Pharmacological Screening of Four Medicinally Important Plants: *Curcuma zedoaria, Nymphoides indica, Drynaria quercifolia* and *Rhynchostylis retusa*

A dissertation submitted to the Department of Pharmacy, East West University as a partial fulfillment for the degree of Bachelor of Pharmacy

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DEDICATON

This Paper is dedicated to

My Parents

Declaration by the Research Candidate

I, **Aunonno Chowdhury**, hereby declare that the dissertation entitled- "Pharmacological Screening of Four Medicinally Important Plants: *Curcuma zedoaria, Nymphoides indica, Drynaria quercifolia* and *Rhynchostylis retusa,*", 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 is a record of original research work carried out by me during Fall 2012- Spring 2013 under the supervision and guidance of **Dr. Chowdhury Faiz Hossain**, Chairperson and Professor, Department of Pharmacy, East West University and **Dr. Shamsun Nahar Khan**, Associate Professor, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/ Diploma/ Fellowship or other similar title to any candidate of any university.

Date: 12/01/2014 **Aunonno Chowdhury**

I.D. # 2008-3-70-043

THESIS CERTIFICATE

This is to certify that the thesis paper entitled- "Pharmacological Screening of Four Medicinally Important Plants: *Curcuma zedoaria*, *Nymphoides indica*, *Drynaria quercifolia* and *Rhynchostylis retusa*," is submitted to the Department of Pharmacy, East West University, Aftabnagar, Dhaka, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by **Aunonno Chowdhury** (I.D. # 2008-3-70-043) under our guidance and supervision and that no part of the proposal has been submitted for any other degree. We further certify that all sources of information and laboratory facilities availed of this connection are duly acknowledged.

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ABSTRACT

In this study, four species of medicinally important plants namely *Curcuma zedoaria* (Zingiberaceae), *Nymphoides indica* (Menyanthaceae), *Drynaria quercifolia* (Polypodiaceae) and *Rhynchostylis retusa* (Orchidaceae), having traditional claims for the treatment of various diseases were investigated for their pharmacological properties. All of the plant extracts were evaluated for antimicrobial activity by using disk diffusion method and agar well dilution method. Among the plant extracts, only *Nymphoides indica* crude extract and its Ethyl acetate fraction showed promising antimicrobial activity against a number of bacterial and fungi strains, which are known to be common pathogens to human. The highest zone diameter was 15 mm against *Streptococcus pyogens* and *Staphylococcus saprophyticus* at a dose of 800 µg for the crude extract of *Nymphoides indica* whereas the ethyl acetate fraction of *Nymphoides indica* showed highest inhibition zone diameter of 10 mm on *Bacillus cereus* at a dose of 200 µg. The VLC fractions of *Curcuma zedoaria* was investigated for analgesic activity by using acetic acid induced writhing method. All the fractions except fraction 3 (ethyl acetate) showed significant reduction ($p<0.05$, and $p<0.01$) of writhing method induced by acetic acid at a dose of 200 mg/kg. Among all the fractions, fraction 2 (DCM) showed highest percentage inhibition of 45.52% reduction in writhing at a dose of 200 mg/kg., which was greater than the inhibition shown by the reference drug Diclofenac (39.74% at a dose of 10 mg/kg). The VLC fractions of *Nymphoides indica* was investigated for anti-inflammatory activity by using Carrageenan induced paw edema method. Fraction 3 (ethyl acetate) of the plant showed better activity than the reference drug Indomethacin (10 mg/kg) at a dose of 400 mg/kg.

Keywords: *Curcuma zedoaria, Nymphoides indica, Drynaria quercifolia, Rhynchostylis retusa,* antimicrobial activity, disk diffusion method, agar well dilution method, analgesic, anti-inflammatory, carrageenan induced paw edema method, acetic acid induced writhing method.

Chapter 1

Introduction

1. Introduction

1.1. Natural Products

Products of natural origin can be called 'Natural Products'. Natural products include: (1) an entire organism (e.g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g., drying), (2) part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ), (3) an extract of an organism or part of an organism, or exudates, and (4) pure compounds (e.g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc.) isolated from plants, animals or microorganisms. Although, in most cases, the term natural products refers to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. Natural products can be from any marine source: plants (e.g., Paclitaxel from *Taxus brevifolia*), animals (e.g., Vitamin A & D from cod liver oil), or microorganisms (e.g., Doxorubicin from *Streptomyces peucetius*).[1]

Over the last century, natural products have been the major sources of chemical diversity for starting materials for driving pharmaceutical companies. Many natural products and synthetically modified natural product derivatives have been developed successfully for clinical use to treat human diseases in almost all therapeutic classes.[2]

The strategies used for the research in the field of natural products have evolved quite significantly over the last few decades. These can be broadly divided into two categories –

1) Older Strategies
    a. Focus on chemistry of compounds from natural sources, but not on activity.
    b. Straightforward isolation and identification of compounds from natural sources followed by biological activity testing (mainly *in vivo*)
    c. Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.
2) Modern Strategies
    a. Bioassay-guided (mainly in vivo) isolation and identification of active "lead" compounds from natural sources.
    b. Production of active compounds in cell or tissue culture, genetic manipulation, natural combinatorial chemistry, and so on.
    c. More focused on bioactivity.

d. Introduction of the concepts of dereplication, chemical fingerprinting and metabolomics.[1]

A generic protocol for the drug discovery from natural products using a bioassay- guided approach is presented in the following figure.

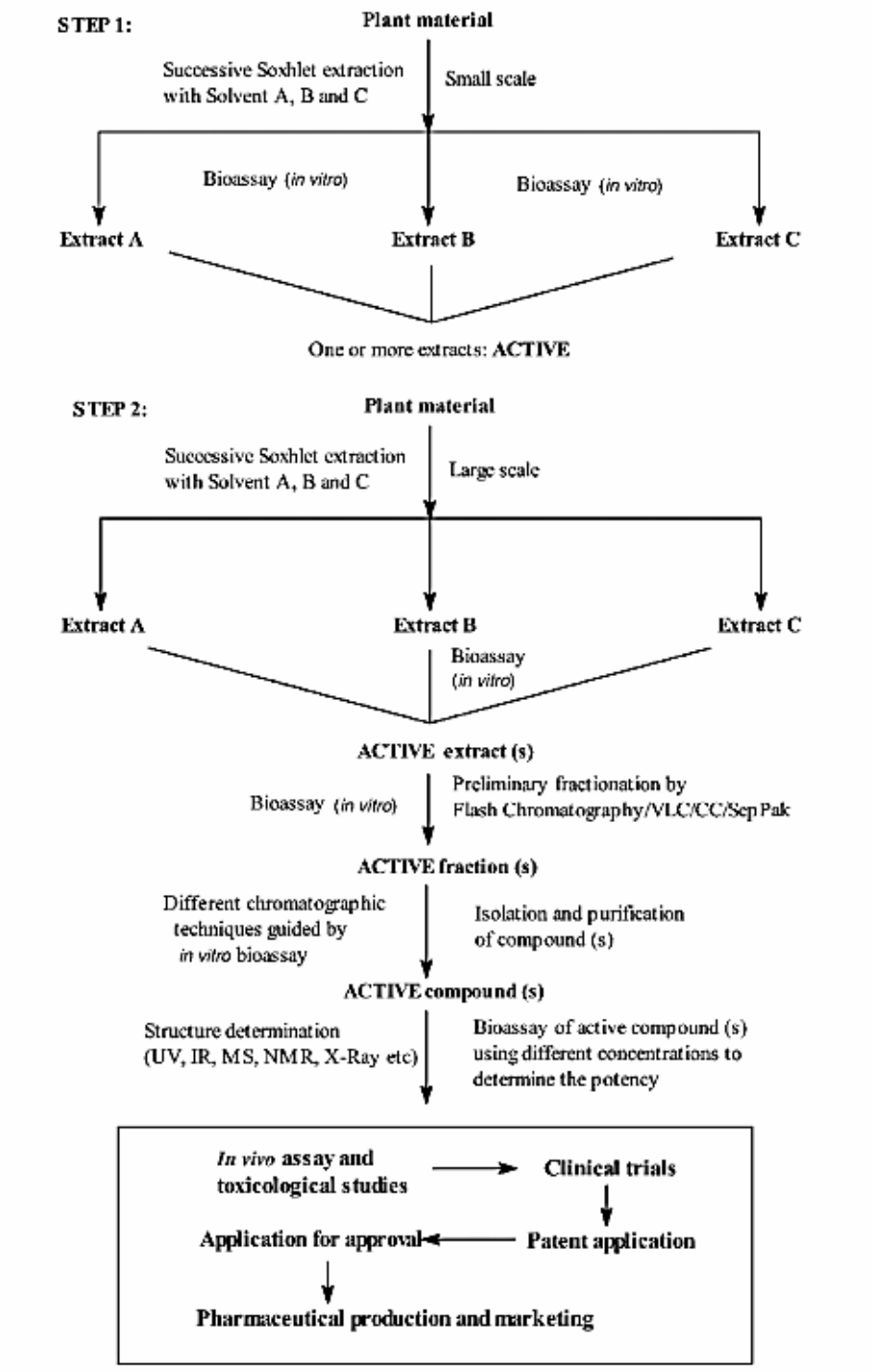

Figure 1: An example of natural product drug discovery process (bioassay- guided approach)[1]

1.2. Natural Products: Historical Perspective

The use of natural products with therapeutic properties is as ancient as human civilization, and for a long time, mineral, plant and animal products were the main sources of drugs.[3] The therapeutic use of plants certainly goes back to the Sumerian civilization, and 400 years before the Common Era, it has been recorded that Hippocrates used approximately 400 different plant species for medical purposes.[1] However, it was not until the early 1800's that the active constituents from plants were isolated. It was at this point that the effectiveness of medicinal natural products began to be attributed to science and not to magic or witchcraft. In 1826, E. Merck produced the first commercially pure natural product, morphine.[4] The thriving of natural product discovery efforts took place after the large scale production of penicillin during World War II. The discovery of streptomycin, gentamicin, tetracycline and other antibiotics stimulated the industry to develop large research programs around natural product discovery, particularly microbial fermentation based technologies. All of the prominent pharmaceutical companies had programs on natural product discovery, and these programs focused not only on anti-microbial and anti-fungal targets, but also on targets other than infectious diseases.[2]

1.3. Natural Products: Present and Future

For thousands of years, nature has been a source of therapeutic agents and a significant number of modern drugs have been developed from natural sources, many based on their use in traditional medicine. Over the last century, a remarkable number of top selling drugs have been derived from natural products (Vincristine from *Catharanthus roseus*, morphine form *Papaver somniferum*, quinine and quinidine from *Cinchona* spp.). Nowadays, approximately 40% of the modern drugs have been developed from natural sources[1]. More precisely, 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60-80% of antibacterial and anti-cancer drugs were from natural origin. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancer had natural origin. In 2001, eight (simvastatin, pravastatin, amoxicillin, clavulanic acid, azithromycin, ceftriaxone, cyclosporine and paclitaxel) of the 30 top-selling medicines were natural products or their derivatives.[5]

In light of all these facts, natural product drug discovery process failed to generate little respect. As drug discovery has emerged into a highly competitive era in which the quality of chemical collections and the time taken from assay to drug development are crucial factors in

the success of a company, 'Combinatorial Chemistry' has become the 'darling' of the pharmaceutical industry, bringing with it the promise of new level of chemical diversity.[6] But this adoption of new strategy by the pharmaceutical companies gained little momentum. Biotechnology companies working in the fields of combinatorial biosynthesis, genetic engineering and metagenomic approaches to identify novel natural product lead molecules have met with limited success. These disappointments have led the pharmaceutical industry to consider whether natural product chemical diversity can or will continue to generate valuable templates for drug development.[2]

Natural products offer a potentially infinite source of chemical diversity unparalleled to any synthetic chemical collection or combinatorial chemistry approach. In addition to that, these potent natural product compounds can have astounding chemical structures that can lead to unexpected, alternative medicinal chemistry programs based on important biological targets.[6] In the past few years, new natural products with a wide variety of chemical classes have been reported in the scientific literatures. Moreover, a total of 19 natural product based drugs were approved for marketing worldwide in between the year 2005 to April 2010, among which 7 being classified as natural products, 10 semi-synthetic natural products and 2 natural product derived drugs.[7]

Table 1: Natural product derived drugs launched during 2005-2010; lead compounds, and therapeutic area.[7]

Year	Trade name	Lead compound	Therapeutic area
2005	Dronabinol (Sativex™)	Dronabinol	Pain
2005	Fumagillin (Flisint™)	Fumagillin	Antiparasitic
2005	Tigecycline (Tygacil™)	Tetracycline	Antibacterial
2005	Zotarolimus (Endeavor™)	Sirolimus	Cardiovascular
2006	Anidulafungin (Eraxis™)	Echinocandin	Anti-fungal
2006	Exenatide (Byetta™)	Exenatide-4	Diabetes
2007	Lisdexamfetamine (Vyvanse™)	Amphetamine	ADHD
2007	Temsirolimus (Torisel™)	Sirolimus	Oncology
2008	Methylnaltrexone (Relistor™)	Naltrexone	Pain
2009	Telavancin (Vibativ™)	Vancomycin	Antibacterial
2009	Romidepsin (Istodax™)	Romidepsin	Oncology
2010	Monobactam aztreonam (Cayston™)	Monobactam aztreonam	Antibacterial

1.4. Phytochemistry

Phytochemistry can be defined as the biochemical study of plants which is concerned with the identification, biosynthesis, and metabolism of chemical constituents of plants, especially used in regard to natural products.[8] Phytochemistry is considered as one of the early subdivisions of organic chemistry. It has been of great importance in the identification of plant substances of medicinal importance.[9] According to a recent survey conducted by the United Nations Commission for Trade and Development (UNCTAD), more than 33 percent of modern drugs and medicinal products are developed from plants. The medicinal and pharmaceutical properties of these plants are due to the chemical substances they produce and store. The usual term used to refer these various chemical substances in plants in *constituents*. The constituents, which possess pharmacological characteristics, are called active constituents. Phytochemistry is concerned with the chemical study of these plant constituents.[10]

1.5. Metabolites

Metabolites are the products of enzyme-catalyzed reactions that occur naturally within cells. To be classified as a metabolite, a compound must meet certain criteria. A summary of the major factors to consider in designating a substance 'metabolite' is given in the following.

i. Metabolites are compounds found inside cells.
ii. Metabolites are recognized and acted upon by enzymes.
iii. The product of a metabolite must be able to enter into subsequent reactions.
iv. Metabolites have a finite half-life and they do not accumulate in cells.
v. Many metabolites are regulators that control the pace of metabolism.
vi. Metabolites must serve some useful biological functions in the cell.[11]

Technically speaking, a compound outside the cell is not considered a metabolite. Glucose, for example, when in the blood is considered a metabolic product excreted from the cell but when inside the cell, glucose is a metabolite because of its vulnerability to chemical change.[11]

A plant cell produces two types of metabolites- 1) Primary metabolites involved directly in growth, development and reproduction (carbohydrates, lipids & proteins) and 2) Secondary metabolites are not required for normal growth and development, but usually have an important ecological function (Alkaloids, terpenoids and phenolics).

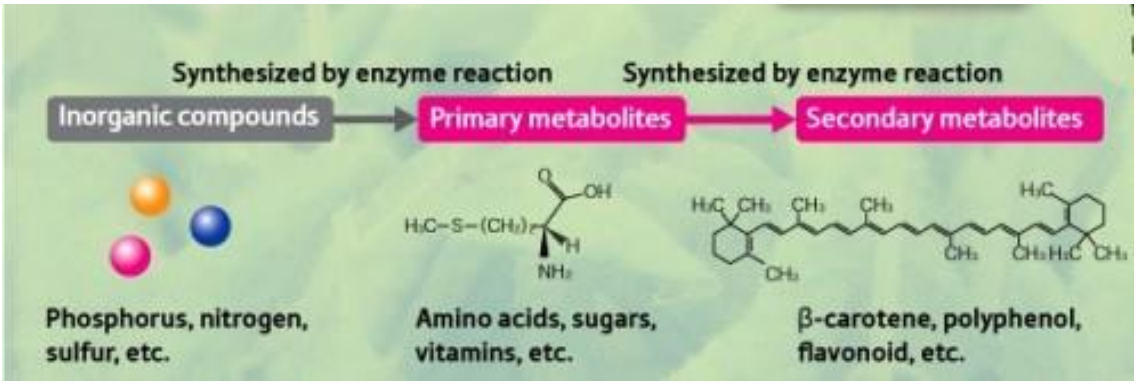

Figure 2: Formation of Primary and Secondary Metabolites

1.5.1. Primary Metabolites

Primary metabolites are compounds that are commonly produced by all plants and that are directly used in plant growth and development. Primary metabolites comprise many different types of organic compounds, including, but not limited to, carbohydrates, lipids, proteins, and nucleic acids. They are usually available universally in the plant kingdom because they are the components or products of fundamental metabolic pathways or cycles such as glycolysis, the Krebs cycle, and the Calvin cycle. Because of the importance of these and other primary pathways in enabling a plant to synthesize, assimilate, and degrade organic compounds, primary metabolites are essential. Examples of primary metabolites include energy rich fuel molecules, such as sucrose and starch, structural components such as cellulose, informational molecules such as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), and pigments, such as chlorophyll. In addition to having fundamental roles in plant growth and development, some primary metabolites are precursors (starting materials) for the synthesis of secondary metabolites.

