# Bioactivity Guided Separation and Purification of Secondary Metabolites of *Nymphoides indica*

A dessertation submitted for the partial fulfillment of the course of Pharmaceutical Research (PHRM 404) of the Department of Pharmacy, East West University for the Degree of Bachelor of Pharmacy

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**Date of Submission:** May 31, 2012

# Dedication

*This Paper Is Dedicated To*

*My Parents*

# Declaration by the research candidate

I, Mahmudul Hasan Chowdhury (ID # 2008-3-70-068), hereby declare that the dissertation entitled **"Bioactivity guided isolation and purification of active principles & Pharmacological evaluation of *Nymphoides indica*",** submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic record of original research work carried out by me during 2011-2012 under the supervision and guidance of Dr. Chowdhury Faiz Hossain, Dean, Faculty of Sciences and Engineering & 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:** 31.05.2012

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# Thesis Certificate

This is to certify that the thesis **"Bioactivity guided isolation and purification of active principles & Pharmacological evaluation of *Nymphoides indica*"** is submitted to the Department of Pharmacy, East West University, Mohakhali, Dhaka, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Mahmudul Hasan Chowdhury (ID # 2008-3-70-068) 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

As a continuing work of Professor Chowdhury Faiz Hossain's research group crude MeOH extract of *Nymphoides indica* showed potent antibacterial effect. The activity was reconfirmed from the 6 gm MeOH extract of *Nymphoides indica*. Highest effect was found in *Salmonella typhi* which was 14 mm diameter of zone of inhibition in dose of 1000 µg/disc. On continuation of the following work large scale of Nymphoides indica collected from Panchagarh. The leaves were crashed and 250 gm of dried crashed leaves were obtained. Extraction of Nymphoides indica leaves (250 gm) at room temperature by maceration with EtOH yielded 42 gm (16.8 % dry weight) extract. The EtOH extract of *Nymphoides indica* was subjected to vacuum liquid chromatography (VLC) and five different fractions were collected using five different solvents; Fractions were- Fraction-1 (0.28 gm) eluted with 5000 ml n-hexane, Fraction-2 (0.63 gm) eluted 2000 ml $CH_2Cl_2$, Fraction-3 (3.97 gm) eluted with 5000 ml EtOAc, Fraction-4 (20.2 gm) eluted with 5000 ml acetone and Fraction-5 (0.74 gm) eluted with 5000 ml MeOH. The antimicrobial effect was further tested by using the VLC fractions. The effect was found in the fractions of n-hexane, $CH_2Cl_2$ and EtOAc. Highest effect was found with EtOAc fraction that was 18 mm diameter of zone of inhibition in *Staphylococcus aureus* in dose of 100 µg/disc. Fraction-2 of VLC fraction was separated by open column chromatography with silica gel which gave and the fractions were collected by monitoring the thin layer chromatography (TLC) which gave 18 fractions. Among them Fr-2-3 & Fr-2-4 yielded a colorless crystal, NIH-3 (17 mg). This compound was UV inactive and charring with MeOH and $H_2SO_4$ (9:1) gave dark black color. Based on $^1$H-NMR analysis, structure of NIH-3 was proposed as shown below (Figure-i). To the best of our knowledge the proposed structure of NIH-3 is novel. The compound may be a triterpenoid and have a structural simillarity with a triterpenoid that was lupeol. Rather than the lupeol, there was an

extra double bond between $C_{12}$ and $C_{13}$ in NIH-3. $^{13}$CNMR, Mass spectroscopy, $^{1}$H-$^{1}$H COSY, HMQC, HMBC and NOSY are required to confirm the proposed structure.

Then Fraction-3 was subjected to open column chromatography and 35 fractions were separated according to different color bands in the column and by monitoring the TLC. Further chemical separation and testing biological activities of these fractions are yet to be done.

**Keywords**: *Nymphoides indica*, triterpenoid, NIH-3, antimicrobial activity, vacuum liquid chromatography, open column chromatography, thin layer chromatography.

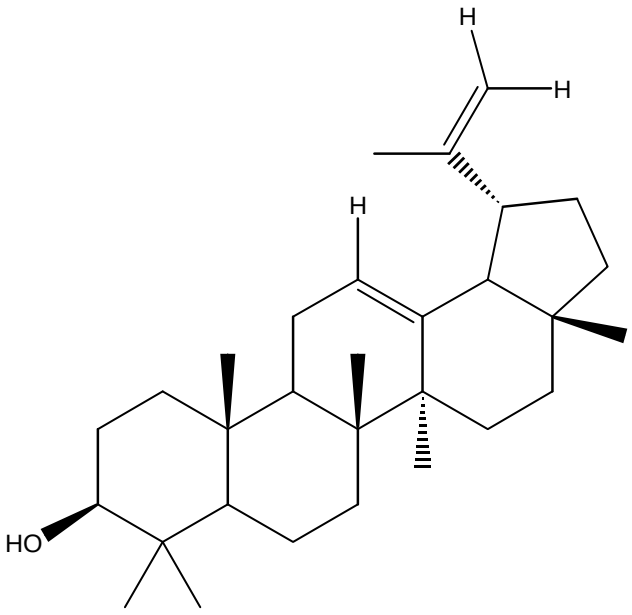
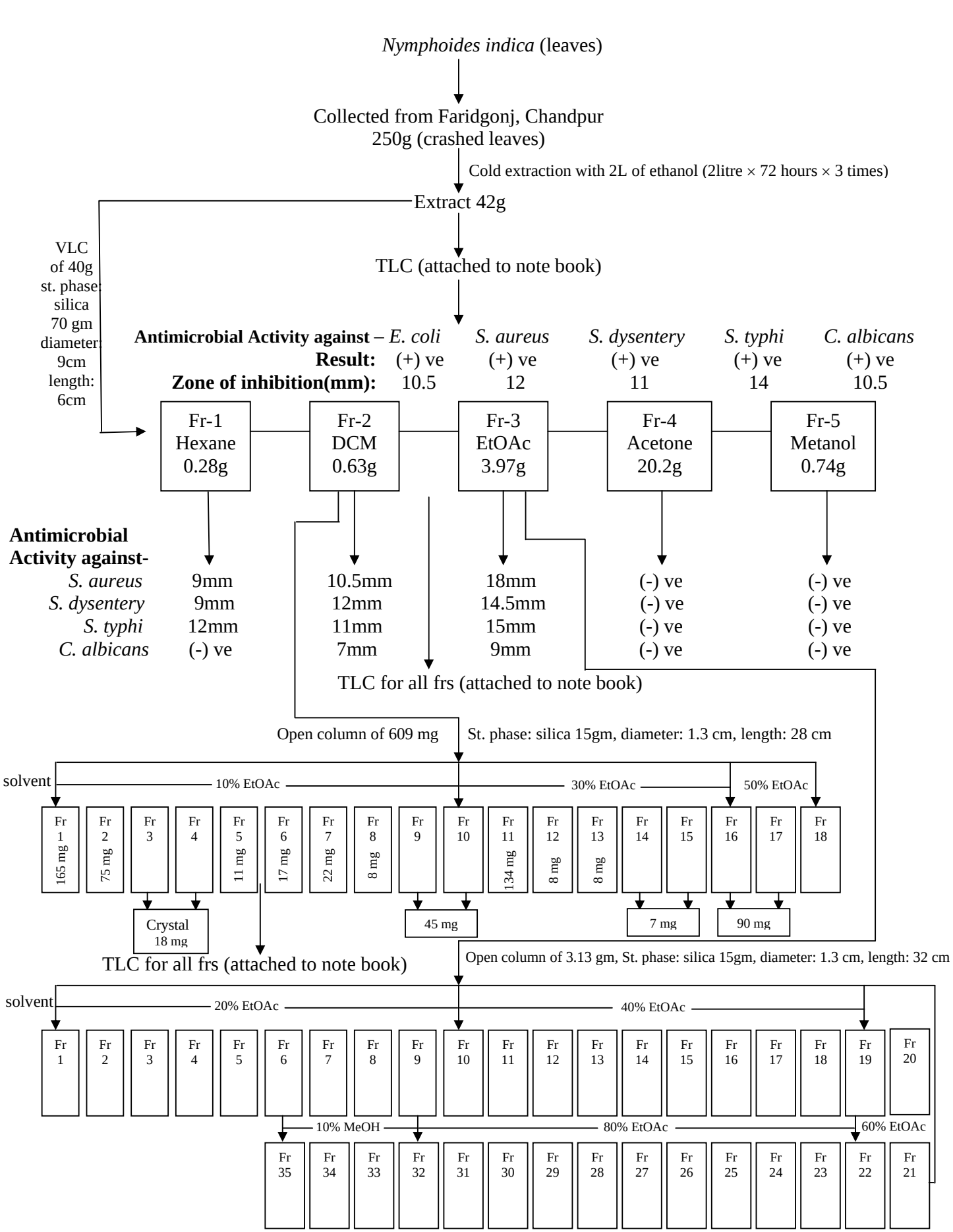



