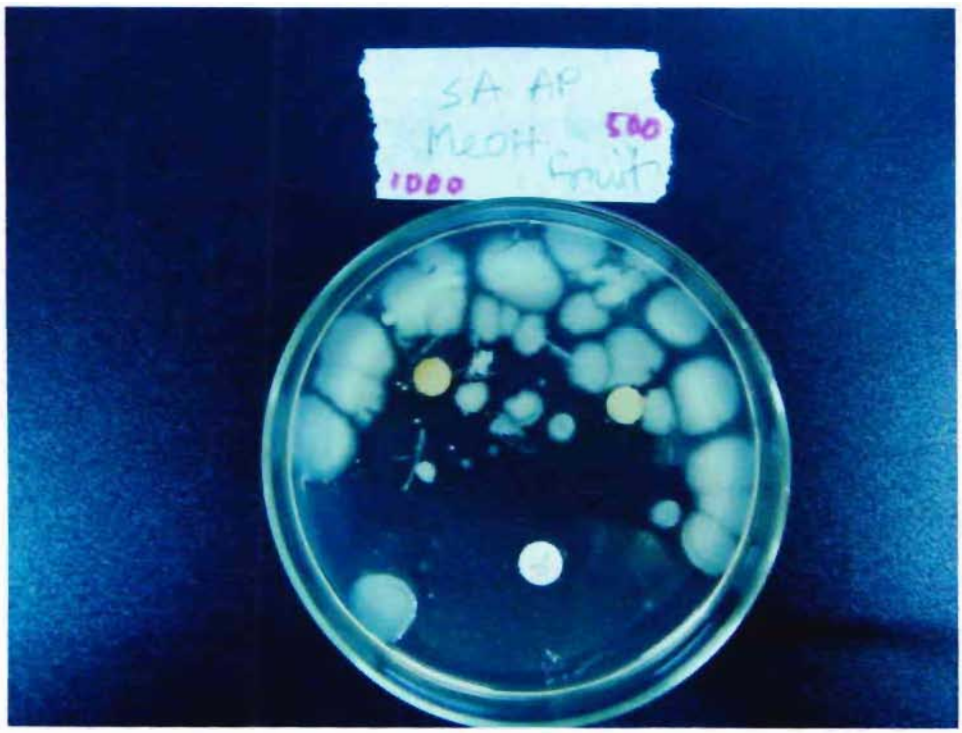


Figure 20: Methanolic crude extracts against *Staphylococcus aureus*

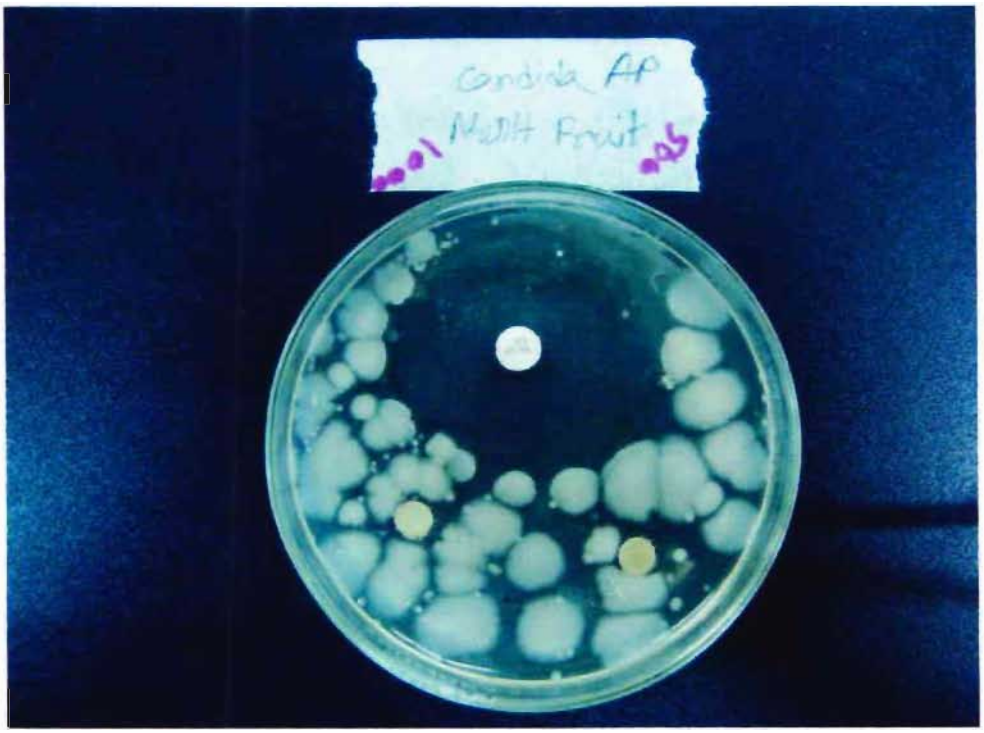


Figure 21: Methanolic crude extracts against *Candida albicans*

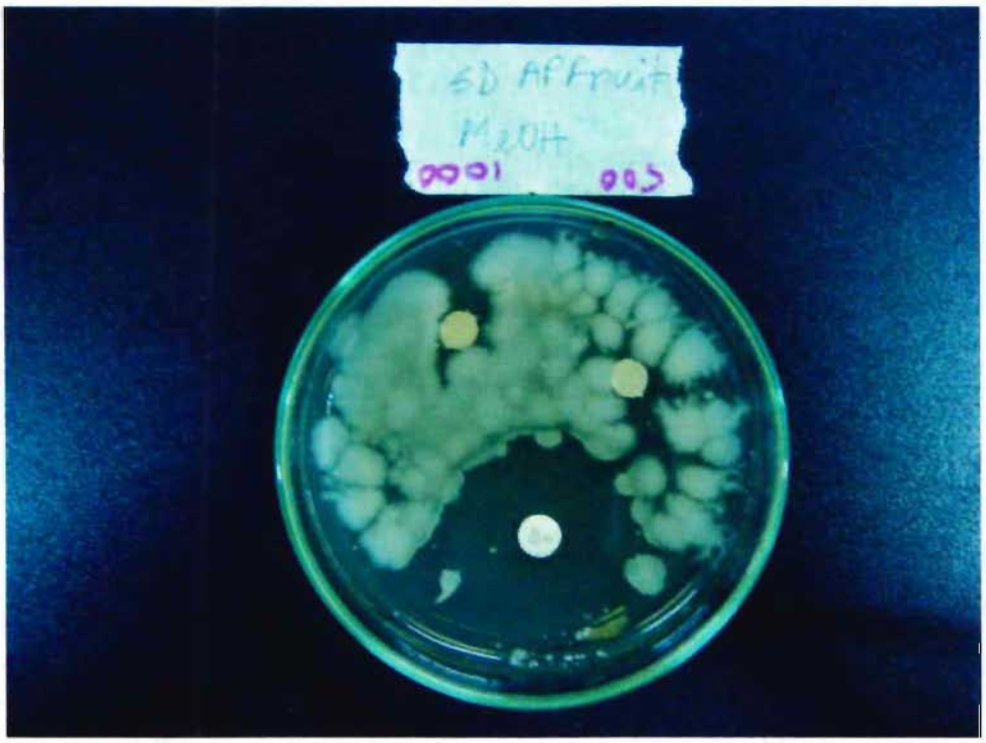


Figure 22: Methanolic crude extracts *Shigella dysentery*

4.3 DISCUSSION

The degree of lethality was directly proportional to the concentration of the extract ranging from significant with the lowest concentration to highly significant with the highest concentration. Maximum mortalities took place at the highest concentration, whereas least mortalities were at lowest concentration. In other words, mortality increased gradually with the increase in concentration of the test samples. The lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the shrimps killed against the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. From test results, it was proved that *Aphanamixis polystachya* methanolic extract of fruit has the ability to provide active cytotoxic effect. LC_{50} values less than 250 µg/ml from crude extracts are considered significantly active and has the potential for further investigation [41].

The anti-microbial susceptibility test results showed very minor presence of that property. Against *Staphylococcus aureus* representing gram positive bacteria, it provided its best anti-microbial effect but compared to Ciprofloxacin, it was very poor. *Shigella dysentery* represented gram negative bacteria and *Candida albicans* represented fungi in the test. The final results showed almost no anti-microbial property against them. So, from the test results, it was proved that *Aphanamixis polystachya* methanolic extract of fruit has no active anti-microbial property.

Chapter Five

CONCLUSION

5 CONCLUSION

The cytotoxic and antimicrobial activities of the methanol fraction of *Aphanamixis polystachya* fruits, found in this study, may enlighten some of the medicinal uses of this plant. These could be of particular interest in attempt to reveal its unexplored efficiency and can be a potential source of chemically remarkable and biologically significant drug candidates.

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