1.5.2. Secondary Metabolites

Secondary metabolites or secondary compounds are compounds that are not required for normal growth and development, and are not made through metabolic pathways common to all plants. Secondary metabolites have a wide range of chemical structures and biological activities. They are derived by unique biosynthetic pathways from primary metabolites and intermediates. In plant kingdom, they are limited to occurrence and may be restricted to particular taxonomic group. These secondary metabolites are synthesized in specialized cells at particular development stages making extraction and purification difficult.

Secondary compounds are grouped into classes based on similar structures, biosynthetic pathways, or the kinds of plants that make them. The largest such classes are the *alkaloids*, *terpenoids*, and *phenolics*. Many thousands of secondary metabolites have been isolated from plants, and many of them have powerful physiological effects in humans and are used as medicines. It is only since the late twentieth century that secondary metabolites have been clearly recognized as having important functions in plants. Examples of secondary metabolites include morphine, caffeine, nicotine, menthol, rubber etc.

1.6. Assay of Natural Products

In order to pinpoint the target compound(s) from a complex natural product extract, it is necessary to carry out chemical, biological, or physical assays. At present, natural product research is more focused on isolating target compounds (assay-guided isolation) rather than trying to isolate all the compounds present in any extract. The target compounds may be of certain chemical classes, have certain physical properties, or possess certain biological activities. Therefore, incorporation of appropriate protocols is necessary in the extraction and isolation protocol.[1]

The following factors should be kept under consideration when carrying out assays of natural products –

a) Samples dissolved or suspended in a solvent different from the parent extraction solvent must be filtered or centrifuged to get rid of any insoluble matter.
b) Positive or negative control should be incorporated in any assay.
c) The assay must be sensitive enough to identify active components in low concentration.
d) In order to prevent acidified or basified samples from interfering with the assay, they should be readjusted to their original pH.[1]

1.6.1. Physical Assay

Physical assays normally offer the comparison of various chromatographic and spectroscopic behaviors, e.g., HPLC, TLC, LC-MS, CE-MS, LC-NMR, and so on, of the target compound with a standard.[1]

1.6.2. Chemical Assay

Chemical assay usually involve different chemical tests for identifying the chemical nature of the compounds, e.g., $FeCl_3$ can be used to detect phenolics, Dragendorff's reagent for alkaloids, 2,2-diphenyl-1-picrylhydrazyl (DPPH) for antioxidant compounds, and many more.[1]

1.6.3. Bioassay

Bioassays can be defined as the use of a biological system to detect properties (e.g., antibacterial, antifungal, anticancer, anti-HIV, antidiabetic, etc.) of a crude extract, chromatographic fraction, mixture, or a pure compound.[1] Bioassays are crucial for the isolation of active compounds from various natural sources. The usual method for isolation of active compounds is the bioassay guided fractionation. The assays can be selected based on the nature and type of activity that is desired to isolate. An ideal bioassay would be highly sensitive to small amounts of active material, selective to the specific bioactivity, cost effective and easy to run or maintain.[12]

Bioassays could involve the use of *in vivo* systems (clinical trials, whole animal experiments), *ex vivo* systems (isolated tissues and organs), or *in vitro* systems (e.g., cultured cells). Among all these, *in vivo* studies are considered to the more relevant to clinical conditions and can also provide toxicity data at the same time. Although, disadvantages of these studies include costs, need for large amount of test compounds/fractions, complex design, and difficulty in mode of action determination. On the other hand, *in vitro* bioassays are faster, and small amount of test samples are required, but might not be relevant to clinical conditions. The trend has now moved from *in vivo* to *in vitro*.[1]

In general, bioassays are broadly classified into two major categories; 1) mechanism based assays involve measurement of the specific activity of the drug towards a specific enzyme, DNA, receptor etc. and 2) cell based assays involve drug-cell interactions with the whole intact cell rather than just isolated system.

Chapter 2

Rationalization of the Work

2. Rationalization of the Work

As discussed in the previous chapter, a range of different approaches has been employed to obtain lead compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial synthesis and molecular modeling. Despite the recent advances in the fields of combinatorial chemistry and other synthetic techniques, natural products and particularly medicinal plants, remain an important source of new drugs and new lead compounds.

The approach for the development of drugs from plant sources depends on the purpose. The selection of a suitable plant for a pharmacological study is very essential and decisive step. There are different ways to select a suitable plant, including traditional use, chemical content, toxicity, randomized selection or a combination of several criteria. The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; which is usually known as ethnobotany or ethnopharmacology. How the plant is used by an ethnic group is a very important consideration. Keeping these selection criteria in mind, I selected four medicinally important plants for my research work.

Curcuma zedoaria is a very well-known ethnomedinical herb that is also used in Ayurveda. Its use in the traditional folk medicine is also well documented. It is used traditionally for the treatment of menstrual disorders, dyspepsia, vomiting and cancer. *Nymphoides indica* is an aquatic plant which has traditional medicinal values for the treatment of fever, dysentery, rheumatism and headache. *Drynaria quercifolia* is a parasitic fern which is very commonly distributed in Bangladesh and it is very widely used by various ethnic groups to treat health problems like chest diseases, cough, hectic fever, dyspepsia, loss of appetite, chronic jaundice, and infectious diseases. *Rhynchostylis retusa* is an orchid species which is commonly used for the treatment of rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations.

Encouraged by these ethnobotanical data on the traditional uses of these plants, I wanted to explore the potential biological activities of the extracts (crude and VLC) which were previously collected as a part of the on-going research project conducted and supervised by Dr. Chowdhury Faiz Hossain.

Thus, I have selected these four medicinally important plants for my research project.

Chapter 3

Introduction to Plants

3. Introduction to Plants

3.1. *Curcuma zedoaria*

3.1.1. Taxonomy

Classification

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division: Magnoliophyta - Flowering plants

Class: Liliopsida - Monocotyledons

Subclass: Zingiberidae

Order: Zingiberales

Family: Zingiberaceae

Genus: *Curcuma* L. - Curcuma

Species: *zedoaria* (Christm.) Roscoe – Zedoary[13]

Synonyms

- *Curcuma longa* (misapplied)
- *Amonum latifolia* Lamik. (1692)
- *Curcuma officinalis* Salisb. (1747)
- *Amomum zadoria* Christm (1779)
- *Amomum zerumbet* Keon (1783)
- *Curcuma zerumbet* Roxb (1810)

Common Names

- Zedoary (English)
- Rose Turmeric (English)
- Er-Jyur (Chinese)
- Kentjur (Indonesian)
- Cedoaria (Spanish)
- Zitwer (German)
- Zedoaire (French)

Local Names

- Shoti,
- Failla,
- Krachura (Sanskrit),
- Gandamatsi (Hindi), and
- Sutha (Bengali).

3.1.2. Description

Curcuma zedoaria, also known as **Zedoary**, is an herb that grows up to 1.2 m in height. This plant has both vertical aerial stems (pseudostems) and horizontal underground stems known as rhizomes, which allow the plant to spread so this species often grows in large clumps. The swollen underground stems are yellow or orange colored inside and are aromatic when crushed. The leaves of Zedoary are oblong and can be up to 81 cm long and 18 cm wide. This species can be recognized by the presence of a purplish hue along the mid vein of the leaf blades. The clusters of flowers are produced in a dense aggregation on an above ground stalk that grows from a leafless underground stem. Green or red tinted bracts at the base of the inflorescence enclose the flowers. The pink, white, or red, upper bracts in the inflorescence contain no flowers but may serve to attract pollinators. The white flower petals are 4.8 cm long and enclose the stamens (pollen producing structures) and ovary (ovule producing structures). Six stamens are present in these flowers although five of these are sterile. The five sterile stamens are fused to form a lip-like structure that is colored and resembles a petal. The ovary of Zedoary is a three-parted capsule and breaks open at maturity to release the seeds. Many seeds are produced in each fruit, each of which is surrounded by a fleshy covering.[14]

3.1.3. Distribution

Zedoary is native to Southeast Asia, although the exact distribution of this species prior to human influence is not known as it has been dispersed along with human migrations throughout its history.[14] It has become naturalized (existing outside of cultivation) in India, Bhutan, Malaysia, Indonesia. In Bangladesh, this species is fairly common at Chittagong, Dhaka, Srimangal and Dinajpur.[15]

3.1.4. Medicinal Uses

The rhizome of Zedoary is used extensively as a medicine largely for its bitter properties. This species was included in the American National Formulary IV under the name Zedoaria. This publication provided instructions for the preparation of bitter tinctures, antiperiodic pills, and antiperiodic tincture. The rhizome is considered to aid digestion, to purify the blood, to provide relief for colic, and for the treatment of colds and infections. The essential oil is an active ingredient in antibacterial preparations. In India the rhizome is chewed to alter a sticky taste in the mouth, and in both Java and India a decoction of the root is used to treat weakness resulting from childbirth. It is combined with pepper, cinnamon and honey and used to treat colds.[14]

3.1.5. Figures of Curcuma zedoaria

Figure 3: ***Curcuma zedoaria***

3.2. *Nymphoides indica*

3.2.1. Taxonomy

Classification

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division: Magnoliophyta - Flowering plants

Class: Magnoliopsida - Dicotyledons

Subclass: Asteridae

Order: Solanales

Family: Menyanthaceae – Buckbean family

Genus: *Nymphoides* Hill - floatingheart

Species: *Nymphoides indica* (L.) Kuntze – water snowflake

Synonyms

- *Limnanthemum indicum* (Linn.) Griseb.
- *Limnanthemum kleinianum* Grisebach
- *Limnanthemum niloticum* Kotschy & Peyr.
- *Limnanthemum senegalensis* (G.Don) N.E.Br.
- *Limnanthemum wrightianum* Grisebach

Common Names

- Water snowflake (English)
- Floating hearts (English)
- Banana plant (English)
- Menyanthe de l'Inde (French)

Local Names

- Kumudini (Hindi)
- Tharo mancha (Manipuri)
- Anthara thaamara (Telegu)
- Chandmala (Bengali)

3.2.2. Description

Nymphoides, indica is a perennial aquatic plant that grows in a large scale during rainy season. It is a species that grows underwater, with the leaves of the plant floating on the surface like water lilies. The flowers are held upright above the leaves. This species prefers warm still or slowly moving water, to about 2 m in depth. *Nymphoides indica* has white flowers with yellow centers. The petals have unusual, feathery edges. The flowers are formed above the floating leaves with 20-40 mm long stalks from about October until May. It gets its common name, of 'Floating hearts' because of the heart-shaped, bright green leaves which lie on the water surface like those of water lilies (*Nymphaea* species). Although they bear some resemblance to water lilies, they are not related. Round, flat, bright green, lily-like leaves (2-8" diameter) cover the water surface somewhat like water lily. Umbels of white, snowflake-like flowers (to 1" diameter) with 5-lobed corollas and yellow centers bloom on stalks above the floating leaves in summer. Corolla lobes are fringed and covered with frilly hairs. Flowers last only one day. Plantlets are produced at the leaves. In mild winter conditions, plants can spread somewhat aggressively.

3.2.3. Distribution

Nymphoides indica is found in tropical areas all over the world. It is be found in pools, pans, marshes and rivers locally and throughout Southern Africa. Other areas where it occurs naturally include Australia, Tropical Africa, New Zealand, China, India, Bhutan, Bangladesh, Indonesia, Panama, Japan, Mexico, Mali, Nepal, Philippines, Romania, Spain, Sri Lanka, Switzerland, Taiwan, and Vietnam. In India, it is reported from most of the states.

3.2.4. Medicinal Uses

Leaf paste of *Nymphoides indica* is applied on forehead to get relief from headache due to bile. Plant decoction is drunk to cure fever and dysentery. Dried plants are dipped in sesame (*Sesamum indicum*) oil for 7 days; the oil is filtered, stored and applied externally to get relief from headache, rheumatism and bile. Leaf paste with turmeric is applied externally to cure scabies; applied on the swelling part of the body to get relief.[16]

3.2.5. Figures of Nymphoides indica

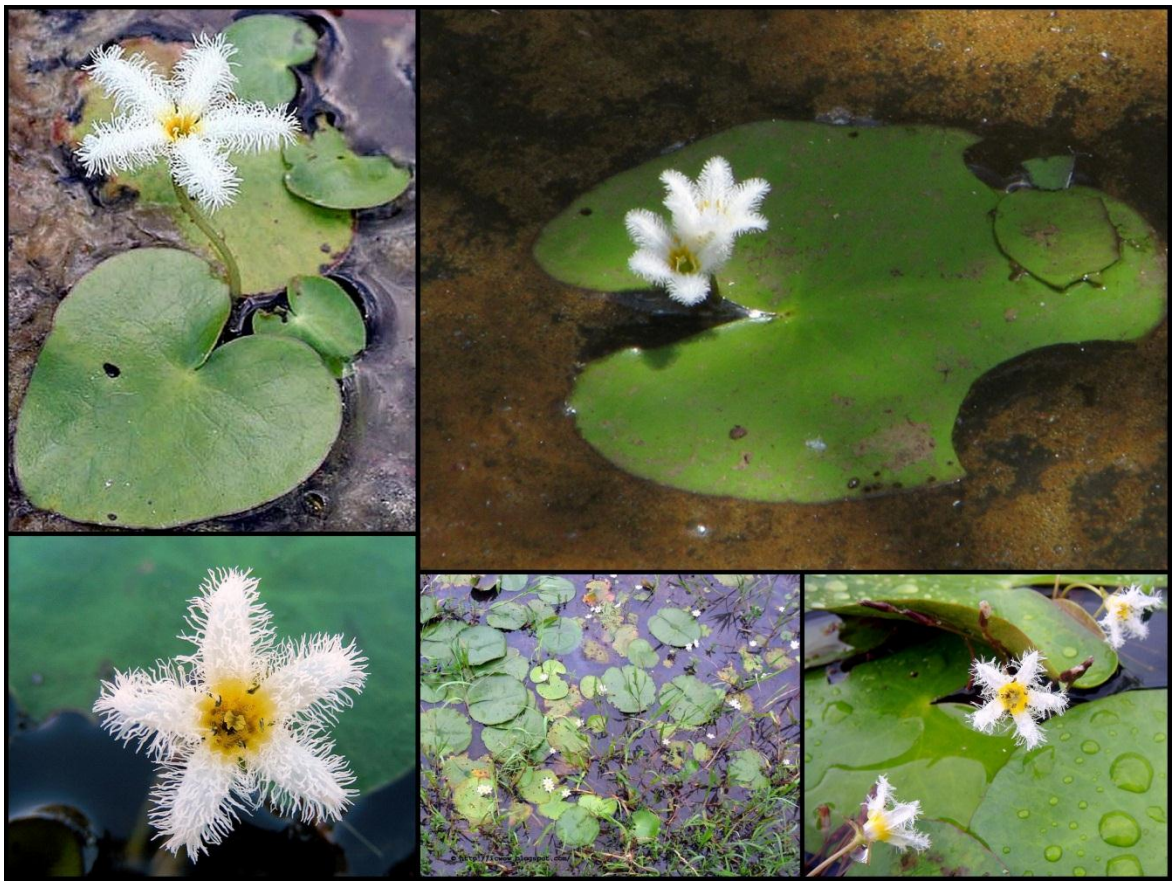

Figure 4: ***Nymphoides indica***

3.3. *Drynaria quercifolia*

3.3.1. Taxonomy

Classification

Kingdom: Plantae - Plants

Subkingdom: Tracheobionta - Vascular plants

Division: Pteridophyta - Ferns

Class: Filicopsida

Order: Polypodiales

Family: Polypodiaceae – Polypody family

Genus: *Drynaria* (Bory) J. Sm.

Species: *Drynaria quercifolia* (L.) J. Sm.

Synonyms

- *Polypodium quercifolium* L.

Common Names

- Oak-leaf basket fern (English)
- Kabkab (Philippines)
- Aswakatri (Sanskrit)

Local Names

- Pankhiraj (Bengali)
- Pankha (Bengali)
- Gurar (Bengali)
- Chilo vasa (Chakma)
- Fo-lo-rere (Marma)
- Nashy- prampap (Murang)

3.3.2. Description

Drynaria quercifolia is an epiphytic fern growing from sea level to medium altitude. It is a large herb with densely scaly woody rhizome. Leaves in adult plants are two types- 1) sessile, cordate-oblong, persistent bract leaves with a pinnately lobed margin and clasping the rhizome to serve as human collectors, and 2) oblong, pinnatisect, normal leaves with a stout, brownish, winged petiole, about a foot or long. Bract leaves are up to 9 inches long and up to 6 inches wide, persistent, clinging to the rhizome as dry, brown, hard structures with a prominent midrib and main lateral veins. Lamina of normal leaves 2-3 feet long and about a foot or so wide pinnatisect each segment being linear with a broad base, tapering apex, and entire margin. Venation is reticulate. Leaves of young plants (up to 2 or 3 years old) are all sessile, simple lanceolate, with an attenuated base and reticulate veination. Sporangia are observed on normal leaves only, in punctiform sori, over the vein plexuses forming two irregular rows between two adjacent main lateral veins of the lobes non-indusiate. Spores are bilateral with very minute sparse echinations on the exine. Gametophytes are cordate with marginal club-shaped unicellular hairs.[17]

3.3.3. Distribution

Drynaria quercifolia is a parasitic fern that is usually distributed in Bangladesh, India and Thailand.[18] It also occurs naturally in places including China, Malaysia, Indonesia, Philippines, New Guinea, Australia, It usually occurs in fairly sunny, humid situations. It sometimes grows in large, spreading colonies, especially in sandstone crevices.