**Figure-i**: NIH-3


*Nymphoides indica* (leaves)
Collected from Faridgonj, Chandpur
250g (crashed leaves)
Cold extraction with 2L of ethanol (2litre × 72 hours × 3 times)
Extract 42g
TLC (attached to note book)
VLC of 40g st. phase: silica 70 gm diameter: 9cm length: 6cm
Antimicrobial Activity against – E. coli S. aureus S. dysentery S. typhi C. albicans
Result: (+) ve (+) ve (+) ve (+) ve (+) ve
Zone of inhibition(mm): 10.5 12 11 14 10.5
Fr-1 Hexane 0.28g
Fr-2 DCM 0.63g
Fr-3 EtOAc 3.97g
Fr-4 Acetone 20.2g
Fr-5 Metanol 0.74g
Antimicrobial Activity against-
S. aureus 9mm 10.5mm 18mm (-) ve (-) ve
S. dysentery 9mm 12mm 14.5mm (-) ve (-) ve
S. typhi 12mm 11mm 15mm (-) ve (-) ve
C. albicans (-) ve 7mm 9mm (-) ve (-) ve
TLC for all frs (attached to note book)
Open column of 609 mg
St. phase: silica 15gm, diameter: 1.3 cm, length: 28 cm
solvent
10% EtOAc
30% EtOAc
50% EtOAc
Fr 1 165 mg
Fr 2 75 mg
Fr 3
Fr 4
Fr 5 11 mg
Fr 6 17 mg
Fr 7 22 mg
Fr 8 8 mg
Fr 9
Fr 10
Fr 11 134 mg
Fr 12 8 mg
Fr 13 8 mg
Fr 14
Fr 15
Fr 16
Fr 17
Fr 18
Crystal 18 mg
45 mg
7 mg
90 mg
TLC for all frs (attached to note book)
Open column of 3.13 gm, St. phase: silica 15gm, diameter: 1.3 cm, length: 32 cm
solvent
20% EtOAc
40% EtOAc
Fr 1
Fr 2
Fr 3
Fr 4
Fr 5
Fr 6
Fr 7
Fr 8
Fr 9
Fr 10
Fr 11
Fr 12
Fr 13
Fr 14
Fr 15
Fr 16
Fr 17
Fr 18
Fr 19
Fr 20
10% MeOH
80% EtOAc
60% EtOAc
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# Chapter-1
# Introduction

## 1. Introduction:

Natural product may be defined as 'Naturally occurring compounds that are end products of secondary metabolism often, they are unique compounds for particular organism or classes of organisms.' A natural product is a chemical compound or substance produced by a living organism - found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design.[1]

Natural products may be extracted from tissues of terrestrial plants, marine organisms or microorganism fermentation broths. A crude (untreated) extract from any one of these sources typically contains novel, structurally diverse chemical compounds, which the natural environment is a rich source of. Chemical diversity in nature is based on biological and geographical diversity, so the travelling around the world obtaining samples to analyze and evaluate in drug discovery screens or bioassays. This effort to search for natural products is known as bioprospecting.[1]

Pharmacognosy derived from two Greek words that are "pharmakon" or drug and "gnosis" or knowledge. Like many contemporary fields of science, Pharmacognosy has undergone significant change in recent years and today represents a highly interdisciplinary science which is one of five major areas of pharmaceutical education. Its scope therefore includes the study of physical, chemical, biochemical and biological properties of drugs, drug substances, or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources.[2]

Pharmacognosy provides the tools to identify, select and process natural products destined for medicinal use. Usually, the natural product compound has some form of biological activity and that compound is known as the active principle - such a structure can act as a lead compound. Many of today's medicines are obtained directly from a natural source. On the other hand, some medicines are developed from a lead compound originally obtained from a natural source. This means the lead compound:

- ✓ can be produced by total synthesis, or
- ✓ can be a starting point (precursor) for a semisynthetic compound, or
- ✓ can act as a template for a structurally different total synthetic compound.

This is because most biologically active natural product compounds are secondary metabolites with very complex structures. This has an advantage in that they are extremely novel compounds but this complexity also makes many lead compounds' synthesis difficult.[1]

Man has been using Plant based medicines in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations since centuries. The plant based indigenous

knowledge was passed down from generation to generation in various parts of the world throughout its history and has significantly contributed to the development of different traditional systems of medicine. Through the use of plants, human beings have taken advantages of the defensive, attractive and medicinal compounds present in leaves, flowers, roots, sap and bark of species around the World. The first record of the use of plant in human medicine was in an Egyptian papyrus of about 1500 B.C. Sucrose the most common and well known natural product has its history of use since the time of Alexander the Great. With the discovery of salicin from willow tree extracts and the development of aspirin in 1899, the art of exploiting natural products became a molecular science. The discovery of penicillin in 1928 and its subsequent development as an anti-infective agent represents another milestone in the history of natural products, and marked the beginning of a new era in drug discovery, in which bacteria and fungi were added to the plant kingdom as sources for biologically active compounds. Isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century and subsequently led to the isolation of early drugs such as cocaine, codeine, digitoxin and quinine etc. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally.

Natural products can contribute to the search for new drugs in three different ways:

- by acting as new drugs that can be used in an unmodified state (e.g., vincristine from Catharanthus roseus).
- by providing chemical ‘‘building blocks’’ used to synthesize more complex molecules (e.g., diosgenin from Dioscorea floribunda for the synthesis of oral contraceptives).
- by indicating new modes of pharmacological action that allow complete synthesis of novel analogs (e.g., synthetic analogs of penicillin from Penicillium notatum).

Natural products will certainly continue to be considered as one of the major sources of new drugs in the years to come because

- they offer incomparable structural diversity.
- many of them are relatively small (<2000 Da).
- they have ‘‘drug-like’’ properties (i.e., they can be absorbed and metabolized).[3]

Recently approved natural-product-based drugs have been described extensively in earlier reviews. They include compounds from plants (including elliptinium, galantamine and huperzine), microbes (daptomycin) and animals (exenatide and ziconotide), as well as synthetic or semi-synthetic compounds based on natural products (e.g. tigecycline,

everolimus, telithromycin, micafungin and caspofungin). They cover a range of therapeutic indications: anti-cancer, anti-infective, anti-diabetic, among others, and they show a great diversity of chemical structures. In UK March 2008, different types of natural products were obtained from different sources were at different stages of development.[4]

**Table-1**

**Drugs based on natural products at different stages of development.**[4]

| Development stage | Plant | Bacterial | Fungal | Animal | Semi-synthetic | Total |
|---|---|---|---|---|---|---|
| Preclinical | 46 | 12 | 7 | 7 | 27 | 99 |
| Phase I | 14 | 5 | 0 | 3 | 8 | 30 |
| Phase II | 41 | 4 | 0 | 10 | 11 | 66 |
| Phase III | 5 | 4 | 0 | 4 | 13 | 26 |
| Pre-registration | 2 | 0 | 0 | 0 | 2 | 4 |
| Total | 108 | 25 | 7 | 24 | 61 | 225 |

Natural products are predominantly being studied for different therapeutic catagories.

**Table-2**

**Therapeutic categories of natural product-derived drugs at different stages of development.**[4]

| Therapeutic area | Preclinical | Phase I | Phase II | Phase III | Pre-registration | Total |
|---|---|---|---|---|---|---|
| Cancer | 34 | 15 | 26 | 9 | 2 | 86 |
| Anti-infective | 25 | 4 | 7 | 2 | 2 | 40 |
| Neuropharmacological | 6 | 3 | 9 | 4 | 0 | 22 |
| Cardiovascular | 9 | 0 | 5 | 6 | 0 | 20 |
| Inflammation | 6 | 2 | 9 | 1 | 0 | 18 |
| Metabolic | 7 | 3 | 6 | 1 | 0 | 17 |
| Skin | 7 | 1 | 2 | 0 | 0 | 10 |
| Hormonal | 3 | 0 | 2 | 1 | 0 | 6 |
| Immunosuppressant | 2 | 2 | 0 | 2 | 0 | 6 |
| Total | 99 | 30 | 66 | 26 | 4 | 225 |

Despite the application of molecular modelling, combinatorial chemistry and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (NCEs). According to a recent survey, 61% of the 877

small-molecule NCEs introduced as drugs worldwide during 1981–2002 was inspired by natural products. These include: natural products (6%), natural products derivatives (27%), synthetic compounds with natural products-derived pharmacophore (5%) and synthetic compounds designed from natural products (natural products mimic, 23%).

Strategies for research in the area 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. Chemotaxonomic investigation.
   d. Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.
2. Modern strategies:
   a. Bioassay-guided (mainly in vitro) isolation and identification of active ‘‘lead’’ compounds from natural sources.
   b. Production of natural products libraries.
   c. Production of active compounds in cell or tissue culture, genetic manipulation, natural combinatorial chemistry, and so on.
   d. More focused on bioactivity.
   e. Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics.
   f. Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.[3]

### 1.1. Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers. Phytochemistry is simply the study of the.chemistry of the constituents of plants, with the aim of identification, isolation and characterization of biologically active compounds, evaluation of their effects in animals and human.[2]

Earth is a planet dominated by plants. The green plant is fundamental to all other life. The oxygen we breathe, the nutrients we consume, the fuels we burn and many of the most important materials we use were produced by plants. Plants represent the first stage in the evolution of living things. In the process of the growth of nature, plants multiplied in number, variety and types. Humanity has identified as many as 7.5 lakhs species of plants"' on earth, of which 5 lakhs are classified as "higher plants" and 2.5 lakhs as "lower plants". The association between plant and man is an age-old process starting from human civilization. There has always been a race between nature and human knowledge. The plants sustain nature and nature sustains them. The interdependence of man and nature increases day by day. Since their evolution, plants became primarily useful for mankind. Realization of the importance of plants in the welfare of humanity prompted their systematic study from different angles.

The process that leads from the plant to a pharmacologically active, pure constituent is very long and tedious, and requires a multidisciplinary collaboration of botanists, ethnobotanists, traditional healers, pharmacognosists, chemists, pharmacologists and toxicologists. This approach involves the following steps-

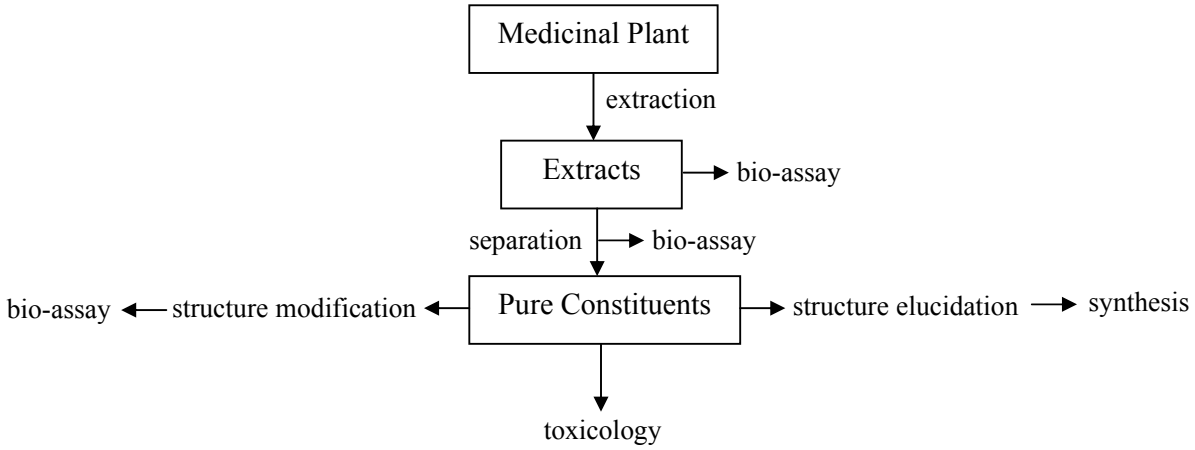



**Figure-1**: Procedure for obtaining the active principles from plants.[2]

- Information of folkloric use by traditional healers and other custodian of this knowledge.
- Collection, proper botanical identification and drying of the plant material.
- Preparation of appropriate extracts and preliminary chromatographic analysis by TLC and HPLC.
- Biological and pharmacological screening of crude extracts.