3.3.4. Medicinal Uses

Drynaria quercifolia is used in traditional medical system by different groups of people to treat various kinds of health problems. It is used as pectoral, expectorant and anthelmintic agent. It is also used in the treatment of chest diseases, cough, hectic fever, dyspepsia, loss of appetite, chronic jaundice and cutaneous affection. Pounded fronds are used as poultice for swellings. Peeled rhizome with sugar is prescribed for urinary disorders and in spermatorrhoea. Tribals in Kalakad Mundanthurai Tiger Reserve, India, used the rhizome of this fern to cure rheumatism. The rhizome of this fern is one of the twelve ingredients of a drug to treat cancer.[19] The rhizome of the plant also has anti-bacterial properties. In different regions of Lakshmipur district of Bangladesh, the rhizome of this plant is used by local people in the treatment of excited mental disorder.[20] Other studies have shown that, the rhizome is also used to treat typhoid fever and it is also used as anti-fertility agent as well as anti-pyretic agent. The tribals of the Kolli hills of Tamil Nadu use the rhizome of the plant as an anti-inflammatory agent.[21] ASEAN center for Biodiversity mentioned in their checklist of medicinal plant in Southeast Asia that rhizome decoction or drink of *Drynaria quercifolia* rhizome uses as antipyretic preparation.[22] *Drynaria quercifolia* is also used topically in traditional Chinese medicine to stimulate hair growth and to treat baldness.[23]

3.3.5. Figures of Drynaria quercifolia

Figure 5: ***Drynaria quercifolia***

3.4. *Rhynchostylis retusa*

3.4.1. Taxonomy

Classification

Kingdom: Plantae - Plants

Division: Magnoliophyta - Flowering plants

Class: Liliopsida

Order: Asparagaceae

Family: Orchidaceae

Subfamily: Epidendroideae

Tribe: Vandeae

Subtribe: Aeridinae

Genus: *Rhynchostylis*

Species: *R. retusa* (L.) Blume

Synonyms

- *Epidendrum retusum* L.
- *Aerides guttata* (Lindl.) Roxb.
- *Aerides praemorsa* Willd.
- *Aerides retusa* (L.) Sw.
- *Anota violacea* (Rchb.f.) Schltr.
- *Epidendrum hippium* Buch.-Ham. ex D.Don.
- *Epidendrum indicum* Poir.
- *Gastrochilus blumei* (Lindl.) Kuntze.
- *Gastrochilus praemorsus* (Willd.) Kuntze.
- *Gastrochilus retusus* (L.) Kuntze.
- *Limodorum retusum* (L.) Sw.
- *Orchis lanigera* Blanco.
- *Rhynchostylis albiflora* I.Barua & Bora.
- *Rhynchostylis praemorsa* (Willd.) Blume.
- *Rhynchostylis violacea* Rchb.f.
- *Saccolabium blumei* Lindl.
- *Saccolabium garwalicum* Lindl.

Common Names

- Foxtail Orchid (English)
- Blunt Rhynchostylis (English)
- Dove flower (English)
- Zuan Hui Lan (Chinese)

Local Names

- Kopou Phool (Bangla)
- Chintaranamu (Telegu)
- Draupadi Mala (Hindi)
- Gajara (Marathi)
- Seetechi Veni (Marathi)
- Seethamudi (Malayalam)

3.4.2. Description

Rhynchostylis retusa is a monopodial, epiphytic orchid species with beautiful flowers arranged in racemose inflorescence. It is an engendered orchid species that grows in moist areas and blossoms during monsoon. The orchid has a bunch consisting of more than 100 pink-spotted white flowers. They have stout, repent, short stem carrying up to 12, curved, fleshy, deeply channeled, keeled, retuse apically leaves and blooms on an axillary pendant to 60 cm long, racemose, densely flowered, cylindrical inflorescence that occurs in the winter and early spring.

3.4.3. Distribution

The plant is found in semi-deciduous and deciduous dry lowland forests woodlands at elevations of sea level to 1,200 m (3,900 ft.), and can be found in Bangladesh, Benin, Burma, Cambodia, China, India, Indonesia, Laos, Malaysia, Nepal, Philippines, Singapore, Sri Lanka, Thailand and Vietnam.

3.4.4. Medicinal Uses

The whole plant preparations of *Rhynchostylis retusa* is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases and external inflammations.[24] It is also used as an emollient and in the treatment of throat inflammation.[25] The plant leaf juice and aerial roots are also used in ear pain and cleaning.[26]

In the Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain.[27] *Rhynchostylis retusa* roots are also used to cure malarial fever.[28] Juice of roots applied to cuts and wounds. Dried flowers are used as insect repellent and to induce vomiting.[29] It is also reported that the plant showed significant antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.[30]

3.4.5. Figures of Rhynchostylis retusa

Figure 6: *Rhynchostylis retusa*

Chapter 4

Literature Review

4. Literature Review

4.1. *Curcuma zedoaria*

4.1.1. Phytochemical Analysis

Curcuma zedoaria is a rich source of essential oils: starch, curcumin (**1**), arabin, gums etc. Makabe *et al*.[31] have isolated more than 10 sesquiterpenes from the rhizome of *C. zedoaria* and were able to structurally characterize 15 such compounds, namely, furanodiene (**2**), furanodienone (**3**), zedorone (**4**), curzerenone (**5**), curzeone (**6**), germacrone (**7**), 13-hydroxygermacrone (**8**), dihydrocurdione (**9**), curcumenone (**10**), and zedoaronediol (**11**).

Phytochemical analysis was carried out by Navarro *et al*.[32] using air-dried rhizomes (3 kg). The powder was extracted twice with dichloromethane at room temperature for five days, and then ethyl acetate and methanol, respectively. The extracts were then concentrated under reduced pressure to give the respective fractions. A part of the dichloromethane fraction (50 g) was chromatographed using a silica gel column eluted with a mixture of hexane-ethyl acetate in increasing polarity. The fraction F1 (3.5 g), obtained from the above, was re-chromatographed over a silica gel column, and when eluted with benzene- acetone (9:1), yielded about 500 mg of compound 1 and 150 mg of compound 2. Spectroscopic data (IR and NMR) confirmed identity of compound 1 as curcumenol (**12**) and compound 2 was a mixture of phytosterols (especially sitosterol and stigmasterol 2:1)

The seasonal variation of curcumenol and dihydrocurdione, two active terpenoids from different parts (roots, mother rhizome, and rugous rhizome) of *C. zedoaria* grown in Brazil was described by Christiane *et al.*[33] The analysis was carried out by high resolution gas chromatography, using external standards for determination. The results showed that both terpenoids are present in all parts studied. However, *C. zedoaria* exhibited about three times more terpenoids in the mother rhizome in autumn than in other parts and seasons studied.

A new eudesmane-type sesquiterpene, zedaorofuran, and six new guaiane or secoguaiane-type sesquiterpenes, 4-epicurcumenol (**49**), neocurcumenol (**51**), gajutsulactones A and B (**32** and **33**) and zedoarolides A and B (**30** and **31**) were isolated from the aqueous acetone extract of zedoaria rhizome together with 36 known sesquiterpenes and two diarylheptanoids. Their steriosturctures were elucidated on the basis of chemical and physiochemical evidence. Two guaiane derivatives were isolated from the rhizomes of *C. zedoaria*. Their structures, zedoalactone A and zedoalactone B (**40**), were established by ^{1}H and ^{13}C NMR spectroscopic

studies and by comparison with closely related compounds.[34] Zedoarol (**13**), 13-hydroxygermacrone and curzeone were isolated and structurally elucidated by Shiobara *et al*.[35] using *C. zedoaria*.

Three sesquiterpenoids, curcumenone, curcumanolide A (**14**) and curcumanolide B (**15**), were isolated from the dried rhizome of *C. zedoaria* by Shiobara *et al*.[36] Ehtyl para-methoxycinnamte (**16**) was isolated from the methalonic extract of *C. zedoaria* by chromatography on neutral alumina and silica gel.[37] In the course of searching for biologically active sesquiterpenoids from the Curcuma genus, two sesquiterpenoids were isolated from the rhizome of *C. zedoaria*. There structures were identified as α- turmerone (**17**) and β- turmerone (**18**). The structural elucidation of these compounds was carried out by comparison of their physical and spectral data with previously reported values.[38] Mau *et al*.[39] isolated essential oils from the rhizomes. They isolated a total of 36 compounds but were only able to structurally characterize epicurzerenone (**19**) and curzerene (**20**).

The essential oil obtained by hydrodistillation of the rhizome of *C. zedoaria* native to north-east India has been analyzed by gas chromatography (GC) and gas chromatography- mass spectroscopy (GC-MS). Thirty seven constituents representing about 87.7% of the total oil have been identified. Curzerenone (22.3%) was the major constituent, followed by 1,8-cineole (**21**) (15.9%) and germacrone (9.0%).[40] The chemical investigation on essential oils of rhizomes of *C. zedoaria*, done by GC and GC-MS, revealed the presence of 1,8-cineole (18.5%), cymene (**57**) (18.42%), α-phellandrene (**27**) (14.9%) and β-eudesmol (**22**) (10.6%).[41]

The essential oil produced by hydrodistillation of *C. zedoaria* leaves was characterized by GC and GC-MS. Twenty three compounds were identified, accounting for 75% of the oil. The oil of *C. zedoaria* was made up mainly of mono- and sesquiterpenoids, monoterpene hydrocarbons (2.3%), oxygenated monoterpenes (26%), sesquiterpene hydrocarbons (38%), and oxygenated sesquiterpenes (13.5%). The major constituent of the leaf oil were α-terpinyl acetate (8.4%), isoborneol (**58**) (7%) and dihydrocurdione (9%).[42]

Chemical analysis of the volatile oil from *C. zedoaria* using GC-MS technique revealed the presence of β- turmerone (19.88%), 1,8-cineole (8.93%) and zingiberene (**23**) (7.84%) as major constituents.[43]

The essential oil of the dried rhizome was isolated using simultaneous steam distillation and solvent extraction and its fractions were prepared by silica gel column chromatography. In total, 36 compounds were identified in the essential oil, including 17 terpenes, 13 alcohols, and 6 ketones. Epicurzerenone and curzerene were found in the first and second highest amounts (24.1 and 10.4%).[39]

Curcumin, dihydrocurcumin (**24**), tetrahydrodemethoxycurcumin and bisdemethoxycurcumin (**34**) were isolated together with bisabolane-type sesquiterpenes, bisacumol (**42**) and bisacurone (**43**), from 80% aqueous acetone extract of the rhizome of *C. zedaoria*. Bioassay-directed fractionation of an ethanol extract from *C. zedaoria* led to the isolation of an active curcuminoid, which was identified as demethoxycurcumin (**59**) by comparison of its ^{1}H and ^{13}C NMR spectra with literature data and by direct comparison with synthetic material. Curcumin and bisdemethoxycurcumin were also isolated.[44]

The variation of curcuminoids in the ethanolic extract of *C. zedoaria* was measured by using high-performance lipid chromatography (HPLC). The analysis was carried out at 425nm using a BDS Hypersil C18 column as a stationary phase, 0.1% acetic acid aqueous solution and acetonitrile as mobile phase. Ethanolic extracts of *C. zedaoria* rhizomes collected from various parts of Thailand contained curcumin, demethoxycurcumin and bisdemethoxycurcumin in the range of 1.46± 0.45 to 5.73±0.11% w/w (average 2.73±1.24% w/w), 3.15±0.15 to 10.98±0.28% w/w (average 7.37±2.71% w/w) and 0.49±0.02 to 2.99±0.20% w/w (average 1.40±0.82% w/w), respectively. The highest average total curcuminoid content in the extracts was found to be 16.83±0.63% w/w while the lowest version was 6.09±1.79 w/w. This information will be beneficial for further standardization of *C. zedaoria* extracts for which the content has not been reported elsewhere.[45]

Two new sesquiterpenoids, named as curcuzederone (**28**) and naringenin (**29**), were isolated and structurally determined by Sohee *et al*.[46] The chemical structures of these compounds were confirmed by interpretation of measured 1D and 2D NMR spectroscopy data and by comparison of these data with published values. Naringenin was isolated for the first time from this plant.

The compound isocurcumenol (**50**) was isolated by Lakshmi *et al*.[47] from the rhizome of *C. zedoaria* which was characterized by the MS and IR spectra significantly inhibited the cell proliferation in human lung, leukemia and murine lymphoma cells. GC-MS profile of the

petroleum ether extract showed isocurcumenol, methyl sterolate, elemene and isolongifolene as the prominent chemical constituents.

The structures of few biologically active compounds that have been isolated from *C. zedoaria* are given in Figure 7 & 8. Table 2 shows the percentage of various phytoconstituents present in *C. zedoaria.*

Table 2: Percentages of various phytoconstituents present in *Curcuma zedoaria*

Source	**Active constituents**	**Percentage (%)**
Oil from *C. zedoaria* rhizome[40]	Curzerenone	22.3
	1,8-cineole	15.9
	Germacrone	9.0
Oil from *C. zedoaria* rhizome[41]	Cymene	18.42
	α- phellandrene	14.90
	β- eudesmol	10.60
Oil from *C. zedoaria* leaves[42]	Monoterpene hydrocarbon	2.3
	Oxygenated monoterpene	20.6
	Sesquiterpene hydrocarbon	38.0
	Oxygenated sesquiterpene	13.5
	α- Terpinyl acetate	8.4
	Isoborneol	7.0
	Dehydrocurdione	9.0
Volatile oil from *C. zedoaria*	Epicurzerenone	24.1
	Curzerene	10.4

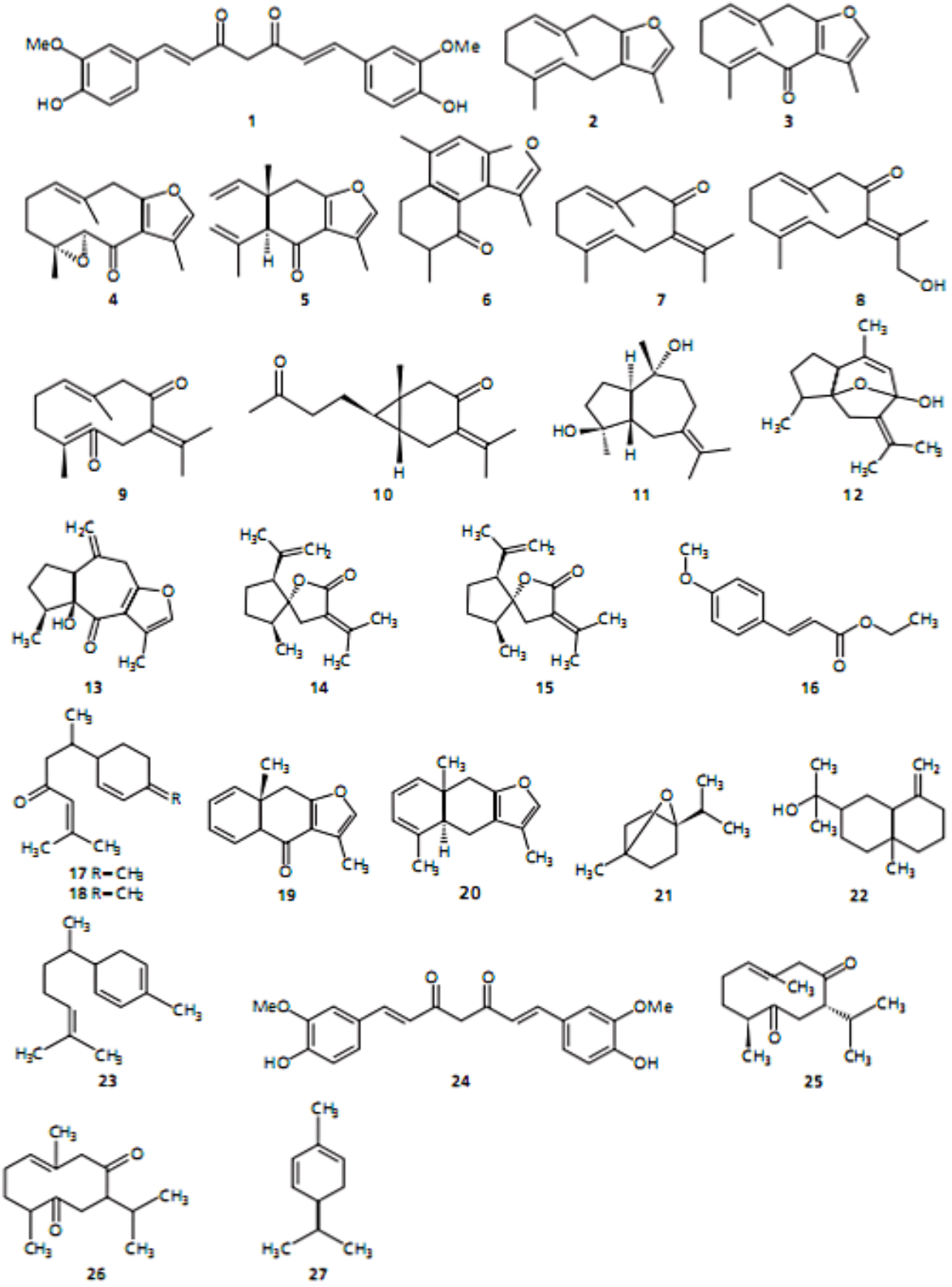

Figure 7: Structures of the biologically active compounds from *C. zedoaria*. **1**) Curcumin; **2**) furanodiene; **3**) furanodienone; **4**) zedorone; **5**) curzerenone; **6**) curzeone; **7**) germacrone; **8**) 13-hydroxygermacrone; **9**) dihydrocurdione; **10**) curcumenone; **11**) zedoaronediol; **12**) curcumenol; **13**) zedoarol; **14**) curcumanolide A; **15**) curcumanolide B; **16**) ethyl para-methoxycinnamate; **17,18**) β- turmerone; **19**) epicurzerenone; **20**) curzerene; **21**) 1,8-cineole; **22**) β- eudesmol; **23**) zingiberene; **24**) dihydrocurcumin; **25**) curdione; **26**) neocurdione; **27**) α- phellandrene.

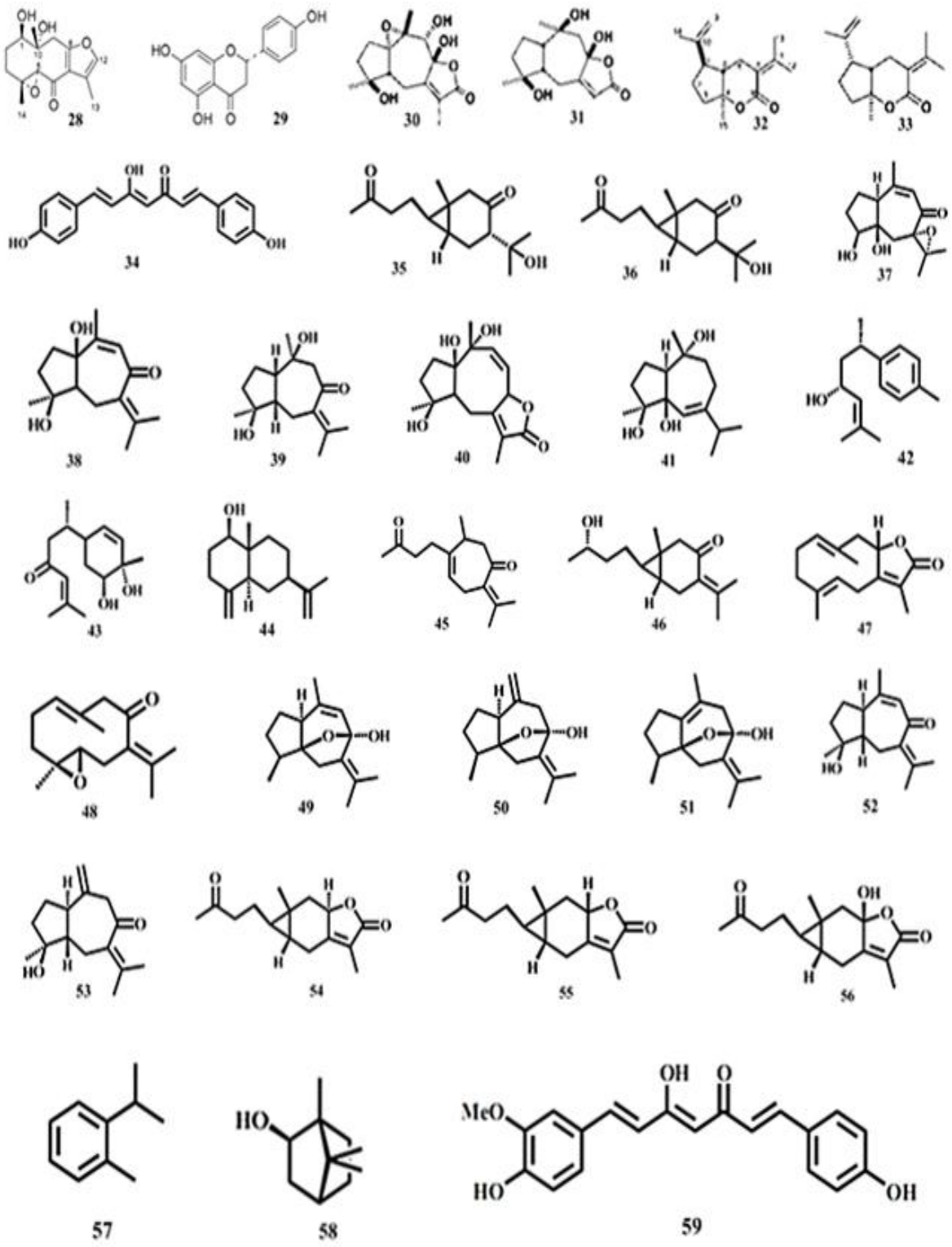

Figure 8: Structures of the biologically active compounds from *C. zedoaria*. **28**) Curcuzederone; **29**) naringenin; **30**) zedoarolide A; **31**) zedoarolide B; **32**) gajutsulactone A; **33**) gajutsulactone B; **34**) bisdemethoxycurcumin; **35**) curcarabranol A; **36**) curcarabranol B; **37**) 7α,11α- epoxy- 5β- hydroxy-9- guaiaen-8-one; **38**) aerugidiol; **39**) isozedoarondiol; **40**) zedoalactone; **41**) alismoxide; **42**)) bisacumol; **43**) bisacurone; **44**) β-dictyopterol; **45**) curcumadione; **46**) 4S-dihydrocurcumenone; **47**) glechomanolide; **48**) (+)-germacrone-4,5-epoxide; **49**) 4-epicurcumenol; **50**) isocurcumenol; **51**) neocurcumenol; **52**) procurcumenol; **53**) isoprocurcumenol; **54**) curcumanolactone A; **55**) curcumanolactone B; **56**) curcumanolactone C; **57**) cymene; **58**) isoburneol; **59**) demethoxycurcumin.

4.1.2. Pharmacological Properties

Several researchers have reported the different biological actions of *Curcuma zedoaria* in various *in vitro* and *in vivo* test models. Different parts of this plant have been found to exhibit antimicrobial, anticancer, anti-allergic, and analgesic activity. These are described in greater detail in the following sections.

Antimicrobial and antifungal activity

The antimicrobial activity of extracts of *C. zedoaria* was tested against six bacterial and two fungal strains using the agar well dilution and broth dilution methods. Petroleum ether, hexane, chloroform, acetone and ethanol extracts exhibited antibacterial as well as antifungal activity as indicated by minimum inhibitory concentration (MIC) values. The MIC values for different strains and extracts ranged from 0.01 to 0.15 mg/ml. The findings also support the use of *C. zedoaria* tubers in traditional medicine for the treatment of bacterial and fungal inspections.[48]

Oils obtained from *C. zedoaria* in Nepal were examined using the petri plate-paper disk method against *Staphylococcus aureus*, *Corynebacterium amycolatum*, *Escherichia coli*, *Candida albicans* and *Aspergillus ochraceus*. *C. zedoaria* oil contained 1,8-cineole (15.8%) and β-eudesmol (10.6%) as major volatile components. All the examined oils indicated antimicrobial activity at similar levels.[49]

On another study, antibacterial activity of essential oils isolated from *Curcuma* species (*C. aromatic*, *C. longa*, and *C. zedoaria*) were examined against four gram negative (*Vibrio cholera*, *Salmonella enteritidis*, exterogenic *Escherichia coli*, enterohaemorrhagic *E. coli*) and two gram positive (*S. aureus* and *Bacillus cereus*) bacteria. The broth dilution method was used for evaluating the antibacterial activity of the essential oils. In the broth dilution method, the MIC of *C. zedaoria* against *B. cereus* was found to be 0.035% v/v.[50]

The antimicrobial activity of *C. zedoaria* extract against different oral microorganisms was compared with the antimicrobial activity of five commercial mouthrinses to evaluate the potential of the plant extract to be incorporated into the formulae for improving or creating antiseptic activity. The results showed that the antimicrobial activity of *C. zedoaria* extract was similar to that of commercial products, and its incorporation into a mouthrinse could be an alternative therapy for improving the antimicrobial efficacy of the oral product.[51]

Extracts from 11 plant species belonging to the family Zingiberaceae were used in a study carried out by Ficker *et al*.[52] for the testing of antifungal activity using disk diffusion bioassays. Among 11 extracts, the extract of *C. zedoaria* was found to have pronounced inhibitory activity against a wide variety of human pathogenic fungi, including strains resistant to the common antifungals amphotericin B and ketoconazole.

Antiamoebic activity

Antiamoebic activity of *C. zedoaria* is also reported. Alcoholic extract of rhizome of *C. zedoaria* was able to inhibit the growth of *Entamoeba histolytica* at a concentration of 1-10 mg/ml.[53]

Analgesic activity

The analgesic activity of *C. zedoaria* rhizomes grown in Brazil was investigated by Navarro *et al*.[32]. From the rhizome's hydroalcoholic extract, different fractions (dichloromethane, ethyl acetate, and methanol) were prepared and tested for analgesic activity along with curcumenol. Aspirin and dipyrone were used as standard drugs. Curcumenol presented promising analgesic effects, being several times more potent than the reference drugs evaluated in the same experimental models. The results indicate that the dichloromethane extract caused a dose-dependent analgesic effect when given by the intraperitoneal route, inhibiting acetic acid induced writhing process in mice. It presented a calculated ID50 (Median infective dose) value of 3.6 mg/kg, with maximum inhibition of 69.5%, being several times more active than reference drugs aspirin and dipyrone, which presented ID50's of 25 and 57 mg/kg, respectively.

Antinociceptive activity

The antinociceptive activity of the dichloromethane extracts from different parts of *Curcuma zedoaria* collected in different seasons was studied using the acetic acid-induced abdominal constriction model in mice. The extracts obtained from mother rhizome collected in autumn and winter at doses of 10 mg/kg intraperitoneally caused considerable antinociceptive activity, inhibiting 91.1% and 93.4% of the abdominal constrictions, respectively, whereas compounds curcumenol and dihydrocurdione caused inhibitions of 64.0% and 46.0% respectively.[33]

Antiallergic activity

The 80% aqueous acetone extract of the rhizomes of *C. zedoaria* cultivated in Thailand (Thai zedoary) was found to inhibit the release of beta-hexosaminidase, as a marker of antigen-IgE mediated degranulation, in RBL-2H3 cells and passive cutaneous anaphylaxis reaction in mice. Among the several curcuminoids isolated from the active acetone fraction, curcumin showed the highest activity against beta-hexosaminidase release having a 50% inhibitory concentration (IC50) of 5.3 µm followed by bisdemethoxycurcumin (IC50 of 11 µm).[54]

Antiulcer activity

The effect of root powder (200 mg/kg) on the volume of gastric juice, gastric pH, total acid, free acid and ulcer index in pyloric-ligated rats was studied. The root powder at a dose level of 200 mg/kg reduced the gastric pH, free acid, total acid and ulcer index significantly and the results were comparable with that of the standard drug omeprazole (30 mg/kg).[55]

Platelet activating activity

Aqueous extract of *C. zedoaria* was studied for its inhibitory effect on platelet activating factor using a radioligand. It was found that *C. zedoaria* inhibited 50.60% platelet activating factor binding to rabbit platelets at a concentration of 200 µg/ml.[56]

Hepatoprotective activity

Hepatoprotective sesquiterpenes were isolated from the aqueous acetone extract of the rhizome of *C. zedoaria*. They showed a potent protective effect on D-galactosamine (D-GaIN)/lipopolysaccharide (LPS)- induced acute liver injury in mice.[57]

Anti-inflammatory activity

C. zedoaria showed promising anti-inflammatory activity in experimental models. Compounds curzeone and dehydrocurdione obtained from the methalonic extract of the rhizomes suppressed 12-*o*-tetradecanoylphorbol-13-acetate (TPA) by 75% and 53%, respectively, at a dose of 1µmol application.[31]

The anti-inflammatory property of methanolic extract of *C. zedoaria* was also studied by Chihiro *et al*.[43] using the adjuvant arthritis mouse model which didn't show any significant activity.

Antivenom activity

Aqueous extract of *C. zedoaria* was investigated for inhibitory activity by binding of anti-cobra venom antibody to antigen of cobra venom by using the 96-well plate enzyme linked immunosorbent assay (ELISA) method. The extract of *C. zedoaria* showed clear inhibitory activity. The extract targeted neurotoxin and protein-degrading enzyme present in venom, thus suggesting use of aqueous extract as antivenom.[58]

Hemagglutinating activity

Hemagglutinating activity has been shown in extract of *C. zedoaria*. Crude proteins obtained by Mg/NP-40 extraction from *Curcuma* species exhibited agglutination activity against rabbit erythrocytes.[59]

Antioxidant activity

The essential oil of *C. zedoaria,* at a dose of 20 mg/ml, showed moderate to good in antioxidant activity, good in reducing power and excellent in scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical but low in chelating effect on ferrousion.[39]

Antihyperglycemic activity

The methanol extract of leaves of *Curcuma zedoaria* showed dose dependent and statistically significant antihyperglycemic activity following glucose loading in mice. At the highest dose tested (400 mg), the extract lowered serum glucose levels by 43.2% versus the reduction of serum glucose levels by 65.5%, obtained with the standard drug glibenclamide, administered at a dose of 10 mg per kg body weight.[60]

Anticancer activity

The inhibitory effect of water extract of *C. zedoaria* on experimental pulmonary metastasis of B16 melanoma cells was investigated. The intake of water extract at doses of 250 and 500 mg/kg for 42 days from 14 days before tumor inoculation significantly reduced the number of metastatic surface nodules in the lung, resulting in an extended life span.[61]

A study conducted by Hong *et al*. found that the methalonic extract of *C. zedoaria* had both anticancer and anti-inflammatory activity. Methalonic extracts of *C. zedoaria* showed potent inhibition of COX-2 activity (>80% inhibition at the test concentration of 10 µg/ml).[62]

Curcuminoids were synthesized and demonstrated to be cytotoxic against human ovarian cancer OVCAR-3 cells. The observed curative dose at 50% (CD50) for curcumin, demethoxycurcumin and bisdemethoxycurcumin was 4.4, 3.8 and 3.1 µg/ml, respectively.[44]

4.2. *Nymphoides indica*

4.2.1. Phytochemical Analysis

The preliminary phytochemical study of *Nymphoides indica* revealed the presence of carbohydrates, glycosides, phenolic compounds, flavonoids, saponins, tannins, gums and mucilage and phytosterols in both alcohol and aqueous extracts.[63]

Madhavan *et al*.[64] stated that the physicochemical parameters of the plant (rhizome and root), viz. percentage of moisture content, total ash, acid insoluble ash, water soluble ash were found to be 8.5, 12.4, 7.4, and 0.5, respectively. The percentage alcohol extract was 36.8 and the aqueous extract was 45.6. The alcohol extract revealed seven phytoconstituents.

A triterpenoid, Bet-20(29)-en-3-ol-28-oic acid, was isolated by Rahman *et al*.[65] from the ethyl extract of *Nymphoides cristatum* (Roxb.) O.kuntze and its structure was elucidated on the basis of spectral evidences. The ethyl acetate extract of *Nymphoides cristatum* (Roxb.) O.kuntze after chromatography over silica gel yielded a pure compound which was obtained as a white crystalline solid having a melting point of 193-195ºC. It produced a single spot on the TLC plate after spraying with vanillin-sulfuric acid and heating. The IR spectrum of the compound showed strong band at 3624 and 1705 cm^{-1} which could be assigned for hydroxyl function. All the proton peaks in ^{1}H NMR data of the compound are in good agreement with those of betulinic acid.

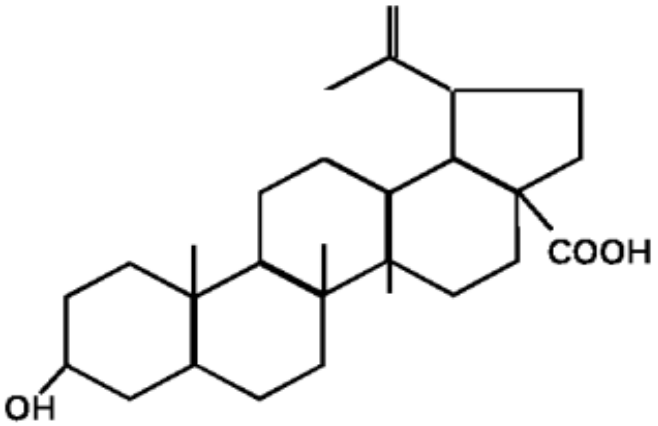

Figure 9: Structure of Bet-20(29)-en-3-ol-28-oic acid

β-sitosterol, betulinic, salicylic and tannic acid were isolated by Madhavan *et al.*[66] for the first time from *N. hydrophylla*. The % yield of β-sitosterol was found to be 24% and the melting point was 136-137ºC.

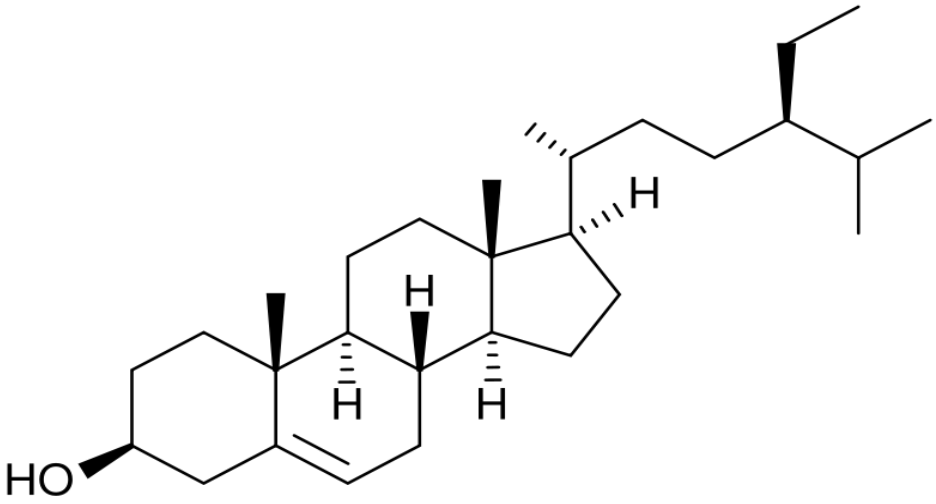

Figure 10: Structure of β-sitosterol

A triterpenoid glycoside, menyanthoside, was isolated by Zbigniew *et al.*[67] from the rhizome of *Menyanthes trifoliate.* It is one of the main saponin of the plant and it's structure was established to be 3-*O*-[β-d-galactopyranosyl(1→4)β-d-glucuronopyranosyl]-28-*O*-[β-d-apiofuranosyl(1→6)β-d-glucopyranosyl]-betulinic acid.