- Several consecutive steps of chromatographic separation, where each fraction obtained has to be submitted to bioassays in order to follow the activity (activityguided fractionation).
- Verification of the purity of the isolated compounds.
- Structure elucidation by chemical and physicochemical methods.
- Partial or total synthesis.
- Preparation of derivatives/analogues for the investigation of structure activity relationships.
- Large scale isolation for further pharmacological and toxicological tests.[2]

**1.2. Metabolites**

Metabolites are the intermediates and products of metabolism. The term metabolite is usually restricted to small molecules. Metabolites have various functions, including fuel, structure and signaling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own (usually as a cofactor to an enzyme), defense, and interactions with other organisms (e.g. pigments, odorants).[5]

**1.2.1. Primary Metabolites**

Primary metabolites are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids and the common amino acids and sugars.

They include compounds such as carbohydrate and lipids- substances essential to the structure and life of the palnt, as well as essential for human nutrition. Carbohydrates are largely made up of sugars- saccaharides. Glucose and fructose are the examples of monosaccharides- they consist of a single saccaharide molecule. Polysaccharides consist of several saccharide molecules linked together. Lipids commonly known as fats- provide a reservoir of fuel for cells. They also form a major component of cell membranes in both plants and animals. A group of lipids known as fatty acids are important for human health. There are some fatty acids that the body can not produce and which must be sourced through the diet. These are known as essential fatty acids.

**1.2.2. Secondary Metabolites**

Secondary metabolites are those metabolites which are often produced in a phase of subsequent to growth, have no function in growth (although they may have survival function), are produced by certain restricted taxonomic groups of microorganisms, have unusual chemicals structures, and are often formed as mixtures of closely related members of a chemical family. A metabolic intermediate or product, found as a differentiation product in

restricted taxonomic groups, not essential to growth and the life of the producing organism, and biosynthesis from one or more general metabolites by a wider variety of pathways than is available in general metabolism. Secondary metabolites are not essential for growth and tend to be strain specific. They have a wide range of chemical structures and biological activities. They are derived by unique biosynthetic pathways from primary metabolites and intermediates.[6]

Plants produce as amazing diversity of low molecular weight compounds. Of the estimated 400,000 – 500,000 plant species around the globe, only a small percentage has been investigated phytochemically and the fraction subjected to biological or pharmacological screening is even lower. The ability to synthesize secondary metabolites has been selected through the course of evolution in different plant lineage when such compounds address specific needs.

- Floral scent volatiles and pigments have evolved to attract insect pollinators and thus enhance fertilization.
- To synthesize toxic chemical has evolved to ward off pathogens and herbivores or to suppress the growth of neighboring plants.
- Chemicals found in fruits prevent spoilage and act as signals (in the form of color, aroma, and flavor) of the presence of potential rewards (sugars, vitamins and flavor) for animals that eat the fruit and thereby help to disperse the seeds.
- Other chemicals serve cellular functions that are unique to the particular plant in which they occur (e.g. resistance to salt or drought).

The majority of secondary metabolites belong to one of a number of families, each of which have particular structural characteristics arising from the way in which they are built up in nature (biosynthesis). The classes of secondary metabolites are:

- Terpenoids(29,000): Terpenes constitute the largest class of secondary metabolites and are united by their common biosynthetic origin from acetyl-coA or glycolytic intermediates. A vast majority of the different terpenes structures produced by plants as secondary metabolites that are presumed to be involved in defense as toxins and feeding deterrents to a large number of plant feeding insects and mammals.

  -monoterpene(1000): Many derivatives are important agents of insect toxicity. It shows strong insecticidal responses (neurotoxin) to insects like beetles, wasps, moths, bees, etc. and a popular ingredient in commercial insecticides because of low persistence in the environment and low mammalian toxicity.[6]

-sesquiterpene(3000): Their role in plant defense characterized by a five membered lactone ring (a cyclic ester) and have strong feeding repellence to many herbivorous insects and mammals. Sesquiterpene also plays primarily regulatory roles in the initiation and maintenance of seed and bud dormancy and plants response to water stress by modifying the membrane properties and act as a transcriptional activator. In addition, it increases the cytosolic calcium concentration and causes alkalinisation of the cytosol.

-diterpenes(1000): They work as skin irritants and internal toxins to mammals. It plays various detrimental roles in numerous plant developmental processes such as seed germination, leaf expansion, flower and fruit set , dry mass and bio mass production, stomatal conductance, $CO_2$ fixation and also known to exert their numerous physiological effects via specific enzymes, the synthesis of which they induce by influencing the basic process of translocation and transcription.

-triterpenes, steroids, saponines(4,000): Several steroid alcohols (sterols) are important component of plant cell membranes, especially in the plasma membrane as regulatory channels and maintain permeability to small molecules by decreasing the motion of the fatty acid chains.

- Phenolics (8,000): Plants produce a large variety of secondary products that contain a phenol group, a hydroxyl functional group on an aromatic ring called Phenol, a chemically heterogeneous group also. They could be an important part of the plants defense system against pests and diseases including root parasitic nematodes.

  -Flavonoids(2000) : One of the largest classes of plant phenolic, perform very different functions in plant system including pigmentation and defense. Two other major groups of flavonoids found in flowers are flavones and flavonols function to protect cells from UV-B radiation because they accumulate in epidermal layers of leaves and stems and absorb light strongly in the UV-B region while letting visible wavelengths throughout uninterrupted.

  -Polyacetylens-1000

  -Polyketides-750

  -Phenylpropanoids-500

- Nitrogen containing: Most of them are biosynthesized from common amino acids. All are of considerable interest because of their role in the anti herbivore defense and toxicity to humans.[6]

  -Alkaloids(12,000): Generally, most of them are toxic to some degree and appear to serve primarily in defense against microbial infection and herbivoral attack. They are usually

synthesized from one of the few common amino acids, in particular, aspartic acid, lysine, tyrosine and tryptophan.

-Non protein amino acids(600): Many plants also contain unusual amino acids called nonprotein amino acids that incorporated into proteins but are present as free forms and act as protective defensive substances. They exert their toxicity in various ways. Some block the synthesis of or uptake of protein amino acid while others can be mistakenly incorporated in to proteins.

-Cyanogenic glycosides(100): They constitute a group of N-containing protective compounds other than alkaloids, release the poison HCN. They are not in themselves toxic but are readily broken down to give off volatile poisonous substances like HCN and $H_2S$ when the plant is crushed.[6]

-Glucosinolates- 100

-Amines- 100

**Table-3**

**The Principle of Phytotherapy.**

| Plant primary metabolites | Plant Secondary metabolites (Plant natural products) |
|---|---|
| 1. Organic compounds produced in the plant kingdom | 1. Organic compounds produced in plant kingdom |
| 2. Have metabolic functions essential for plant growth and development | 2. Don't have apparent functions involved in plant growth and development |
| 3. Produced in every plant | 3. Produced in different plant families, in specific groups of plant families or in specific tissues, cells or developmental stages throughout plant development. |
| 4. Include carbohydrates, amino acids, nucleotides, fatty acids, steroids and lipids. | 4. Include terpenoids, special nitrogen metabolite (including, non-protein amino acids, amines, cyanogenic glycosides and alkaloids) and phenolics. |

# Chapter-2

# Preparation of Plant Material

## 2. Preparation of Plant Material:

### 2.1. Selection

Any plant species and plant parts, collected randomly, can be investigated using available phytochemical methods. However, a more targeted approach is often preferred to a random selection. The plant material to be investigated can be selected on the basis of some specific traditional ethnomedical uses. Extracts prepared from plants and used as traditional remedies to treat certain diseases are more likely to contain biologically active components of medicinal interest. Alternatively, the plant can be selected based on chemotaxonomical data. This means that if species or genera related to the plant under investigation are known to contain specific compounds, then the plant itself can be expected to contain similar compounds.[3]

Another approach is to select the plant with a view to investigate a specific pharmacological activity. Additionally, work can be carried out on a particular group of natural products, a plant family, or on plants from a specific country or local area. Some plants can be selected following a combination of approaches. The use of literature databases early in the selection process can provide some preliminary information on the type of natural products already isolated from the plant and the extraction methods employed to isolate them.[3]

### 2.2. Collection and Identification

The whole plant or a particular plant part can be collected depending on where the metabolites of interest (if they are known) accumulate. Hence, aerial (e.g., leaves, stems, flowering tops, fruits, seeds, bark) and underground (e.g., bulbs, tubers, roots) parts can be collected separately. Only healthy specimens should be obtained, as signs of contamination (fungal, bacterial, or viral) may be linked to a change in the profile of metabolites present. Collection of plant material can also be influenced by other factors such as the age of the plant and environmental conditions (e.g., temperature, rainfall, amount of daylight, soil characteristics, and altitude). In some cases, it can be challenging, if not hazardous. This is particularly true if the targeted plant is a species of liana indigenous to the canopy (60m above ground level) of a remotely accessible area of the rain forests. It is important to take these issues into account for recollection purposes to ensure a reproducible profile (nature and amount) of metabolites.[3]