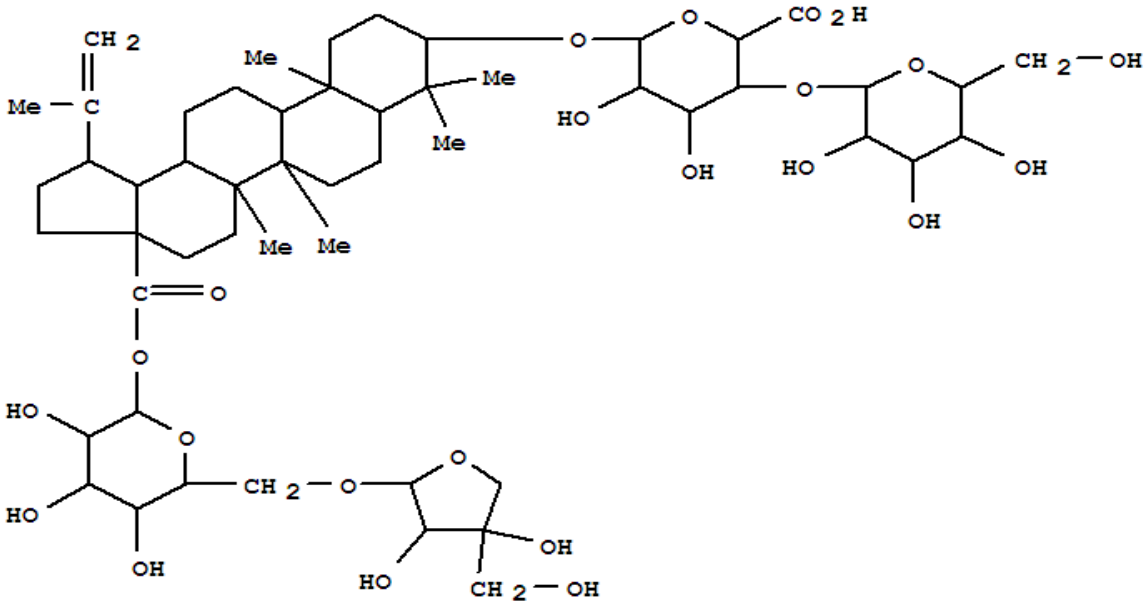

Figure 11: Structure of menyanthoside

Anti-inflammatory compounds were isolated from *Menyanthes trifoliate* by Huang *et al.*[68] Eight compounds were isolated by column chromatography and preparative TLC from the methanol extract of the rhizomes of the plant. These compounds are betulinic acid, betulin, loganin, foliamenthin, sucrose, loganetin, α-lupeol and β-lupeol.

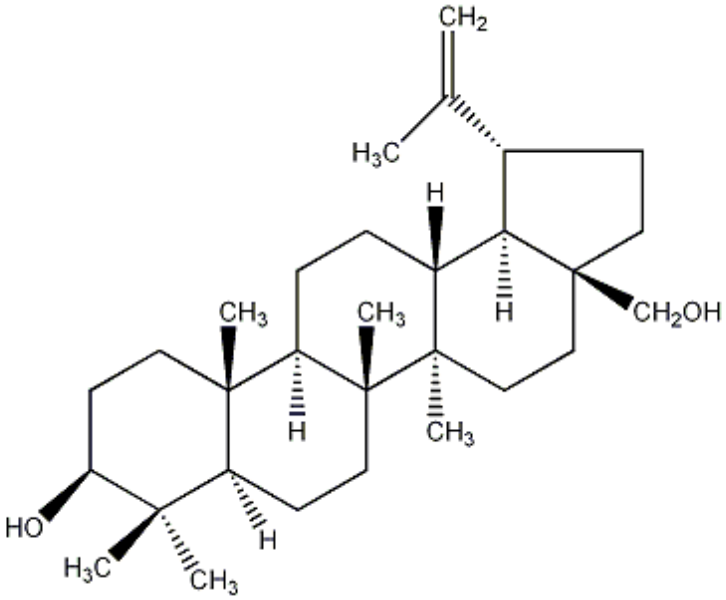

Figure 12: Structure of betulin

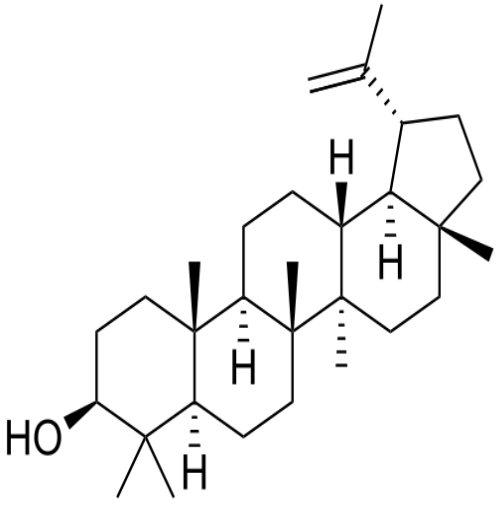

Figure 13: Structure of lupeol

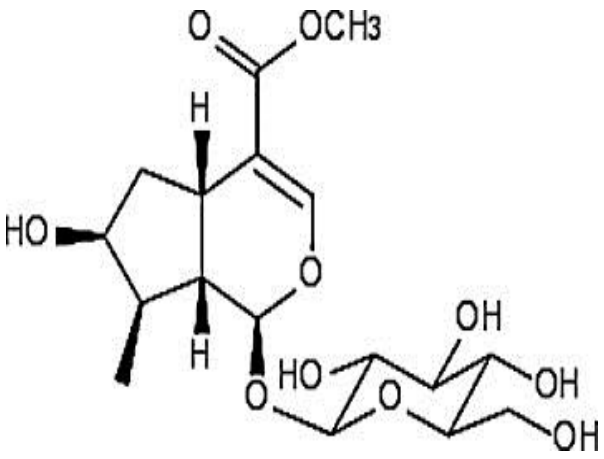

Figure 14: Structure of loganin

4.2.2. Pharmacological Properties

Anticonvulsant and Anti-epileptic Activity

The anti-convulsant effect of aqueous and alcohol extract of roots and rhizomes of *Nymphoides indica* (L.) Kuntze was evaluated by Madhavan *et al*.[63] using Maximum Electroshock Convulsion (MEC) and Pentylenetetrazole (PTZ) induced convulsion methods in *swiss albino* mice. *Nymphoides indica* extracts exhibited significant anticonvulsant activity against both the models. The oral administration of alcohol and aqueous extract at the dose level of 300 and 600 mg/kg reduced the duration of extensor phase and time taken for recovery in MEC induced convulsion model. Similarly both extracts delayed the onset of convulsion and reduced the time taken for recovery in PTZ induced convulsions. The alcohol extract was found to be more effective and the effects of alcohol and aqueous extract on extensor and onset of convulsion was dose dependent.

Nymphoides indica is used as an admixture along with *Nymphoides macrospermum* and as a substitute of Ayurvedic drug Tagara (*Valerlana jatamansi* Jones) in the treatment of various diseases like epilepsy, anemia, jaundice, tuberculosis to name a few.[64]

Antimicrobial Activity

A triperpenoid bet-20(29)-en-3-ol-28-oic acid was isolated by Rahman *et al*.[65] from ethyl acetate extract of *Nymphoides cristatum* and the compound was screened for antimicrobial activity against a number of pathogenic bacteria and fungi. The compound showed significant antibacterial activity against a wide range of gram positive and gram negative bacteria and produced zone of inhibition in between 15-23 mm. It showed highest inhibitory activity against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Shigella sonnei* and the MIC values against these organisms were 64, 128, 64 and 32 µg/ml respectively. The compound also showed activity against the tested pathogenic fungi.

Cytotoxic Activity

A triperpenoid bet-20(29)-en-3-ol-28-oic acid was isolated by Rahman *et al*.[65] from ethyl acetate extract of *Nymphoides cristatum* and the compound was screened for the cytotoxic activity by the brine shrimp lethality bioassay. The compound showed positive result, indicating the biological activity. The 50% mortality (LC_{50}) of the compound was found to be 15.39 µg/ml and 95% confidence limits is 8.87-26.68.

Anti-inflammatory Activity

The anti-inflammatory activities on *Menyanthes trifoliate* was investigated by Tunon *et al*.[69] The plant extract inhibited carrageenan-induced rat paw edema ($ID_{50} \approx 1.7$ g/kg p.o.) and ethyl phenylpropiolate-induced rat ear edema (32% at 2.0 g/kg p.o.) in a dose-dependent manner. Further studies revealed that *Menyanthes trifoliate* inhibited both fMLP-induced exocytosis ($IC_{50} = 0.16$ mg/ml) and elastase activity ($IC_{50} = 0.16$ mg/ml). According to these results it is likely that the activity shown in the PAF-test is at least partly due to an inhibition of elastase. *Menyanthes trifoliate* showed only minor hemolytic properties at the concentrations used in the PAF- and fMLP-tests, suggesting that the cells in these tests are undamaged. The decoction also inhibited the biosynthesis of LTB_4 ($IC_{50} \approx 0.73$ mg/ml) and prostaglandins ($IC_{50} = 0.37$ mg/ml) *in vitro* in a concentration-dependent way. However, at concentrations where the decoction is active in the LTB_4-test, it also possesses hemolytic properties.

Hepatoprotective Activity

The hepatoprotective effect of *Nymphoides cristatum* was investigated in an experimental model by Niranjan *et al*.[70] The albino rats were treated with 50% ethanol extract of the whole plant and callus of *Nymphoides cristatum* extracts at a dose of 500 mg/kg body weight orally. After intraperitoneal administration of CCl_4 (2 ml/kg), significant protective effects from this hepatotoxin was expressed. Activities of serum enzymes were significantly lower in *N. cristatum* treated rats. In the histopathological observation, liver damage induced by hepatotoxin was markedly improved in *N. cristatum* treated rats. These results demonstrated that *N. cristatum* has a protective effect against experimental liver damage.

4.3. *Drynaria quercifolia*

4.3.1. Phytochemical Analysis

Preliminary phytochemical analysis showed the presence of catechin, coumarins, flavonoids, phenolics, saponin, steroids, triterpenes, and tannin in *Drynaria quercifolia*. TLC studies revealed the presence of β-amyrin, β-sitosterol and catechin. Total phenolic compound of *D. quercifolia* was determined as 244 mg/g. The presence of the flavanone glycoside, naringin in *D. quercifolia* was established by HPLC and quantified as 0.048%.[21]

Table 3: Preliminary phytochemical screening of *Drynaria quercifolia* rhizome extracts.[22]

Test	Hexane	$CHCl_3$	MeOH	Water
Alkaloids	-	-	-	-
Coumarins	+	+	+	-
Flavonoids	-	+	+	+
Lignans	+	+	+	+
Phenolics	-	-	+	+
Proteins	-	-	+	-
Saponins	-	-	+	-
Starch	-	-	+	-
Steroids	+	+	+	+
Sugars	-	-	+	+
Tannins	-	-	+	+
Triterpenes	+	+	+	+

+, present; -, absent

Ramesh *et al.*[22] isolated friedelin (**1**) (yield: 0.15% on dried weight), epifriedelinol (**2**) (0.1%), β-amyrin (**3**) (0.09%), β-sitosterol (**4**) (0.18%) from hexane and $CHCl_3$ combined extracts of *Drynaria quercifolia*. β-sitosterol 3-β-D-glucopyranoside (**6**) (0.24%) and naringin (**5**) (0.09%) were isolated from MeOH extract.

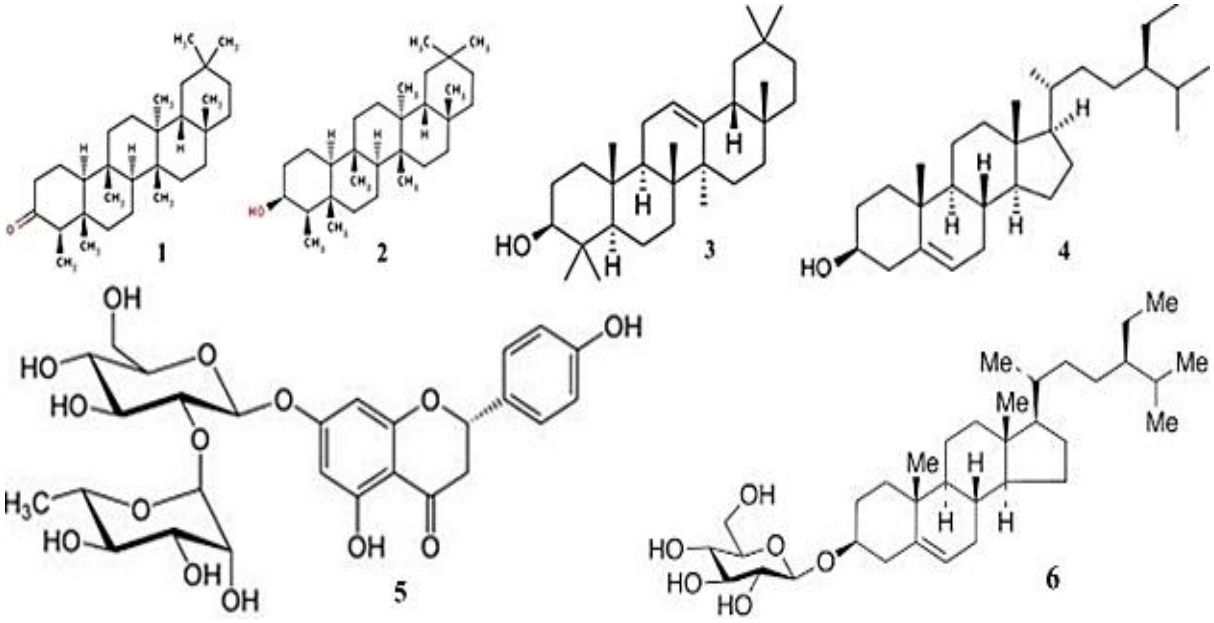

Figure 15: Structures of some isolated compounds from *D. quercifolia*. **1)** friedelin; **2)** epifriedelinol; **3)** β-amyrin; **4)** β-sitosterol; **5)** naringin; **6)** β-sitosterol 3-β-D-glucopyranoside**.**

3,4-dihydroxybenzoic acid (**7**) and acetyl lupeol (**8**) were isolated by Khan *et al*.[71] from the rhizome of *Drynaria quercifolia* through bioassay-guided investigations. 3,4-dihydroxybenzoic acid was obtained as brownish color needle like crystal with melting point 199-200ºC. In solubility test, the compound was sparingly soluble in water and freely soluble in ethyl acetate, methanol and acetone. The liquid chromatography/electrospray- mass spectroscopy (LC/ES-MS) in the positive ion mode of the compound showed molecular $[M+H]^+$ peak at m/z 154.8 corresponding to a molecular formula of $C_7H_6O_4$. The IR spectrum exhibited bands at 1240, 1375, 1739, 2877, 2908 and 2985 cm^{-1}. The ^{1}HNMR, ^{13}CNMR, HSQC and HMBC spectral data of the compound was in good agreement with spectral data of 3,4-dihydroxybenzoic acid published in literature. Acetyl lupeol was obtained as needle like crystal with melting point 218-220ºC. The liquid chromatography/electrospray- mass spectroscopy (LC/ES-MS) in the positive ion mode of the compound showed molecular $[M+H]^+$ peak at m/z 469.5 corresponding to a molecular formula of $C_{32}H_{52}O_2$. The IR spectrum exhibited bands at 1240, 1650, 1700, 1735 and 3065 cm^{-1}. The ^{1}HNMR, ^{13}CNMR, HSQC and HMBC spectral data of the compound was in good agreement with spectral data of acetyl lupeol acid published in literature.

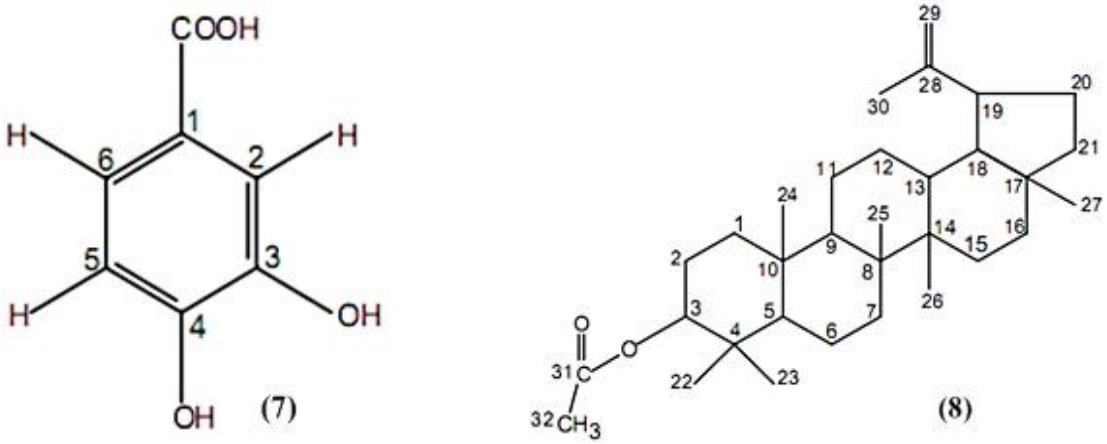

Figure 16: Structure of isolated compounds from *D. quercifolia*. **7)** 3,4-dihydroxybenzoic acid; **8)** acetyl lupeol

A new natural product, namely propinqualin, whose structure was established as (-)-epiafzelechin-3-*o*-beta-D-allopyranoside isolated from rhizomes of *Drynaria propinqua*. 4-o-beta-D-glucopyranoside and sucrose were also isolated from *Drynaria propinqua*. Fern-9(11)-ene, hop-22(29)-ene and cyclolaudenol were structurally elucidated from rhizome of Drynariae.[72]

4.3.2. Pharmacological Properties

A great number of pharmacological evaluations of *Drynaria quercifolia* have been reported till date. These are described in greater detail in the following section.