It should be stressed that the plant must also be identified correctly. A specialized taxonomist should be involved in the detailed authentication of the plant (i.e., classification into its species, genus, family, order, and class). Any features relating to the collection, such as the

name of the plant, the identity of the part(s) collected, the place and date of collection, should be recorded as part of a voucher (a dried specimen pressed between sheets of paper) deposited in a herbarium for future reference.[3]

**2.3. Drying and Grinding**

If the plant is known to contain volatile or thermolabile compounds, it may be advisable to snap–freeze the material as soon as possible after collection. Once in the laboratory, the collected plants are washed or gently brushed to remove soil and other debris. Frozen samples can be stored in a freezer (at -20$^{o}$C) or freeze-dried (lyophilized). It is usual to grind them subsequently in a mortar with liquid nitrogen. Extracting the pulverized residue immediately or storing it in a freezer to prevent any changes in the profile of metabolites is advisable.[3]

It is, however, a more common practice to leave the sample to dry on trays at ambient temperature and in a room with adequate ventilation. Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. Plant material should be sliced into small pieces and distributed evenly to facilitate homogenous drying. Protection from direct sunlight is advised to minimize chemical reactions (and the formation of artifacts) induced by ultraviolet rays. To accelerate the drying process (especially in countries with high relative humidity), the material can be dried in an oven. This can also minimize enzymatic reactions (e.g., hydrolysis of glycosides) that can occur as long as there is some residual moisture present in the plant material. The dried plant material should be stored in sealed containers in a dry and cool place. Storage for prolonged periods should be avoided, as some constituents may decompose.[3]

The aim of grinding (i.e., fragmentation of the plant into smaller particles) is to improve the subsequent extraction by rendering the sample more homogenous, increasing the surface area, and facilitating the penetration of solvent into the cells. Mechanical grinders (e.g., hammer and cutting mills) are employed conveniently to shred the plant tissues to various particle sizes. Potential problems of grinding include the fact that some material (e.g., seeds and fruits rich in fats and volatile oils) may clog up the sieves and that the heat generated may degrade thermolabile metabolites.[3]

**Figure-2:** Drying the plant

**Figure-3:** Blending the plant

### 2.4. Range of Extraction Methods

Solvent extraction relies on the principle of either ‘‘liquid–liquid’’ or ‘‘solid–liquid’’ extraction. In solid–liquid extraction, the plant material is placed in contact with a solvent. While the whole process is dynamic, it can be simplified by dividing it into different steps. In the first instance, the solvent has to diffuse into cells, in the following step it has to solubilize the metabolites, and finally it has to diffuse out of the cells enriched in the extracted metabolites. In general, extractions can be facilitated by grinding (as the cells are largely destroyed, the extraction relies primarily on the solubilization of metabolites) and by increasing the temperature (to favor solubilization). Evaporation of the organic solvents or freeze-drying (of aqueous solutions) yields dried crude extracts. Different types of extraction methods are-

❖ Maceration

This simple, but still widely used, procedure involves leaving the pulverized plant to soak in a suitable solvent in a closed container at room temperature. The method is suitable for both initial and bulk extraction. Occasional or constant stirring of the preparation (using mechanical shakers or mixers to guarantee homogenous mixing) can increase the speed of the extraction. The extraction ultimately stops when an equilibrium is attained between the concentration of metabolites in the extract and that in the plant material. After extraction, the residual plant material has to be separated from the solvent. This involves a rough clarification by decanting, which is usually followed by a filtration step. Centrifugation may be necessary if the powder is too fine to be filtered. To ensure exhaustive extraction, it is common to carry out an initial maceration, followed by clarification, and an addition of fresh solvent to the marc. This can be performed periodically with all filtrates pooled together.[3]

The main disadvantage of maceration is that the process can be quite time-consuming, taking from a few hours up to several weeks. Exhaustive maceration can also consume large volumes of solvent and can lead to the potential loss of metabolites and/or plant material.[3]


**Figure-4**: Extraction by macerating the plant in the suitable solvent

❖ Soxhlet Extraction

Soxhlet extraction is used widely in the extraction of plant metabolites because of its convenience. This method is adequate for both initial and bulk extraction. The plant powder is placed in a cellulose thimble in an extraction chamber, which is placed on top of a collecting flask beneath a reflux condenser. A suitable solvent is added to the flask, and the set up is heated under reflux. When a certain level of condensed solvent has accumulated in the thimble, it is siphoned into the flask beneath.[3]

The main advantage of Soxhlet extraction is that it is a continuous process. As the solvent (saturated in solubilized metabolites) empties into the flask, fresh solvent is recondensed and extracts the material in the thimble continuously. However, the main disadvantage of Soxhlet

extraction is that the extract is constantly heated at the boiling point of the solvent used, and this can damage thermolabile compounds and/or initiate the formation of artifacts.[3]

- ❖ Ultrasound-assisted solvent extraction.
- ❖ Percolation
- ❖ Pressurized solvent extraction
- ❖ Extraction under reflux and steam distillation.[3]

**2.5. Selection of an Extraction Method and Solvents**

The ideal extraction procedure should be exhaustive (i.e., extract as much of the desired metabolites or as many compounds as possible). It should be fast, simple, and reproducible if it is to be performed repeatedly. The selection of a suitable extraction method depends mainly on the work to be carried out, and whether or not the metabolites of interest are known.[3]

Extraction processes can employ water-miscible or water-immiscible solvents. The solvent selected should have a low potential for artifact formation, a low toxicity, a low flammability, and a low risk of explosion. Additionally, it should be economical and easily recycled by evaporation. These issues are particularly important in the case of bulk extraction where large volumes of solvents are employed. The main solvents used for extraction include aliphatic and chlorinated hydrocarbons, esters, and lower alcohols.[3]

**Table-4**

**Physicochemical Properties of Some Common Solvents Used in Natural Products Extraction.**

| Solvent | Polarity index | Boiling Point ($^{o}$C) | Viscosity (cPoise) | Solubility in water (% w/w) |
|---|---|---|---|---|
| n-Hexane | 0.0 | 69 | 0.33 | 0.001 |
| Dichloromethane | 3.1 | 41 | 0.44 | 1.6 |
| n-Butanol | 3.9 | 118 | 2.98 | 7.81 |
| iso-propanol | 3.9 | 82 | 2.30 | 100 |
| n-Propanol | 4.0 | 92 | 2.27 | 100 |
| Chloroform | 4.1 | 61 | 0.57 | 0.815 |
| Ethyl acetate | 4.4 | 77 | 0.45 | 8.7 |
| Acetone | 5.1 | 56 | 0.32 | 100 |
| Methanol | 5.1 | 65 | 0.60 | 100 |
| Ethanol | 5.2 | 78 | 1.20 | 100 |
| Water | 9.0 | 100 | 1.00 | 100 |

Extractions can be either ‘‘selective’’ or ‘‘total.’’ The initial choice of the most appropriate solvent is based on its selectivity for the substances to be extracted. In a selective extraction, the plant material is extracted using a solvent of an appropriate polarity following the principle of ‘‘like dissolves like.’’ Thus, nonpolar solvents are used to solubilize mostly lipophilic compounds (e.g., alkanes, fatty acids, pigments, waxes, sterols, some terpenoids, alkaloids, and coumarins). Medium-polarity solvents are used to extract compounds of intermediate polarity (e.g., some alkaloids, flavonoids), while more polar ones are used for more polar compounds (e.g., flavonoid glycosides, tannins, some alkaloids). Water is not used often as an initial extractant, even if the aim is to extract water-soluble plant constituents (e.g., glycosides, quaternary alkaloids, tannins). A selective extraction can also be performed sequentially with solvents of increasing polarity. This has the advantage of allowing a preliminary separation of the metabolites present in the material within distinct extracts and simplifies further isolation.[3]

In an extraction referred to as ‘‘total,’’ a polar organic solvent (e.g., ethanol, methanol, or an aqueous alcoholic mixture) is employed in an attempt to extract as many compounds as possible. This is based on the ability of alcoholic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium- to low-polarity constituents. The ‘‘total’’ extract is evaporated to dryness, redissolved in water, and the metabolites re-extracted based on their partition coefficient (i.e., relative affinity for either phase) by successive partitioning between water and immiscible organic solvents of varying polarity.[3]

Specific protocols during which the pH of the extracting aqueous phase is altered to solubilize selectively groups of metabolites (such as acids or bases) can also be used. For instance, these are applied to the extraction of alkaloids (which occur mostly as water-soluble salts in plants). On treating the plant material with an alkaline solution, the alkaloids are released as free bases that are recovered following partition into a water-immiscible organic solvent. Subsequent liquid–liquid extractions and pH modifications can be performed to separate the alkaloids from other nonalkaloidal metabolites. Alternatively, alkaloids can be extracted from the plant material in their salt form under acidic conditions. However, one drawback of the acid–base treatment is that it can produce some artifacts and/or lead to the degradation of compounds.[3]

Finally, single solvents or solvent mixtures can be used in extraction protocols. When a solvent mixture is necessary, a binary mixture (two miscible solvents) is usually employed. In a Soxhlet extraction, it is preferable to use a single solvent simply because one of the solvents

in the mixture may distill more rapidly than another. This may lead to a change in the solvent proportions in the extracting chamber.[3]

**2.6. Fractionation**

A crude natural product extract is literally a cocktail of compounds. It is difficult to apply a single separation technique to isolate individual compounds from this crude mixture. Hence, the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid–liquid extraction or they may be the contiguous eluate from a chromatography column, e.g., vacuum liquid chromatography (VLC), column chromatography (CC), size-exclusion chromatography (SEC), solid-phase extraction (SPE), etc. For initial fractionation of any crude extract, it is advisable not to generate too many fractions, because it may spread the target compound over so many fractions that those containing this compound in low concentrations might evade detection. It is more sensible to collect only a few large, relatively crude ones and quickly home in on those containing the target compound. For finer fractionation, often guided by an on-line detection technique, e.g., ultraviolet (UV), modern preparative, or semipreparative high-performance liquid chromatography (HPLC) can be used.[3]

**2.7. Isolation**

The most important factor that has to be considered before designing an isolation protocol is the nature of the target compound present in the crude extracts or fractions. The general features of the molecule that are helpful to ascertain the isolation process include solubility (hydrophobicity or hydrophilicity), acid–base properties, charge, stability, and molecular size. If isolating a known compound from the same or a new source, it is easy to obtain literature information on the chromatographic behavior of the target compound, and one can choose the most appropriate method for isolation without any major difficulty. However, it is more difficult to design an isolation protocol for a crude extract where the types of compounds present are totally unknown. In this situation, it is advisable to carry out qualitative tests for the presence of various types of compounds, e.g., phenolics, steroids, alkaloids, flavonoids, etc., as well as analytical thin-layer chromatography (TLC) or HPLC profiling. The nature of the extract can also be helpful for choosing the right isolation protocol. For example, a MeOH extract or fractions from this extract containing polar compounds are better dealt with using reversed-phase HPLC (RP-HPLC). Various physical properties of the extracts can also be determined with a small portion of the crude extract in a series of small batch-wise experiments. Some of these experiments are summarized below-

- Hydrophobicity or hydrophilicity: An indication of the polarity of the extract as well as the compounds present in the extract can be determined by drying an aliquot of the mixture and trying to redissolve it in various solvents covering the range of polarities, e.g., water, MeOH, acetonitrile (ACN), EtOAc, DCM, $CHCl_3$, petroleum ether, n-hexane, etc. The same information can be obtained by carrying out a range of solvent partitioning, usually between water and EtOAc, $CHCl_3$, DCM, or n-hexane, followed by an assay to determine the distribution of compounds in solvent fractions.
- Acid–base properties: Carrying out partitioning in aqueous solvents at a range of pH values, typically 3, 7, and 10, can help determine the acid–base property of the compounds in an extract. It is necessary to adjust the aqueous solution or suspension with a drop or two of mineral acid or alkali (a buffer can also be used), followed by the addition of organic solvent and solvent extraction. Organic and aqueous phases are assessed, preferably by TLC, for the presence of compounds. This experiment can also provide information on the stability of compounds at various pH values.
- Charge: Information on the charge properties of the compound can be obtained by testing under batch conditions, the effect of adding various ion exchangers to the mixture. This information is particularly useful for designing any isolation protocol involving ion exchange chromatography.
- Heat stability: A typical heat stability test involves incubation of the sample at ~90$^{o}$C for 10 min in a water bath followed by an assay for unaffected compounds. It is particularly important for bioassay-guided isolation, where breakdown of active compounds often leads to the loss or reduction of biological activity. If the initial extraction of natural products is carried out at a high temperature, the test for heat stability becomes irrelevant.
- Size: Dialysis tubing can be used to test whether there are any macromolecules, e.g., proteins, present in the extract. Macromolecules are retained within the tubing, allowing small (<2000 amu) secondary metabolites to pass through it. The necessity of the use of any SEC in the isolation protocol can be ascertained in this way.[3]

**2.8. Chromatographic Technique**

The chromatographic techniques used in the isolation of various types of natural products can be broadly classified into two categories: classical or older, and modern.

Classical or older chromatographic techniques include:

- ✓ Thin-layer chromatography (TLC).
- ✓ Preparative thin-layer chromatography (PTLC).
- ✓ Open-column chromatography (CC).

- ✓ Flash chromatography (FC).

Modern chromatographic techniques are:

- ✓ High-performance thin-layer chromatography (HPTLC).
- ✓ Multiflash chromatography.
- ✓ Vacuum liquid chromatography (VLC).
- ✓ Chromatotron.
- ✓ Solid-phase extraction.
- ✓ Droplet countercurrent chromatography (DCCC).
- ✓ High-performance liquid chromatography (HPLC).
- ✓ Hyphenated techniques (e.g., HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR).[3]

### 2.8.1. Principle of Thin Layer Chromatography

TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the $R_f$ of a compound is compared with the $R_f$ of a known compound (preferably both run on the same TLC plate).[8]

A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.

As the solvent moves past the spot that was applied, equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates. (The plate itself contains a fluorescent dye which glows everywhere except where an organic compound is on the plate).[8]

The uses of TLC are-

1. To determine the number of components in a mixture.
2. To determine the identity of two substances.
3. To monitor the progress of a reaction.

4. To determine the effectiveness of a purification.
5. To determine the appropriate conditions for a column chromatographic separation.
6. To monitor column chromatography.[9]

### 2.8.2. Principle of Open Column Chromatography

In column chromatography, silica gel is used as a stationary phase, and an organic solvent (less polar than the silica gel) is used as the mobile phase. Column chromatography is carried out in a glass tube clamped vertically with the initial mixture placed at the top. Organic solvents run past this mixture on their way down the column. Because of silica gel's stronger affinity for the more polar components, the components with the lower polarity will descend (elute) first through the column. During the chromatography, the polarity of the mobile phase can be slowly increased by varying the solvent mixture used. As a result, increasingly polar components will elute.[9]

As in any other chromatography it is important to introduce the mixture in as concentrated volume as possible to avoid overlapping of the components as they elute. Below the column, pre-weighed flasks are used to collect the solvent with the various components (fractions). Normally, flasks are changed when the solvent mixture is changed. Each flask is then warmed to evaporate the solvent, and then reweighed, and the amount of component in the fraction is recorded.[9]

### 2.8.3. Principle of Vacuum Liquid Chromatography

In this process, a Buchner filter funnel fitted with glass frit (10-20 μm porosity) is dry packed with sorbent (10-40 μm of TLC grade, e.g. 60G silica gel). The sorbent is allowed to settle by gentle tapping under gravity. Sample will be applied in the upper portion of the sorbent. Then vacuum is applied via a vacuum pump. The vacuum is released, solvent of low polarity is poured quickly onto the surface of the adsorbent and then vacuum is re-applied. When the eluent is through, the column is sucked dry and is ready for loading. The sample in a suitable solvent is applied directly to the top of the column and is drawn gently into the packing by applying the vacuum. The column is developed with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the polarity, pulling the column dry between each fraction collected (this helps to avoid channeling).

Fractions are collected in a round bottomed flask or in a suitable separatory funnel. The use of separatory funnel avoids the problem of changing the flask for each fraction.

In general the height of the sorbent should not exceed 5 cm. For small scale operation (<100mg) a column of 0.5-1.0 cm i.d. and 4 cm height is appropriate; for 0.5-1.0 g, the

dimensions 2.5×4 cm are suitable; for 1-10 g, 5×5 cm; larger amount are best separated in a 250 ml sintered glass filter funnel packed to a height of 5 cm.

### 2.9. Quantification

The yield of compounds at the end of the isolation and purification process is important in natural product research. An estimate of the recovery at the isolation stage can be obtained using various routine analytical techniques that may involve the use of a standard. In bioassay-guided isolation, the compound is monitored by bioassay at each stage, and a quantitative assessment of bioactivity of the compound is usually carried out by serial dilution method. Quantitative bioactivity assessment provides a clear idea about the recovery of the active compound(s) and also indicates whether the activity results from a single or multiple components. During the isolation process, if the activity is lost or reduced to a significant level, the possible reasons could be as follows:

- The active compound has been retained in the column.
- The active compound is unstable in the conditions used in the isolation process.
- The extract solution may not have been prepared in a solvent that is compatible with the mobile phase, so that a large proportion of the active components precipitated out when loading on to the column.
- Most of the active component(s) spread across a wide range of fractions, causing undetectable amounts of component(s) present in the fractions.
- The activity of the extract is probably because of the presence of synergy among a number of compounds, which, when separated, are not active individually.[3]

### 2.10. Structure Elucidation

In most cases of extraction and isolation of natural products, the end point is the identification of the compound or the conclusive structure elucidation of the isolated compound. However, structure elucidation of compounds isolated from plants, fungi, bacteria, or other organisms is generally time consuming, and sometimes can be the ‘‘bottleneck’’ in natural product research. There are many useful spectroscopic methods of getting information about chemical structures, but the interpretation of these spectra normally requires specialists with detailed spectroscopic knowledge and wide experience in natural product chemistry. With the remarkable advances made in the area of artificial intelligence and computing, there are a number of excellent automated structure elucidation programs available that could be extremely useful. If the target compound is known, it is often easy to compare preliminary spectroscopic data with literature data or to make direct comparison with the standard sample.

However, if the target compound is an unknown and complex natural product, a comprehensive and systematic approach involving a variety of physical, chemical, and spectroscopic techniques is required. Information on the chemistry of the genus or the family of plant or microbe under investigation could sometimes provide additional hints regarding the possible chemical class of the unknown compound. The following spectroscopic techniques are generally used for the structure determination of natural products:

1. Ultraviolet-visible spectroscopy (UV-vis): Provides information on chromophores present in the molecule. Some natural products, e.g., flavonoids, isoquinoline alkaloids, and coumarins, to name a few, can be primarily characterized (chemical class) from characteristic absorption peaks.

2. Infrared spectroscopy (IR): Determines different functional groups, e.g.,-C=O, -OH, -$NH_2$, aromaticity, and so on, present in a molecule.

3. Mass spectrometry (MS): Gives information about the molecular mass, molecular formula, and fragmentation pattern. Most commonly used techniques are: electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS), electrospray ionization mass spectrometry (ESIMS), and fast atom bombardment mass spectrometry (FABMS).

4. NMR: Reveals information on the number and types of protons and carbons (and other elements like nitrogen, fluorine, etc.) present in the molecule, and the relationships among these atoms. The NMR experiments used today can be classified into two major categories:

a. One-dimensional techniques: 1HNMR,13CNMR,13CDEPT

b. Two-dimensional techniques: 1H-1H COSY,1H-1H DQF-COSY, 1H-1H COSY-lr,1H-1H NOESY,1H-1H ROESY,1H-1H TOCSY (or HOHAHA),1H-13C HMBC,1H-13C HMQC,1H-13C HSQC, HSQCTOCSY, and the like.