Antimicrobial Activity

The methanol extract of *Drynaria quercifolia* showed broad and concentration-dependent antibacterial activity as reported by Ramesh *et al*.[22]. Significant zone of inhibition was recorded at a dose concentration of 50 mg/ml against *Chromobacterium violaceum*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi*, *Vibrio cholera*, *Vibrio parahaemolyticus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Aeromonas hydrophil.*

Six different organic solvents such as ethanol, methanol, petroleum ether, hexane, benzene and chloroform were used by Kandhasamy *et al*.[73] to extract the bioactive compounds from the rhizome of *Drynaria quercifolia* to screen the antibacterial activity against infectious disease including causing bacterial pathogens such as *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Salmonella marscence, Staphylococcus aureus* and *Bacillus subtilis* by agar dilution method. The ethanolic extract of *D. quercifolia* was more active against 80% of the organisms tested. It was followed by methanolic extract (70%), benzene (50%) and chloroform extract (40%) in inhibiting the growth of organisms tested. Petroleum ether and hexane extract of *D. quercifolia* did not show any antibacterial activity against any of the pathogenic tested. Among the bacteria tested, gram-negative bacteria were more susceptible to the crude extracts compared to gram-positive bacteria. Among gram positive bacteria, *S. aureus* was susceptible to ethanolic (11 mm), methanolic (10 mm) and chloroform (13 mm) extracts whereas *B. subtilis* was susceptible to methanolic extract of *D. quercifolia* alone.

Antibacterial study was carried out by Mithraja *et al*.[19] on clinically isolated Urinary Tract Infecting (UTI) bacteria by disc diffusion method. Among the six extracts tested against eight different UTI bacteria, acetone extract was effective against *Enterococcus faecalis* and *Streptococcus pyogenes*, while ethanol extract was effective against *Pseudomonas aeruginosa*.

3.4-dihydroxybenzoic acid was isolated by Khan *et al*.[71] from the rhizome of *Drynaria quercifolia* which showed significant antibacterial activity against four gram-positive and six

gram-negative bacteria. The MIC values of 3.4-dihydroxybenzoic acid against tested gram-positive and gram-negative bacteria ranged from 8-32 and 16- 64 µg/ml, respectively These MIC values indicate the potency of the isolated compound against gram-positive bacteria is higher than that of gram-negative bacteria.

Preliminary studies were conducted by Shokeen *et al.*[74] on three plants including *Drynaria quercifolia* to determine activity against *Neisseria gonorrhoeae*. The extracts of *D. quercifolia* caused inhibition of *Neisseria gonorrhoeae* clinical isolates and World Health Organization (WHO) strains, more so than the multidrug resistant *Neisseria gonorrhoeae*.

Antidermatophytic Activity

Antidermatophytic activity of different extracts of *D. quercifolia* was assayed by Batool *et al.*[75]. Agar dilution and disk diffusion methods were used to determine antidermatophytic against infectious disease causing pathogenic fungi such as *Trichophyton mentagrophytes, Microsporum canis, M. gypseum, T. rubrum* and *Epidermophyton floccosum*. The ethanol extarct of the dried rhizome of *D. quercifolia* did not show inhibitory activity up to concentration of 20 mg/ml. The solvents of acetone, methanol and water also did not show any efficacy for extraction from *D. quercifolia* but di-ethyl ether with semi-polarity gave clear zone to antifungal activity compounds.

Anthelmintic Activity

Anthelmintic activity of *Drynaria quercifolia* was evaluated by Kulkarni *et al.*[76] using adult earthworms and piperazine citrate was used as a standard. Various doses (2.5, 5, 10, 25, 50 mg/ml) of alcoholic extracts of leaves and rhizomes of *Drynaria quercifolia* were used. At all the tested doses, both extracts caused paralysis and also death of the worms. Though time taken for each concentration to paralyse and kill the parasite is comparatively longer than that for piperazine citrate, the results are highly significant ($P<0.01$). The activity also confirms dose dependent nature of the extract.

Anti-inflammatory and Analgesic Activity

Drynaria quercifolia was used to evaluate anti-inflammatory and analgesic effects using carrageenan-induced paw edema/cotton pellet-induced granuloma in Wistar rats and acetic acid- induced writhing/formalin-induced paw licking test in *swiss albino* mice respectively.

Oral administration of *Drynaria quercifolia* at all doses (125, 250 and 500 mg/kg) significantly inhibited the carrageenan-induced paw edema in rats in a dose dependent manner, i.e. 80.00%, 83.53% and 85.88% respectively. *Drynaria quercifolia* also produced a significant dose dependent inhibition of granuloma formation. At 500 mg/kg, *Drynaria quercifolia* produced 55.56% inhibition in the exudative phase and 62.83% in the proliferative phase. *Drynaria quercifolia* also significantly attenuated acute and delayed phases of formalin-induced pain and acetic acid- induced writhing episode in mice.[21]

Antipyretic Activity

The antipyretic effect of petroleum ether and ethyl acetate soluble fractions of ethanol extract of the rhizome of *Drynaria quercifolia* was investigated by A. Khan *et al*.[77] Intraperitoneal administration of petroleum ether and ethyl acetate soluble fractions of ethanol extract of the rhizome of *Drynaria quercifolia* at a dose of 80 mg/kg body weight were shown to significantly reduce the elevated body temperature of rabbit, which was compared with aspirin (standard) and solvent used.

Anti-oxidant Activity

The anti-oxidant activity of different fractions of *Drynaria quercifolia* was measured by the DPPH free radical scavenging activity. The concentration of petroleum ether soluble fraction, carbon-tetrachloride fraction, ethyl acetate soluble fractions and aqueous soluble fraction needed for 50% scavenging (IC_{50}) of DPPH was found to be 161.68 µg/ml, 62.98 µg/ml, 38.25 µg/ml, 124.39 µg/ml, respectively. The positive control used as Butyl hydroxyl toluene (BHT) and for which the IC_{50} values were found to be 35.52 µg/ml.[78]

Cytotoxic Activity

Evaluation of cytotoxic activity of *Drynaria quercifolia* was done using the brine-shrimp lethality bioassay. The carbon-tetrachloride soluble fraction showed the greatest cytotoxic activity with a LC_{50} value of 30.31 µg/ml.[78]

Moderate cytotoxic activity of *Drynaria quercifolia* was also reported by Runa *et al.*[79]. Compared to vincristine sulfate, the LC_{50} values of crude methanolic extract, chloroform, carbon-tetrachloride, pet-ether and aqueous soluble fractions of *Drynaria quercifolia* leaves were found to be 12.45, 14.95, 13.02, 15.83 and 7.612 µg/ml, respectively.

Neuropharmacological Activity

The neuropharmacological effect of petroleum ether and ethyl acetate soluble fractions of ethanol extract of the rhizome of *Drynaria quercifolia* were studied in mice by intraperitoneal administration. The tests used were determination of effect on duration of diazepam-induced sleep, determination of effect on nikethamide-induced toxicity, light dark test and force swimming test. The duration of diazepam-induced sleep was extended by administration of these fractions. Nikethamide at high dose cause death of mice and time to cause death of mice was delayed by administration of these fractions. In light dark test and force swimming test, these fractions were given diazepam type effect. In all experiment it was observed that that petroleum ether fraction is more active than ethyl acetate fraction.[20]

4.4. *Rhynchostylis retusa*

4.4.1. Phytochemical Analysis

Preliminary phytochemical screening of *Rhynchostylis retusa* revealed the presence of tannins, alkaloids, steroids, terpenes, flavonoids, triterpenoids, coumarins, flavones, flavanones, anthrocyanins, quinones and carbohydrates.[80]

Table 4: Phytochemical analysis of methanol extract of *Rhynchostylis retusa*[80]

Phytoconstituents	**Whole plant extract**
Tannins	+
Alkaloids	+
Saponins	-
Cardiac glycosides	-
Steroids	+
Terpenes	+
Flavonoids	+
Coumarins	+
Phenols	-
Quinones	+
Proteins	+
Carbohydrates	+

+, positive; -, negative

4.4.2. Pharmacological Activity

Antimicrobial Activity

Aqueous fresh and dried leaves extract of *Rhynchostylis retusa* were investigated for antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*. Maximum antibacterial activity was exhibited by fresh leaves extract (22.0, 20.8, 22.4 and 20.0 mm against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi* respectively) while dried leaf extract showed vey less activity.[81]

Evaluation of antifungal activity of *Rhynchostylis retusa* was carried out by Radhika *et al*.[82] Hexane, chloroform and methanol extracts of this orchid were used to determine antifungal activity against six phytopathogens. Among these extracts, chloroform and methanolic extracts showed highest zone of inhibition than the hexane extract.

Cytotoxic Activity

Various solvent extracts of *Rhynchostylis retusa* was screened by the brine shrimp lethality assay in order to determine the cytotoxic potential of the plant. Methanol extract showed significant toxicity to the brine shrimp and exhibited potent activity.[82]

Analgesic and Anti-inflammatory Activity

The methanolic leaf extract of *Rhynchostylis retusa* (L.) Blume was evaluated for analgesic and anti-inflammatory activities in mice. The analgesic activity was studied using acetic acid induced writhing and the anti-inflammatory activity was studied on carrageenan and formaldehyde induced paw edema. The extract showed 28.84% and 35.81% inhibition of acetic acid induced writhing at doses of 200 mg/kg and 400 mg/kg, respectively. 7.80%, 8.67% and 14.32% mean inhibition of carrageenan induced paw edema at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg, respectively, and significant ($p<0.01$, $p<0.001$) anti-inflammatory activity of formaldehyde induced mice paw edema at doses of 200 mg/kg and 400 mg/kg. The result shows that the extract exerts dose dependent analgesic and anti-inflammatory activities.[83]

Chapter 5

Materials and Methods

5. Materials and Methods

5.1. Equipments

Spatula, mortar and pestle, large beaker (1000 ml), small beaker (50 ml), pipette, appendrof micropipette (Eppendrof, Germany), appendrof micropipette tip, appendrof tube, petri dishes, cork borer, distilled water, glass container for agar, glass container for isotonic solution, gas burner, forceps, masking tape, filter paper (Whatman 40), permanent marking pen, inoculating loop, aluminium foil paper, test tube, test tube holder, autoclave (Hydroclave MC8, Barnstead International, USA), laminar air flow (EQU/03-EHC, ESCO, USA), incubator (BK 4266), hot air oven (YCO-N01, Germany Industrial Corporation), analytical balance (ELH 3000, Shimadzu, Japan), refrigerator, vortex machine, dropper, microorganism spreading glass rod, UV lamp, hot plate, glass rod, funnel, cotton, pencil, scale, 5 ml vial, mice oral needle, 1m insulin syringe (50 units), container.

5.2. Drugs and Chemicals

Nutrient agar (Himedia Laboratories, India), agar powder (BDH Laboratory, England), sodium chloride (Merck, Germany), acetic acid (Sigma-Aldrich Laborchemikalien Gmbh, Germany), nutrient broth, amoxicillin (Square Pharmaceuticals Limited, Bangladesh), diclofenac (Square Pharmaceuticals Limited, Bangladesh), indomethacin (Beximco Pharmaceuticals Limited, Bangladesh), carrageenan, carboxy methyl cellulose (CMC), tween 80.

5.3. Solvents

Ethyl acetate, acetone, methanol, ethanol, dichloromethane, acetic acid, chloroform, dimethylsulfoxide (DMSO), distilled water.

5.4. Plant Materials

All the plant materials were collected by research students of Dr. Chowdhury Faiz Hossain as part of an on-going research project.

1) *Curcuma zedoaria*: The whole plant of *Curcuma zedoaria* was collected from Panchagar district of Bangladesh in November 2012 by Fahima Hasan. The whole plant with leaves, stems and roots were collected and identified by experts in

Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (Accession No. 37516) has been deposited for further reference. 1000 gram dried powder of *Curcuma zedoaria* was extracted with methanol at room temperature (2500 ml × 72 hours × 3 times) and gave 136.15g crude extract. The crude extract was then subjected to Vacuum Liquid Chromatography (VLC) and five different fractions were yielded: hexane (fraction 1, yield: 14.7g), dichloromethane (fraction 2, yield: 20.28g), ethyl acetate (fraction 3, yield: 16.5g), acetone (fraction 4, yield: 18.3g) and methanol (fraction 5, yield: 9.87g).

2) *Nymphoides indica*: The whole plant of *Nymphoides indica* was collected from Panchagar district of Bangladesh by Mahmudul Hasan Chowdhury. The plant was identified by Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (Accession No. 35611) has been deposited for further reference. 250g dried powder leaves of *Nymphoides indica* was extracted with ethanol at room temperature (2000 ml × 72 hours × 3 times) and gave 42g crude extract. The crude extract was then subjected to Vacuum Liquid Chromatography (VLC) and five different fractions were yielded: hexane (fraction 1, yield: 0.28g), dichloromethane (fraction 2, yield: 0.63g), ethyl acetate (fraction 3, yield: 3.97g), acetone (fraction 4, yield: 20.2g) and methanol (fraction 5, yield: 0.74g).

3) *Drynaria quercifolia*: The whole plant of *Drynaria quercifolia* was collected from Panchagar district of Bangladesh by Md. Al Amin. Dried powder leaves of *Drynaria quercifolia* was extracted with methanol at room temperature (2000 ml × 72 hours × 3 times) and gave 36.674g crude extract. Dried powder rhizomes of *Drynaria quercifolia* was also extracted with methanol at room temperature (2000 ml × 72 hours × 3 times) and gave 47.3g crude extract.

4) *Rhynchostylis retusa*: The whole plant of *Rhynchostylis retusa* was collected from Panchagar district of Bangladesh by Md. Al Amin. 80g crashed leaves of *Rhynchostylis retusa* was extracted with methanol at room temperature (400 ml × 72 hours × 3 times) and gave 5.00g crude extract.

5.5. Evaluation of antimicrobial activity

5.5.1. Microorganisms

Eight gram-positive bacteria (*Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Sarcina lutea, Streptococcus pyogens, Staphylococcus saprophyticus, Betahaemolytic streptococci*), nine gram-negative bacteria (*Salmonella paratyphi, Salmonella typhi, Vibrio parahemolyticus, Vibrio mimicus, Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa, Shigella boydii, Klebsiella pneumoniae*), three fungi (*Saccharomyces cereveceae, Candida albicans, Aspergillus niger*); in total twenty microorganisms were used for the antimicrobial test. They were grown in Muller-Hinton agar at 37ºC and maintained on nutrient agar slant at 4ºC.

5.5.2. Culture media

Nutrient agar (NA) was used for the activation of all bacteria and fungi. In order to prepare this agar solution, at first required amount of agar (2.8% w/v) was measured and poured into a clean bottle. Distilled water is then added to make up the required volume. The bottle is then placed into the autoclave machine for 15 minutes.

Besides Nutrient agar, Muller-Hinton agar (MHA) was also used for the antimicrobial test. The formula for preparing 100 ml MHA is given in Table 5.

Table 5: Formula of Muller-Hinton agar (100 ml)

Ingredient	**Quantity (gram)**
Beef extract	0.325
Acid hydrolysate of casein	1.75
Starch	0.15
Agar	2.00

5.5.3. Bacterial solution preparation

A suspension of organism is prepared for the antimicrobial test. The suspension should be freshly made prior to the experiment in order to get reliable results and it is best to make subcultures of the organisms prior to the day of experiment. Four or five isolated colonies are taken out by using a sterile inoculating loop from the culture. These colonies are then suspended in 2 ml of sterile saline solution (0.9g NaCl in 1000 ml distilled water). Suspension of these test organisms was prepared using vortex machine in order to achieve uniform distribution of the organisms in the suspension. The prepared solution was compared to 0.5 McFarland standards. It can be done either concentrating or diluting the suspension,

5.5.4. McFarland standard

Barium sulfate suspension, which is used to compare bacterial density, is known as McFarland standard. This standard is used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. It is considered that a 0.5 McFarland standard contains 1×10^8 CFU/ml of tested organism.

In order to prepare 0.5 McFarland standard, a suspension of barium chloride and sulfuric acid was prepared where 0.5 ml aliquot of a 0.048 mole/liter $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.18 mole/liter of H_2SO_4 (1% v/v) was added with continuous stirring. The turbidity of the suspension was measured by spectrophotometer and at wavelength of 625 nm; the absorbance should be 0.08 to 0.13 for the 0.5 McFarland standards. Then the suspension was stored in a dark place at room temperature and used to determine the turbidity of bacterial suspension.

5.5.5. Disk diffusion method

In disk diffusion method, a filter paper disk impregnated with a chemical is placed on agar. The chemical will diffuse from the disk into the agar. This diffusion will place the chemical in the agar only around the disk. If an organism is placed on the agar, it will not grow in the area around the disk given that it is susceptible to the chemical. This area of no growth around the disk is known as a “zone of inhibition”.