In addition to the above-mentioned spectroscopic techniques, X-ray crystallographic techniques provide information on the crystal structure of the molecule, and polarimetry offers information on the optical activity of chiral compounds.[3]

**2.11. Assays**

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

The following basic points should be borne in mind when carrying out assays of natural products:

- Samples dissolved or suspended in a solvent different from the original extraction solvent must be filtered or centrifuged to get rid of any insoluble matter.
- Acidified or basified samples should be readjusted to their original pH to prevent them from interfering with the assay.
- Positive and negative controls should be incorporated in any assay.
- Ideally, the assay should be at least semiquantitative, and/or samples should be assayed in a series of dilutions to determine where the majority of the target compounds resides.
- The assay must be sensitive enough to detect active components in low concentration.

Physical assays may involve 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 known standard.

Chemical assays involve various chemical tests for identifying the chemical nature of the compounds, e.g., $FeCl_3$ can be used to detect phenolics, and so on.

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. 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). In vivo studies are more relevant to clinical conditions and can also provide toxicity data at the same time. Disadvantages of these studies are costs, need for large amount of test compounds/fractions, complex design, patient requirement, and difficulty in mode of action determination. In vitro bioassays are faster (ideal for HTS), and small amounts of test compounds are needed, but might not be relevant to clinical conditions. The trend has now moved from in vivo to in vitro. Bioassays available today are robust, specific, and more sensitive to even as low as picogram amounts of test compounds.[3]

### 2.12. Synthesis of Natural Product

Once a natural product compound has been screened for biological activity, isolated, purified, its structure identified, and the pharmacological profile refined, the journey is not over. The molecule may turn out to be too complex in nature and too expensive to be synthesized. Any given natural product compound may possess unacceptable physicochemical, pharmacodynamic, pharmacokinetic, or bioavailability properties or demonstrate excessive

toxicity and will therefore require optimization of its chemical structure. Optimization involves a dissection of the lead molecule and the synthetic addition, removal, replacement, or modification of substituent groups so as to enhance the utility and efficacy of the molecule. The synthesis of a complicated molecule is a very difficult task since every group and atom must be placed in a proper position and with the correct stereochemistry.[7]

Despite all of the knowledge and achievements that have been gained and the advances that have been made, classical synthetic organic chemistry will not alone unlock and open the potential of natural products to the pharmaceutical marketplace. Instead, the future lies in the synergistic union of classical organic chemistry with microbiology, biochemistry, combinatorial chemistry, and other fields to provide new synthetic strategies to generate natural-product-based drugs. It should be recognized that combinatorial chemistry is a perfect match for high-throughput screening because of its ability to produce large numbers of compounds in a short period of time. The promise of combinatorial chemistry to deliver more drug candidates within a shorter period of time has remained unfulfilled.[7]

# Chapter-3

# Introduction to plant

## 3. Introduction to plant:

### 3.1. *Nymphoides indica*

*Nymphoides indica*, common name is White Water Fringe, Water snowflake. Nymphoides species are widespread around the world and are a group of plants that grow underwater with their leaves floating on the water surface like water lillies. The flowers are held upright above the leaves. *Nymphoides indica* is native to northern Australia and is freely flowering from spring into autumn. This species prefers warm, still or slowly moving water the green rounded glossy leaves are 6cm across and the attractive fringed white flowers with yellow centres are 2cm wide. This nymphoides species is especially suited for growing in water bowls, tubs and small ponds in protected areas. In warmer areas of Australia they can be grown in larger ponds and dams.

- Cultural notes:
  - ✓ Size - Height above water surface up to 20cm × 60cm wide
  - ✓ Flower - attractive fringed white flower with yellow centre
  - ✓ Moisture - water from 20cm to 1m deep

Suitability: Decorative Water Containers, Small Ponds, Large Water Gardens, Mass Displays, Farm Dams, Wetland Regeneration, Aquariums.

#### 3.1.1. Scientific Classification

Kingdom: Plantae
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Solanales
Family: Menyanthaceae
Genus: Nymphoides
Species: *Nymphoides indica*

**Figure-5**: Full plant of *Nymphoides indica*

**Figure-6**: Flower of *Nymphoides indica*

**Figure-7**: Leaves of *Nymphoides indica*


**Figure-8**: Leaves with flower of *Nymphoides indica*

### 3.1.2. Synonyms

Limnanthemum calycinum Miquel; Limnanthemum ecklonianum Grisebach; Limnanthemum esquirolii H. Léveillé; Limnanthemum fraserianum Grisebach; Limnanthemum humboldtianum (Kunth) Grisebach; Limnanthemum humboldtianum (Kunth) Grisebach var. parviflorum Grisebach; Limnanthemum indicum (L.) Grisebach; Limnanthemum indicum (L.) Thwaites; Limnanthemum kleinianum Grisebach; Limnanthemum niloticum Kotschy & Peyritsch; Limnanthemum orbiculatum (G. Don) Grisebach; Limnanthemum thunbergianum Grisebach; Limnanthemum wightianum Grisebach; Menyanthes indica L.; Nymphoides eckloniana (Grisebach) O. Kuntze; Nymphoides humboldtiana (Kunth) O. Kuntze; *Nymphoides indica* (L.) O. Kuntze subsp. occidentalis A. Raynal; Nymphoides orbiculata (G. Don) Kuntze; Nymphoides thunbergiana (Grisebach) O. Kuntze; Trachyspermum humboldtianum (Kunth) House; Villarsia eglandulosa Griffith; Villarsia glandulosa Griffith; Villarsia humboldtiana Kunth; Villarsia indica (L.) Ventenat; Villarsia nymphaeifolia Fraser; Villarsia orbiculata G. Don; Villarsia rheedei Kosteletzky; Villarsia senegalensis G. Don; Villarsia simsii G. Don; Villarsia swartzii G. Don.

### 3.1.3. Botanical Description

Nymphaeaceae and Menyanthaceae family plants are classified into a different community. This community is termed as Rooted-floating plants. These plants root deeply in the soil and float leaves and flower on the water surface. To accomplish this, most plants have very long stalks for both leaf and flower, and a stem that remains under water, sometimes beneath the soil; a few plants have long stems rather than long stalks. This community is one of the most dominant in the haors. It is comprised of about 15 plant species.

These plants are also susceptible to seasonal water level fluctuations. In the permanent beels they can survive and regenerate for the whole year. But in seasonally flooded areas, the rhizomes or seeds remain buried under the soil during the dry season and then start sprouting with the arrival of water. As water levels increases, they then elongate their stems or leaf and floral stalks. They typically start flowering on a large scale when the water starts receding just after the peak flood. Almost all the plants of this community can propagate vegetatively as well as sexually. *Nymphoides indica* are mainly found in Balai Haor.

### 3.1.4. Traditional use

Over the past few decades, herbal medicines have been accepted universally and they put the impact on both world health and international trade. Now a days more than 50% of all modern drug in clinical use are of natural product. According to WHO, more than 80% people in developing countries depending on traditional medicinesfor their primary health needs.[15]

Whole plant of *Nymphoides indicum* Kuntze (*Limnanthemum cristatum*) used as hepatoprotective medicinal plant.[15]

In Papua New Guinea, the plant is known as misuanto (Hatarn), auggi meritz (Sougb), and used to promote pregnancy. In Vietnam, the plant is used to reduce fever, to invigorate, as a carminative, and as an antiscorbutic.

### 3.1.5. Morphological Characteristics

*Nymphoides indica* posses different morphological characteristics which were revealed by different researcher. From their research experience-

*Nymphoides indica* is perennial rhizomatous free floating herbs rooting at nodes, rhizome elongated horizontally, cone-like. Leaves ovate-orbicular to orbicular, deeply cordate at base, coarsely crenate. Umbels of more than 20 flowers. Corolla white, throat yellow; lobes 5-7, linear-lanceolate to oblong-lanceolate, densely fimbriate within, with multicellular, cottony outgrowths. Seeds globose, occasionally lenticular, smooth, pale yellowish-white, shining.[17]

*Nymphoides* one of the most diverse and widespread floating-leaved groups are approximately consist of 50 *Nymphoides* species worldwide. Some (e.g. *Nymphoides indica* ) have rather broad distributions, but most are restricted to a single continent or even to a local geographic region. Flowers are borne above water, but the capsular fruits develop underwater. The flowers of most species are pentamerous with yellow or white petals that can be glabrous or ornamented with hairs or laciniate wings. Seeds differ among species in their shape (globose or elliptical, some compressed laterally), size (from 0.4 mm to over 5 mm), surface

ornamentation (smooth or tuberculate, some carunculate), and number (from one or two to over 100 per fruit). Two major types of inflorescence architecture exist in *Nymphoides*, and these differ by their relative internode elongation. Floral axes are either expanded (having internodes that elongate between pairs of flowers) or condensed (with each cluster of several flowers supported by a single floating leaf). The temperate Eurasian species *N. peltata* produces a unique third inflorescence type, in which two leaves support each of several successive flower clusters on an axis with elongated internodes. The condensed inflorescence morphology characterizes the majority of *Nymphoides* species throughout the range of the genus, whereas expanded-inflorescence species occur only in Australia and tropical Asia. A recent phylogenetic study of *Nymphoides* , which sampled 12 species, inferred a basal grade of expanded-inflorescence species (and *N. peltata* ) toward a clade of species with condensed inflorescences. Four *Nymphoides* species (*N. aquatica* , *N. cordata* , *N. krishnakesara* , *N. macrosperma* ) are dioecious, having both flowers and individuals that are unisexual. Still another condition characterizes the gynodioecious *N. cristata* , a species in which individuals produce either pistillate or hermaphroditic flowers.[11]

Of the Australian endemics, only 4 species that were not restricted to the floodplains, including *Nymphoides spongiosa* and *Nymphoides subacuta*, were restricted to the Northern Territory. The non-endemic nature of the flora was considered to reflect the many opportunities for exchange of species with other landmasses during recent geological times. Within the Kakadu Region, one of the ten community for the distribution of plant include *Nelumbo-Nymphoides* herbland, a mixed community dominated by the water lilies *Nelumbo nucifera* and *Nymphoides indica* that occur in permanently and semi-permanently wet areas. On sites that remain flooded for 10-11 months *Nymphoides indica* and *Nymphaea macrosperma* may be present.[12]

*Nymphoides indica* (L.) Kuntze is one of the species in which water-level fluctuations affect the growth of juvenile plants. *Nymphoides indica* is an endangered species in Asia and the sub-continental region and the failure of sexual reproduction will decrease the genetic diversity of the populations. At the juvenile stage, exposed conditions were required for seedling growth and the young seedlings died under submergence. Seedling establishment was successful after occasional water drawdown or along the water margin according to the period and pattern of water-level fluctuation. Dessication associated with prolonged drawdown may be fatal to the survival of the seedlings. Thus the pattern and period of water level fluctuation are essentially important to successful germination and survival of seedlings.[13]

Clonal diversity within and among populations of aquatic macrophytes is often low because recruitment occurs predominantly through vegetative reproduction. *Nymphoides indica* (L.) Kuntze is a typical heterostylous plant with dimorphic herkogamy and intramorph- and self incompatibility, its chromosome number has been reported as diploid. Pollination occurs via insects. The flying distance from flower to flower is small and the species can set seeds only when different floral morphs coexist in a population. Vegetative reproduction allows the species to maintain populations, even when these contain only one floral morph. Because of the short flying distance of *Nymphoides indica* pollinators, legitimate pollination usually occurs between neighboring, randomly distributed different floral morphs.[14]

# Chapter-4

# Literature Review

## 4. Literature Review:

### 4.1. Phytochemical Analysis

Maceration of the rhizome exhibit the following elements-

- ✓ Group of parenchyma cells with starch grains.
- ✓ Fiber with broad lumen and pointed ends.
- ✓ Tracheids which are narrow and with spiral thickenings.
- ✓ Vessels with scalariform and spiral thickenings.
- ✓ Sclereids of different size and shape (polymorphic), thick walled, with many arms.

Maceration of the root exhibit-

- ✓ Epidermal peel made up of compactly arranged parenchyma cells.
- ✓ Parenchyma cells of various shapes, thin walled.
- ✓ Fibers which are long, broad with pointed ends.
- ✓ Vessels of different size and shape, with pitted thickenings, drum shaped, barrel shaped or with short drawn out ends.[17]

The sections of rhizome when treated with iodine solution turned blue indicating the presence of starch grains whereas the root sections did not turn blue indicating the absence of starch grains. Both rhizome and root sections when treated with phloroglucinol and HCl turned pink indicating the presence of lignin; on treating with ferric chloride solution, both root and rhizome sections turned black showing the presence of tannin; with Dragendroff's reagent, sections of both rhizome and root did not show reddish brown colour indicating the absence of alkaloids; with conc. HCl, root sections did not exhibit effervescence indicating the absence of crystals. The pet. ether, benzene, chloroform, acetone, ethanol and aqueous extracts of *Nymphoides indica* were analyzed. It was found that no alkaloids and volatile oils were present in any of extracts, phytosterols were present in pet. ether, acetone and ethanol extracts, phenols and tannins were present in acetone, ethanol and aqueous extracts while saponins, carbohydrates, glycosides, flavonoids, proteins, amino acids, gums and mucilage were present in ethanol and aqueous extracts.[17]

The alcohol extract revealed 7 phytoconstituents. At 254 nm, spots with $R_f$ values of 0.07, 0.12, 0.16, 0.23, 0.28, 0.35, 0.57 were observed, of these, spots at $R_f$ 0.12, 0.57 were the most pronounced whereas spot at $R_f$ 0.35 was less pronounced and spots at $R_f$ 0.07, 0.16, 0.23, 0.28 were least pronounced. At 366 nm, 9 phytoconstituents were revealed at $R_f$ 0.04, 0.08, 0.13, 0.17, 0.23, 0.30, 0.35 0.48, and 0.57, of these, spots at $R_f$ 0.17, 0.30 were the most pronounced whereas spots at $R_f$ 0.23, and 0.35 were less pronounced and spots at $R_f$ 0.04, 0.08, 0.13, 0.48, 0.57 were least pronounced. At 425nm, 6 phytoconstituents at $R_f$ 0.04, 0.08,

0.17, 0.23, 0.31, 0.35 were revealed. Of these, spot at $R_f$ 0.17 was the most pronounced whereas spots at $R_f$ 0.31, and 0.35 were less pronounced and spots at 0.04, 0.08, 0.23 were least pronounced. The aqueous extract revealed 9 phytoconstituents at 254 nm; spots with $R_f$ 0.13, 0.18, 0.24, 0.29, 0.34, 0.39, 0.49, 0.58, and 0.65 were observed, of these, spots at $R_f$ 0.18, 0.49, and 0.58 were the most pronounced whereas spots at $R_f$ 0.34, 0.39, 0.65 were less pronounced and spots at $R_f$ 0.13, 0.24, and 0.29 were least pronounced. At 366 nm, 5 phytoconstituents having $R_f$ 0.12, 0.23, 0.32, 0.42, and 0.47 were observed, of these spots at $R_f$ 0.23, 0.42 were the most pronounced whereas spots at $R_f$ 0.32, and 0.47 were less pronounced while the spot at $R_f$ 0.12 was least pronounced.[17]

The Ethyl-acetate extract of Nymphoides cristatum O. kuntze after chromatography over silica gel yielded a pure compound Bet-20 (29)-en-3-ol-28-oic acid which was obtained as a white crystalline solid having melting point 193-195$^oC$. The IR spectrum of the compound showed strong band at 3624 and 1705 $cm^{-1}$ which could be assigned for hydroxyl function. The spectrum also revealed stretching at 1641 and 884 $cm^{-1}$ ($C=CH_2$). The mass spectrum displayed a highest ion peak ($M^+$) at m/z 456 corresponding to $C_{30}H_{48}O_3$. Other peaks included at m/z 408, 218, 208, 203 and 189. The 1H NMR spectrum of compound showed six singlets at δ 0.823 (3H, Me-24), 0.862 (3H, Me-25), 0.916 (3H, Me-27), 0.963 (3H, Me-23), 0.987 (3H, Me-26) and 1.634 (3H, Me-30), each integrating for three protons which was assigned for three methyl groups in the compound. A carbinylic proton at C-3 was assigned by the presence of characteristic signal at δ 3.12 (1H, m, $W_{1/2}$=6.8 Hz, H-3). The carbinylic proton is highly de-shielded due to the OH substituent at C-3. Two doublets At δ 4.543 and 4.675 (1H each, J=2 Hz) represents two protons at C-29 ($C=CH_2$). A single proton at C-19 was assigned by the double triplet (dt) at the δ 2.497 (J=10.5 and 4.2 Hz). All the proton peaks in 1H NMR data of the compound are in good agreement with those of betulinic acid.[16]

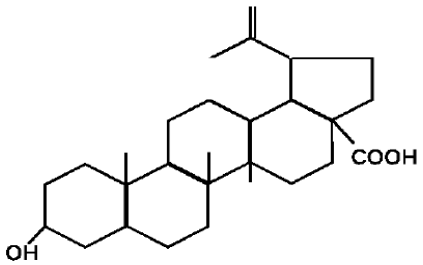



**Figure-9:** Bet-20 (29)-en-3-ol-28-oic acid

*Nymphoides indica* is a floating leaved attached plant occurring in constructed wetlands in Queensland, Australia. Floating leaved attached plants had slightly higher phosphorus concentrations but significantly greater nitrogen concentrations. Most of the floating leaved

species produced adventitious roots into the water column and would therefore be able to absorb dissolve nutrients directly from the water as well as from the sediment. Maximum nutrient concentration (mg/g) of *Nymphoides indica* in case of phosphorus is 16.6 $mgP.g^{-1}$ and for nitrogen is 40.4 $mgN.g^{-1}$.[18]

*Menyanthes trifoliata* is common in the marshes and bogs of Europe but can also be cultivated in shallow waters. *Menyanthes trifoliate* extracts contain betulinic acid in the root and rhizome but not found in the leaves.[19]

Structures that are isolated from Menyantheaceae are given below-

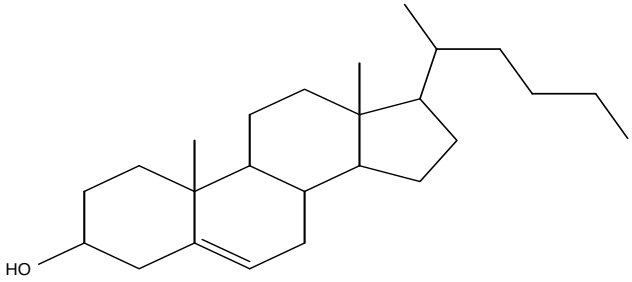



**Figure-10:** Nymphyol

1HNMR spectra of nymphyol is given below:

1HNMR (δ, $CDCl_3$, 500 MHz): 0.63 (3H, s, H-18), 0.84 (3H, t, J = 6.5 Hz, H-25), 0.93 (3H, d, J = 6.5 Hz, H-21), 1.02 (3H, s, H-19), 3.54 (1H, m, H-3), 5.37 (1H, s, H-6).