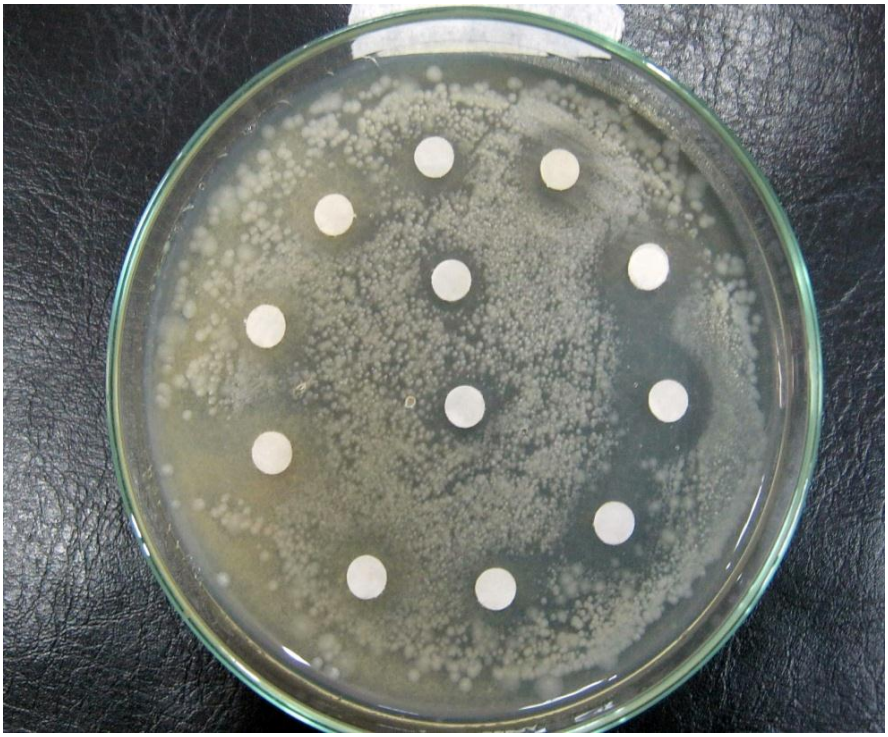

Figure 17: Disk diffusion method

The antimicrobial activity of the test samples (Plant extracts, standard drugs and negative control) were assayed by the standard disk diffusion method. The tested samples were mixed with MeOH and DMSO in a composition of 80:20 ratios. DMSO facilitates the solubilization of the sample and methanol will increase solubility further. Then the samples were injected into sterilized disk of 6mm in diameter. Nutrient agar (NA) was sterilized at 121ºC by using autoclave and after the withdrawal from the autoclave; it was cooled to 60-70ºC. Then it was transferred to sterilized petri dishes with a diameter of 9 cm (15 ml). The depth of agar is an important issue which can interfere with the size of the zone of inhibition. Shallow layer of agar will produce a larger zone of inhibition than a deeper layer. The bacterial solution was then prepared by suspending the microorganisms in an isotonic solution of 0.9% NaCl. Vortex of these solutions was done for 2 minutes in order to completely disperse the organisms in the NaCl solution. The turbidity of the prepared bacterial solution must be like as 0.5 McFarland standards. From this solution, 100 µl was transferred to the agar containing petri dishes and distributed homogenously. Disks injected with test samples were placed on the solid agar medium. Petri dishes were incubated at 37ºC for 24 hour. On each plate, a reference standard (amoxicillin 20 µg/disk) was used and control (5 µl MeOH: DMSO in a ratio of 80:20/disk) was applied in the plate. At the end of the period, inhibition zones formed on the NA were evaluated in millimeters.

5.5.6. Agar well diffusion method

The method can be differentiated from disk diffusion method is such a way that in agar well diffusion method, a cork borer is used to make a small well in the NA and the extract is directly applied into the well rather than the use of filter paper. If an organism is placed on the agar, it will not grow in the area around the well given that it is susceptible to the chemical. This area of no growth around the well is known as "zone of inhibition".

In this method, agar was poured on the plate and was allowed to dry. Then freshly prepared bacterial solution (100 µl) was poured in the plate and the solution was spread uniformly throughout the plate with the help of a sterile glass rod spreader. With the help of a sterile cork borer, well of 0.5 cm diameter was made in the agar plate. Depending on the plate size, 9-12 were done and since different doses or fractions were applied, appropriate labeling was done or the well can be done by following special pattern to identify which well contains which dose or fraction. The wells were then filled with test samples (100 µl each) and the plates were allowed to stay for 1-2 hour at room temperature. Finally, the plates were incubated at 37ºC for 18-24 hours and resulting diameters of zone of inhibition was measured afterwards.

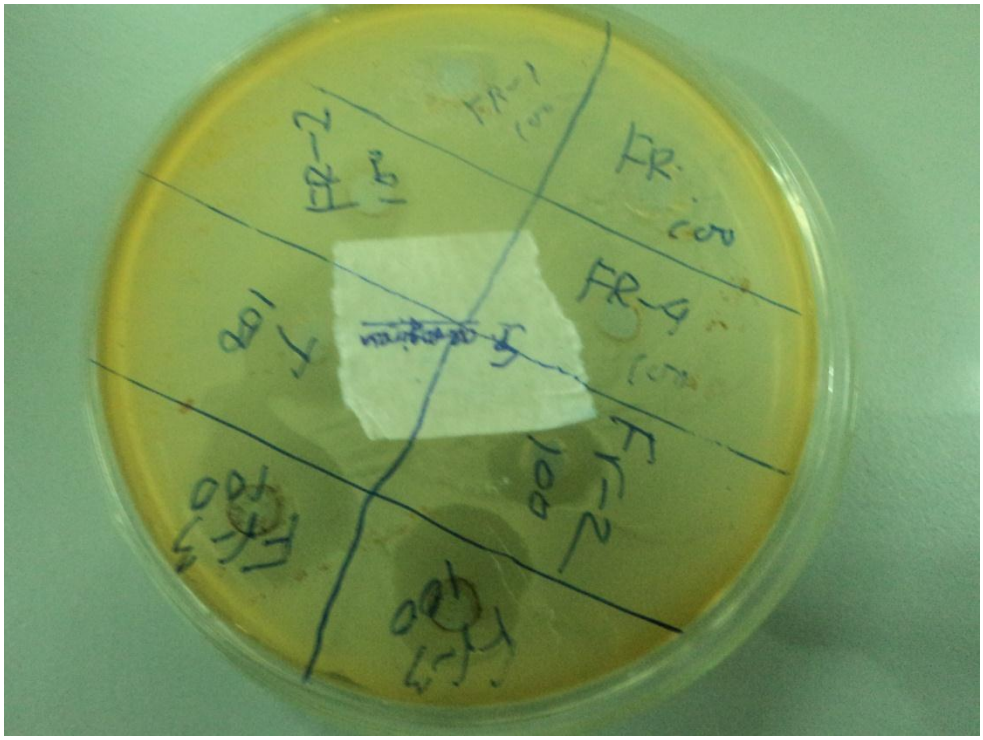

Figure 18: Agar well diffusion method

5.6. Animals

Swiss albino mice of either sex, weighing 22-28g, obtained from the Animal Resource Division, International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB), were used to evaluate the anti-inflammatory and analgesic activity of the plant extracts. All animals were kept in standard environmental condition, had free access to standard food (ICDDRB formulated) and water *ad libitum.*

5.7. Evaluation of anti-inflammatory activity

5.7.1. Carrageenan induced mice paw edema method

Carrageenan induced mice paw edema method described by Okokon *et al*.[84] was used with slight modification to determine the anti-inflammatory activity of the plant material. *Swiss albino* mice, weighing 22-28g (collected from the animal house of ICDDRB), were divided into several groups (at least 5) in order to perform the test. According to this method, Group-I (negative control group) receives 0.5% carboxymethyl cellulose (CMC) suspension and Group-II (positive control group) receives reference drug (indomethacin 10 mg/kg, p.o.). Rest of the groups received two doses of test samples. Subsequently, 1h after treatment, 0.05 ml of 1% suspension of carrageenan in normal saline was injected into the sub-planter region of left hind paw to induce edema. The paw edema was measured initially at 0, 1, 2, 3, 4, 5 hour time interval after carrageenan injection. Then the difference between the initial and subsequent values yielded the actual edema that was compared with control. Following formula was used to calculate the inhibition of inflammation:

$$\% \text{ inhibition} = 100\ (V_c - V_t/V_c)$$

Where V_c represents mean edema in control and V_t mean edema in group treated with drug and test extract.

5.8. Evaluation of analgesic activity

5.8.1. Acetic acid induced writhing method

The analgesic activity of the test samples (plant extracts and fractions) was evaluated using acetic acid induced writhing method in mice described by Zulkifer *et al*.[85] In this method, pain sensation is created by administrating acetic acid intraperitoneally (i.p.) to the

experimental animals. Diclofenac sodium (10 mg/kg) was used as a positive control. Test samples were administered orally at doses of 200 mg/kg body weight to the *swiss albino* mice following an overnight fast. Test samples (aqueous suspensions of the extract with 1% Tween 80) and vehicle (1% Tween 80 in water) were administered orally 30 minutes prior to intraperitoneal administration of 0.7% v/v acetic acid solution (0.1 ml/10gm) and diclofenac sodium (positive control) was administered 15 minutes prior to acetic acid injection. The animals were then placed on an observation table. Each mouse of all groups was observed individually for counting the number of writhing they made in 10 minutes commencing just 5 minutes after the intraperitoneal administration of acetic acid solution. Full writhing was not accomplished always by the animal, as sometimes the animal did start to give writhing but could not able to complete it. This incomplete writhing was counted as half-writhing. Two half-writhings were eventually considered as a full writhing. The number of writhes in each tested group was compared to that of a control group, while diclofenac sodium (10 mg/kg) was used as a reference substance (positive control).

Chapter 6

Results and Discussions

6. Results and Discussions

6.1. *Curcuma zedoaria*

6.1.1. Evaluation of antimicrobial activity

Disk diffusion method

Antimicrobial activity of the crude extract of *Curcuma zedoaria* has been evaluated in vitro against nine bacterial species and three yeasts (Table 6) which are known to cause infections in humans. As summarized in the table, the plant extract showed very little antimicrobial activity at a dose of 500 µg/disk and 1000 µg/disk against tested microorganisms. The highest inhibition zone diameter was 9 mm on *Vibrio mimicus* at a dose of 1000 µg and the lowest inhibition diameter was 5 mm on *Candida albicans* at a dose of 1000 µg. The reference drug amoxicillin showed highest zone of inhibition of 15 mm on *Vibrio parahemolyticus* and lowest zone of inhibition of 7 mm on *Candida albicans* at 20 µg/disk.

Table 6: Antimicrobial activities of *Curcuma zedoaria* using disk diffusion method

Samples	**Conc. (µg/disk)**	**Inhibition zone diameter (mm)**											
		Tested microorganisms											
		B.s	*S.t*	*V.p*	*V.m*	*S.a*	*S.d*	*S.l*	*S.b*	*P.a*	*S.c*	*C.a*	*A.n*
C. zedoaria (Crude)	500	5	-	-	7	6	5	6	5	6	5	-	-
	1000	7	-	-	9	8	7	8	7	7	7	5	-
Amoxicillin	20	13	12	15	14	10	14	12	13	10	9	7	8
Negative	20	-	-	-	-	-	-	-	-	-	-	-	-

- = no inhibition.

B.s = Bacillus subtilis; S.t = Salmonella typhi; V.p = Vibrio parahemolyticus; V.m = Vibrio mimicus; S.a = Staphylococcus aureus; S.d = Shigella dysenteriae; S.l = Sarcina lutea; S.b = Shigella boydii; P.a = Pseudomonas aeruginosa; S.c = Saccharomyces cereveceae; C.a = Candida albicans; A.n = Aspergillus niger.

6.1.2. Evaluation of Analgesic activity

Acetic acid induced writhing method

The analgesic activity of the VLC fractions of *Curcuma zedoaria* has been evaluated using acetic acid induced writhing method in mice. Table 7 and figure 19 show the effect of the VLC fraction extracts of *Curcuma zedoaria* on acetic acid induced writhing in mice. All the fractions except fraction 3 (ethyl acetate) showed significant reduction ($p<0.05$, and $p<0.01$) of writhing method induced by acetic acid at a dose of 200 mg/kg. Among all the fractions, fraction 2 (DCM) showed highest percentage inhibition of 45.52% reduction in writhing. The reference drug diclofenac showed 39.74% inhibition at a dose of 10 mg/kg.

Table 7: Effect of VLC fractions of *Curcuma zedoaria* on acetic acid induced writhing test in mice

Treatment	**Dose, Route**	**No. of inhibition**	**Percent inhibition**
Control (1% Tween 80 in water)	0.4ml/mouse, p.o.	53.60±2.11	-------
Diclofenac	10mg/kg, p.o.	32.3±1.72***	39.74
Fraction 1 (n-hexane)	200mg/kg, p.o.	33.1±1.62***	38.25
Fraction 2 (DCM)	200mg/kg, p.o.	29.2±0.49***	45.52
Fraction 3 (Ethyl acetate)	200mg/kg, p.o.	45.4±1.81*	15.30
Fraction 4 (Acetone)	200mg/kg, p.o.	37.4±2.78***	28.36
Fraction 5 (Methanol)	200mg/kg, p.o.	42.7±1.67**	20.37

Results are represented as Mean ± SEM, (n=5), *p<0.05, **p<0.01, Dunnett's test as compared to control

In acetic acid induced writhing method, sensation of pain is caused by abdominal constrictions associated with irritation of peritoneal cavity by acetic acid. Prolonged acetic acid induced irritation lead increase levels of prostaglandins (PGE_2 and $PGF_{2\alpha}$) biosynthesis via cyclooxygenase (COX) and lipoxygenase products in peritoneal fluids followed by

increased levels of free arachidonic acid secretion from tissue phospholipids.[85] The analgesic agents reduces the number writhing preferably by inhibition of synthesis of prostaglandins and lipoxygenase products. Fraction 2 (DCM) of *Curcuma zedoaria* showed significant analgesic action compared to the reference drug diclofenac by reducing the number of acetic acid induced writhing in mice at a dose of 200mg/kg. Thus, the results indicate that the significant pain reduction by the DCM fraction of the plant might be due to the presence of peripherally active analgesic principles. Further investigations are required in order to isolate the analgesic active compound of the fraction.

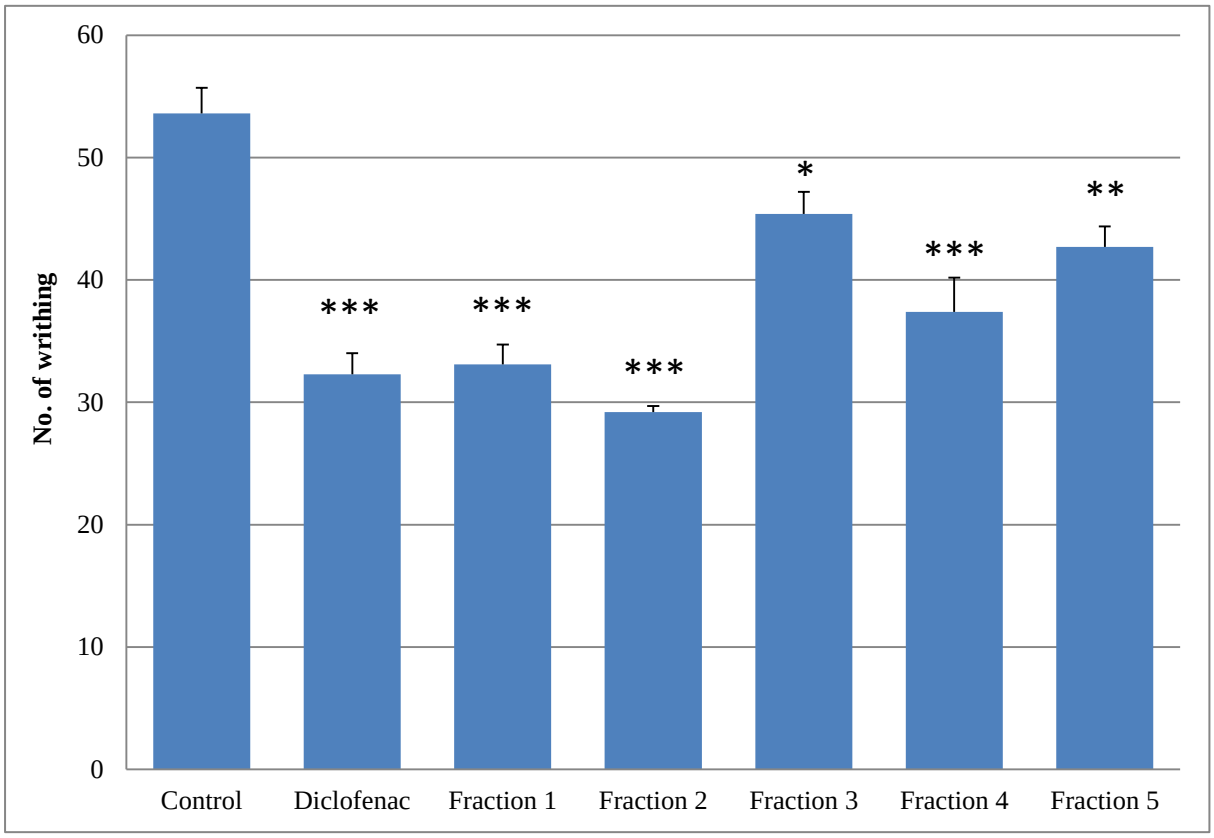

Figure 19: Effect of the VLC fractions of *Curcuma zedoaria* on acetic acid induced writhing in mice (n=5 for each group). Data are presented as mean±SEM using one-sample T test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, when compared to negative control. p value is determined by using oneway ANOVA followed by Dunnett's multiple comparison test. Graphical presentation was done using Microsoft Excel 2010.

6.2. *Nymphoides indica*

6.2.1. Evaluation of antimicrobial activity

Disk diffusion method

Antimicrobial activity of the crude extract of *Nymphoides indica* has been evaluated in vitro against thirteen bacterial species and three yeasts (Table 8) which are known to cause infections in humans. As summarized in the table, the plant extract showed promising antimicrobial activity against a number of pathogenic organisms at a dose of 400 µg/disk and 800 µg/disk against tested microorganisms. The highest inhibition zone diameter was 15 mm on *Streptococcus pyogens* and *Staphylococcus saprophyticus* at a dose of 800 µg and the lowest inhibition diameter was 6 mm on *Bacillus subtilis* at a dose of 400 µg. The reference drug amoxicillin showed highest zone of inhibition of 20 mm on *Bacillus subtilis* and lowest zone of inhibition of 10 mm on *Candida albicans* at 20 µg/disk

Table 8: Antimicrobial activities of *Nymphoides indica* using disk diffusion method

Sample	**Conc. (µg/disk)**	**Inhibition zone diameter (mm)**															
		Tested microorganisms															
		B.s	*V.p*	*V.m*	*S.a*	*E.c*	*S.d*	*P.a*	*S.l*	*S.b*	*S.c*	*C.a*	*A.n*	*K.p*	*S.p*	*S.s*	*B.c*
N. indica (Crude)	400	6	12	11	10	10	12	10	10	10	9	7	10	11	9	12	8
	800	8	14	13	12	12	14	12	12	13	11	9	13	12	15	15	10
A	20	20	15	15	17	18	17	14	12	16	12	10	14	14	16	18	14
N	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = no inhibition; A = Amoxicillin; N = Negative

B.s = Bacillus subtilis; V.p = Vibrio parahemolyticus; V.m = Vibrio mimicus; S.a = Staphylococcus aureus; E.c = Escherichia coli; S.d = Shigella dysenteriae; P.a = Pseudomonas aeruginosa ; S.l = Sarcina lutea; S.b = Shigella boydii; S.c = Saccharomyces cereveceae; C.a = Candida albicans; A.n = Aspergillus niger; K.p = Klebsiela pneumoniae; S.p = Streptococcus pyogens; S.s = Staphylococcus saprophyticus; B.c = Betahaemolytic streptococci.

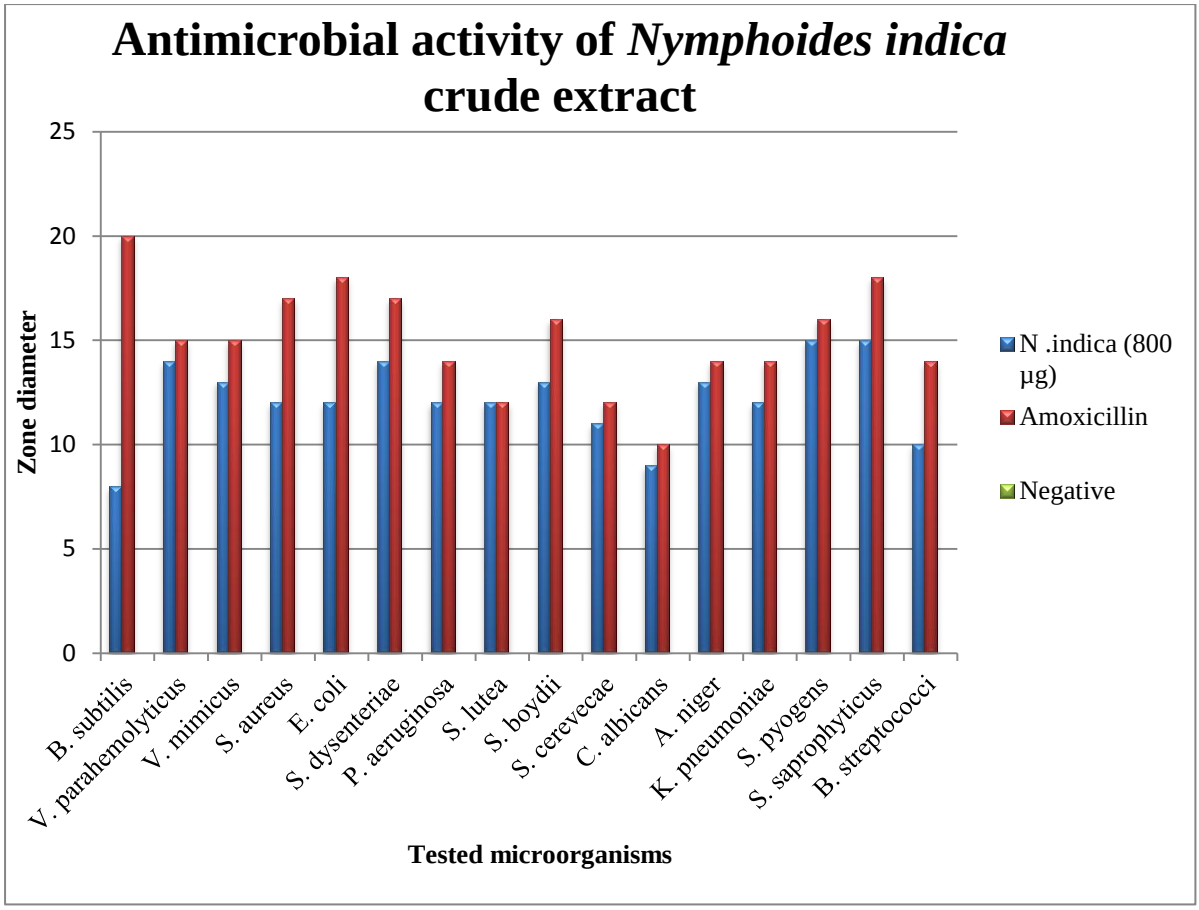

Figure 20: Evaluation of antimicrobial activity of *Nymphoides indica* crude extracts

Encouraged by the antimicrobial activity of the crude extract, the VLC fractions of *Nymphoides indica* has also been evaluated using disk diffusion method in vitro against thirteen bacterial species and three yeasts (Table 9) which are known to cause infections in humans. Fraction 2 (DCM), fraction 3 (Ethyl acetate), fraction 4 (acetone) and fraction 5 (methanol) were screened for antimicrobial activity. As summarized in the table, fraction 3 showed antimicrobial activity at a dose of 100 µg/disk and 200 µg/disk against tested microorganisms. The highest inhibition zone diameter was 10 mm on *Bacillus cereus* at a dose of 200 µg and the lowest inhibition diameter was 5 mm on *Vibrio mimicus* at a dose of 100 µg. The reference drug amoxicillin showed highest zone of inhibition of 20 mm on *Bacillus subtilis* and lowest zone of inhibition of 10 mm on *Candida albicans* at 20 µg/disk.

Table 9: Antimicrobial activities of VLC fractions of *Nymphoides indica*

Sample	**Conc. (µg/disk)**	**Inhibition zone diameter (mm)**						
		Tested microorganisms						
		B.c	*S.t*	*V.m*	*P.a*	*S.l*	*C.a*	*S.p*
Fraction 2 (DCM)	100	n.t	-	n.t	-	-	n.t	n.t
	200	n.t	7	n.t	7	7	n.t	n.t
Fraction 3 (Ethyl Acetate)	100	9	-	5	-		-	8
	200	10	-	7	-		8	9
Fraction 4 (Acetone)	100	-	-	-	-		-	-
	200	-	-	-	-		-	-
Fraction 5 (Methanol)	100	-	-	-	-		-	-
	200	-	-	-	-	-	-	-
Amoxicillin	10	10	12	11	12	13	18	12
Negative	20	-	-	-	-	-	-	-

- = no inhibition; n.t = not tested

B.c = Bacillus cereus; S.t = Salmonella paratyphi; V.m = Vibrio mimicus; P.a = Pseudomonas aeruginosa; S.l = Sarcina lutea; C.a = Candida albicans; S.p = Streptococcus pyogens.

The results show that the Ethyl acetate fraction of *Nymphoides indica* has potential antimicrobial activity. Therefore, further research should be carried out to isolate potential antimicrobial agent from the extract.

6.2.2. Evaluation of anti-inflammatory activity

Carregeenan induced mice paw edema method

The potential anti-inflammatory activity of VLC fractions of *Nymphoides indica* were investigated by the carrageenan induced mice paw edema method on mice. The anti-inflammatory activity was tested on three extracts; fraction 3 (ethyl acetate), fraction 4 (acetone) and fraction 5 (methanol). The effect of these extracts (200 mg/kg and 400 mg/kg) in carrageenan induced paw edema in mice is shown in table 10 and 11. Fraction 3 prevented the formation of edema induced by carrageenan in both doses with percent mean inhibition of 5.19% at 200 mg/kg and 8.59% at 400 mg/kg after 5 hours of fraction 3 administration as compared to the control vehicle treated group. Indomethacin (10 mg/kg) showed 7.8% mean inhibition. On carrageenan induced acute inflammation model, fraction 3 produced better inhibition of paw edema than indomethacin (positive) at a dose of 400 mg/kg.

Table 10: Anti-inflammatory activity of VLC fractions of *Nymphoides indica* and Indomethacin on carrageenan induced edema in the left hind-limb of mice.

Treatment	**Dose (mg/kg)**	**Edema diameter (mm) after carrageenan**					
		0hr	**1hr**	**2hr**	**3hr**	**4hr**	**5hr**
Control	10 ml/kg	4.38±0.16	4.02±0.06	4.24±0.18	4.16±0.11	4.12±0.58	4.20±0.14
Indomethacin	10	4.14±0.05	3.74±0.11	3.88±0.04*	3.84±0.02**	3.82±0.02**	3.84±0.02**
Fraction 3	200	4.22±0.11	3.88±0.04	4.02±0.04	3.90±0.03*	3.96±0.05	3.90±0.04*
	400	4.08±0.05	3.64±0.14*	3.88±0.05*	3.88±0.02**	3.78±0.06***	3.78±0.09***
Fraction 4	200	4.02±0.05	3.98±0.02	4.18±.010	4.14±0.06	4.12±0.06	4.16±0.05
	400	4.28±0.06	3.84±0.07	4.24±0.07	4.14±0.06	4.10±0.06	3.88±0.04**
Fraction 5	200	4.18±0.05	4.02±0.05	4.18±0.02	4.14±0.02	4.12±0.02	4.06±0.04
	400	4.34±0.10	3.96±0.07	4.10±0.03	4.14±0.06	4.06±0.06	4.02±0.05

Results are represented as Mean±SEM, n=5 mice, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. One way ANOVA followed by Dunnett's multiple comparison tests. Statistically significant when compared to control.

Table 11: Percent inhibition of paw edema exhibited by VLC fractions of *Nymphoides indica*

Treatment	Dose (mg/kg)	Percent inhibition (%) at various time intervals					
		1hr	2hr	3hr	4hr	5hr	Mean of % inhibition
Indomethacin	10	6.97	8.49	7.69	7.28	8.57	7.8
Fraction 3	200	3.48	5.19	6.25	3.88	7.14	5.19
	400	9.45	8.50	6.73	8.25	10.00	8.59
Fraction 4	200	0.99	1.41	0.48	0.00	0.95	0.77
	400	4.48	0.00	0.48	0.48	7.62	2.61
Fraction 5	200	0.00	1.42	0.48	0.00	3.33	1.05
	400	1.49	3.30	0.48	1.46	4.29	2.20

Fraction 3 showed better activity than the reference drug Indomethacin (10 mg/kg) at a dose of 400 mg/kg. These results indicate the presence of potential anti-inflammatory principles in fraction 3 of *Nymphoides indica*.

6.3. *Drynaria quercifolia* (rhizomes)

6.3.1. Evaluation of antimicrobial activity

Agar well diffusion method

Antimicrobial activity of the crude extract of the rhizome of *Drynaria quercifolia* has been evaluated in vitro using agar well diffusion method against seven bacterial species and three yeasts (Table 12) which are known to cause infections in humans. As summarized in the table, the plant extract showed very little antimicrobial activity at a dose of 200 µg/disk and 400 µg/disk against tested microorganisms. The highest inhibition zone diameter was 8mm on *Salmonella typhi* and *Saccharomyces cereveceae* at a dose of 400 µg and the lowest inhibition diameter was 5 mm on *Klebsiella pneumoniae* at a dose of 200 µg. The reference drug amoxicillin showed highest zone of inhibition of 14 mm on *Vibrio mimicus* and lowest zone of inhibition of 9 mm on *Staphylococcus aureus, Sarcina lutea, Candida albicans and Aspergillus niger* at 25 µg/disk.

Table 12: Antimicrobial activities of *Drynaria quercifolia* (rhizomes)

Sample	Conc. (µg/disk)	Inhibition zone diameter (mm)									
		Tested microorganisms									
		S.t	*V.p*	*V.m*	*S.a*	*S.l*	*K.p*	*P.a*	*S.c*	*C.a*	*A.n*
D. quercifolia (rhizomes)	200	6	-	-	-	-	5	-	6	6	-
	400	8	-	-	-	-	7	6	8	7	-
Amoxicillin	25	10	11	14	9	9	12	11	12	9	9
Negative	20	-	-	-	-	-	-	-		-	-

- = no inhibition.

S.t = Salmonella typhi; V.p = Vibrio parahemolyticus; V.m = Vibrio mimicus; S.a = Staphylococcus aureus; S.l = Sarcina lutea; P.a = Pseudomonas aeruginosa; K.p = Klebsiella pneumoniae; S.c = Saccharomyces cereveceae; C.a = Candida albicans; A.n = Aspergillus niger.

6.4. *Drynaria quercifolia* (leaves)

6.4.1. Evaluation of antimicrobial activity

Disk diffusion method

Antimicrobial activity of the crude extract of the leaves of *Drynaria quercifolia* has been evaluated in vitro using disk diffusion method against seven bacterial species and one yeast (Table 13) which are known to cause infections in humans. As summarized in the table, the plant extract showed very little antimicrobial activity at a dose of 400 µg/disk and 800 µg/disk against tested microorganisms. The extract did not show any activity at a dose of 200 µg/disk on any of the tested microorganisms. The highest inhibition zone diameter was 9 mm on *Pseudomonas aeruginosa* at a dose of 800 µg and the lowest inhibition diameter was 3 mm on *Escherichia coli* and *Saccharomyces cereveceae* at a dose of 400 µg. The reference drug amoxicillin showed highest zone of inhibition of 14 mm on *Bacillus subtilis* and lowest zone of inhibition of 8 mm on *Saccharomyces cereveceae* at 25 µg/disk.

Table 13: Antimicrobial activities of *Drynaria quercifolia* (leaves)

Sample	**Conc. (µg/disk)**	**Inhibition zone diameter (mm)**							
		Tested microorganisms							
		B.s	*S.t*	*S.p*	*S.a*	*E.c*	*K.p*	*P.a*	*S.c*
D. quercifolia (leaves)	200	-	-	-	-	-	-	-	-
	400	-	5	-	-	3	6	7	3
	800	-	7	-	-	5	7	9	5
Amoxicillin	25	14	12	12	10	9	11	11	8
Negative	20	-	-	-	-	-		-	-

- = no inhibition.

B.s = Bacillus subtilis; S.t = Salmonella typhi ; S.p= Salmonella para typhi; S.a = Staphylococcus aureus; E.c = Escherichia coli; K.p = Klebsiella pneumoniae; P.a = Pseudomonas aeruginosa; S.c = Saccharomyces cereveceae.

6.5. *Rhynchostylis retusa*

6.5.1. Evaluation of antimicrobial activity

Agar well diffusion method

Antimicrobial activity of the methanolic extract of *Rhynchostylis retusa* has been evaluated in vitro using disk diffusion method against six bacterial species and one yeast (Table 14) which are known to cause infections in humans. As summarized in the table, the plant extract showed very little antimicrobial activity at a dose of 200 µg/disk, 400 µg/disk and 800 µg/disk against tested microorganisms. The highest inhibition zone diameter was 9 mm on *Pseudomonas aeruginosa* at a dose of 800 µg and the lowest inhibition diameter was 4 mm on *Klebsiella pneumoniae* at a dose of 200 µg and *Staphylococcus aureus* at a dose of 400 µg. The reference drug amoxicillin showed highest zone of inhibition of 14 mm on *Salmonella typhi* and lowest zone of inhibition of 8 mm on *Saccharomyces cereveceae* at 10 µg/disk.

Table 14: Antimicrobial activities of *Rhynchostylis retusa*

Sample	**Conc. (µg/disk)**	**Inhibition zone diameter (mm)**						
		Tested microorganisms						
		S.t	*S.a*	*S.d*	*E.c*	*K.p*	*P.a*	*S.c*
R. retusa	200	-	-	-	-	4	-	-
	400	-	4	-	-	7	6	-
	800	4	6	-	5	8	9	-
Amoxicillin	10	14	9	10	10	11	12	8
Negative	20	-	-	-	-	-		-

- = no inhibition.

S.t = Salmonella typhi ; S.a = Staphylococcus aureus; S.d = Shigella dysenteriae; E.c = Escherichia coli; K.p = Klebsiella pneumoniae; P.a = Pseudomonas aeruginosa; S.c = Saccharomyces cereveceae.

Chapter 7

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7. References

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