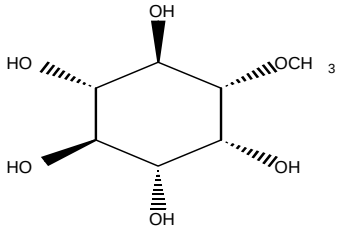



**Figure-11:** L-(+)-bornesitol

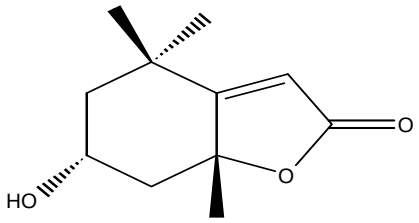



**Figure-12:** Loliolide

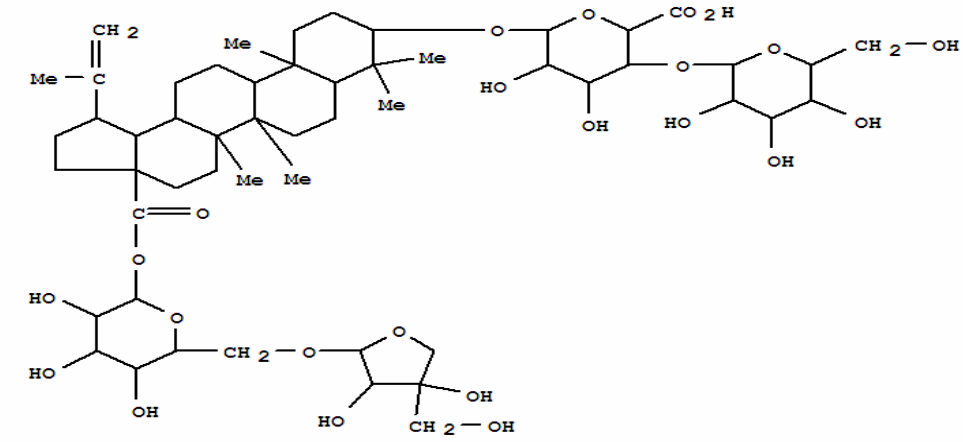



**Figure-13:** Menyanthoside

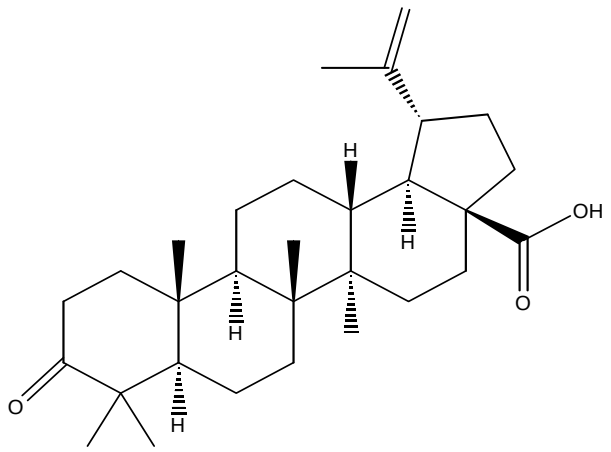



**Figure-14:** Betulonic acid

The isolated compound found from fraction-DCM-3,4 may have the almost same structural similarity with a triterpene which is lupeol (molecular formulae is $C_{30}H_{47}O$). The structure of the lupeol is-

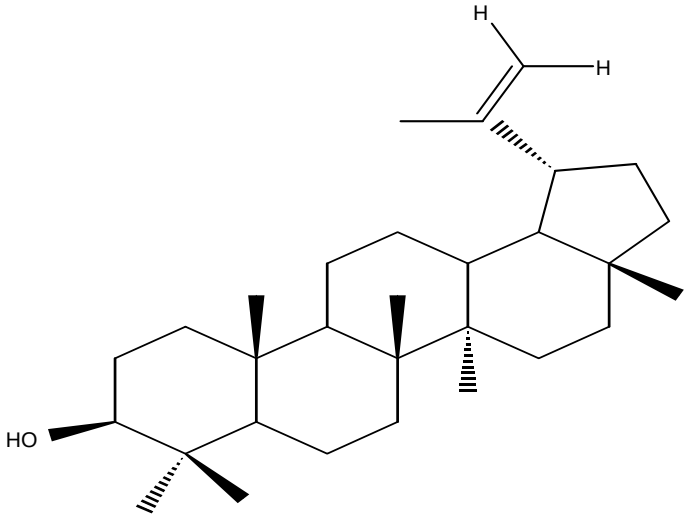



**Figure-15**: Lupeol

The NMR spectra of lupeol was given below

1HNMR (δ, $CDCl_3$, 400 MHz): 4.67 (s, H-29), 4.55 (s, H-29), 3.77 ( m, H-3), 2.35 (m, H-19), 1.28 (3H, s, H-30), 1.02 (3H, s, H-24), 0.98 (3H, s, H-23), 0.84 (3H, s, H-26), 0.77 (3H, s, H-27), 0.68 (3H, s, H-25).

13CNMR (δ, $CDCl_3$, 400 MHz): 38.78 (C-1), 26.00 (C-2), 78.99 (C-3), 37.19 (C-4), 55.35 (C-5), 18.02 (C-6), 34.33 (C-7), 40.87 (C-8), 50.48 (C-9), 38.10 (C-10), 20.97 (C-11), 25.19 (C-12), 38.64 (C-13), 42.85 (C-14), 26.19 (C-15), 29.71 (C-16), 47.99 (C-17), 48.35 (C-18), 47.67 (C-19), 150.88 (C-20), 29.89 (C-21), 33.35 (C-22), 27.48 (C-23), 15.38 (C-24), 16.01 (C-25), 15.60 (C-26), 14.57 (C-27), 59.91 (C-28), 109.34 (C-29), 19.33 (C-30).[15]

### 4.2. Pharmacological Evaluation

*Nymphoides indica* rhizome and roots are bitter, used as antiscorbutic and febrifuge, also as a substitute for chiretta to treat fever and jaundice. From *Nymphoides indica* a drug is isolated known as Tagara, used in Ayurvedic medicine for the treatment of diseases like anemia, epilepsy, fever, jaundice, mental disorders, tuberculosis and it is also used as a general and brain tonic.[17]

*Nymphoides macrospermum* is used in place of Tagara for the same ayurvedic preparations under the same formulations. The alcohol extract of *Nymphoides macrospermum* was tested for its anticonvulsant and sedative activities. Alcohol extract of *Nymphoides macrospermum* at doses of 500 and 750 mg/kg body weight, significantly reduced the severity and increased the latency of convulsions induced by pentylenetetrazole (PTZ) and also reduced the time taken for recovery. However, *Nymphoides macrospermum* up to a dose of 750 mg/kg body weight did not exhibit any significant effect on pentobarbitone-induced hypnosis. The sedative effect of the plant extract was confirmed by a significant reduction in locomotor activity, when tested using an actophotometer. The possible mechanism may be due to the activation of N-methyl D-aspartate receptor (NMDA) is also involved in the initiation and generalization of PTZ-induced seizures. Drugs that block glutamatergic excitation mediated by NMDA receptors have an anticonvulsant property against PTZ-induced seizures.[26]

*Nymphoides indica* (L.) Kuntze leaf paste 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.[20]

*Nymphoides hydrophylla* (L.) Kuntze leaf juice drops are applied against eye diseases. Leaf paste is used as an antidote for scorpion sting and snakebite. Seed powder with honey is taken orally as an anthelmintic.[20]

*Nymphoides indica* (L.) Kuntze whole plant juice (20-35 ml) is taken empty stomach twice daily for a week, for curing fever and jaundice by Rabha, Santal and Jayantipur tribes.[21]

*Nymphoides indica* plant paste is applied as bandage in cut and injuries as coolant and for early healing. Dried rhizome paste along with little honey is taken in diuresis.[22]

*Nymphoides stellata* fresh petiole paste mixed with *Cuminum cyminum* L. powder, common salt, milk butter and is taken against dysmenorrhoea twice a day for 2-3 days.[22]

*Nymphoides indica* (L.) Kuntze fruit and stems are used. Ripe fruits are taken raw specially by the children.[24]

*Nymphoides aquatica* (J. F. Gmelin) Kuntze consist in the banana lily extract which is used as a weak antioxidant.[25]

The isolated Bet-20 (29)-en-3-ol-28-oic acid terpenoid from *Nymphoides cristatum* has significant antimicrobial and cytotoxic activity. The compound was dissolved in MeOH to get concentration of 200 μg per 10 μl. Antibacterial test are carried out by disc diffusion method in presence of reference standard Kanamycin (30 μg/disc). 22 mm of highest zone of inhibition was found in the compound against *Escherichia coli* and lowest in 14 mm against *Klebsiella species*. In case of antifungal test concentration of the compound was 400 μg/disc and the reference standard was Cotrimazole. The highest zone of inhibition was found in *Hensinela califomica* was 14 mm and the lowest in 10 mm against *Phizopus arizae*.[16]

The cytotoxic effect of the compound was evaluated by $LC_{50}$ of brine shrimp lethality test. The compound showed positive result. It was dissolved in DMSO and the 50% mortality ($LC_{50}$) of the compound was found to be 15.39 μg/ml and 50% confidence limit 8.87-26.68. A regression equation, $Y = 3.06 + 1.63X$ and $X^2$ value 0.215 were observed in comparison with galic acid as a standard.[16]

The hepatoprotective effect of *N.cristatum* was investigated in albino rats. 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 $CCl_4$ (2ml/kg) intra peritoneal administration, significant protective effects from this hepatotoxin was expressed. This protection was evidenced by comparing the SGOT (Serum glutamate oxaloacetate transaminase), SGPT (Serum glutamate pyruvate transaminase), ALP (alkaline phosphatase), total bilirubin and histopathological examination in rats treated and untreated with *N. cristatum*. 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.[27]

*Nymphoides cristatum* plant juice is used in fever and jaundice.[23]

*Menyanthes trifoliata* leaf contains a skin beneficial amount of actives which are inhibitors of one or more of MMP-1 (Matrix Metalloproteinases) and scavengers of peroxynitrite. It also includes treating the skin for signs of chronological or pre-mature aging.[19]

# Chapter-5

# Materials and Methods

## 5. Materials and methods:

### 5.1. Drug and chemicals

Acetic acid was obtained from Sigma-Aldrich Laborchemikalien Gmbh, Germany. Amoxicillin was purchased from Sanofi Aventis Pharma, Bangladesh. Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich Laborchemikalien Gmbh, Germany. Vernier caliper was obtained from Shanghai China. Micrometer screw gauge was obtained from BMA Bhaban. Organic solvents are obtained from Merck, Germany.

Nutrient agar (NA) was obtained from Techno Pharmchem, Bahdurgarh, India. Microscopic observation was done by using Carl Zeiss microscope, USA. TLC were run on Merck TLC plates precoated with Si60 F254 with visualization by spraying with 1: 9 $H_2SO_4$ in MeOH and heating. Open Column Chromatography was done by using Silica gel 60 (0.063- 0.020 mm), Merck, Germany. The UV visible spectrum was obtained by using an SHIMADZU UV-1700 Pharma Spec UV-VIS Spectrophotometer.

### 5.2. Equipments

Spatula, Small beaker (50 ml), Analytical balance, 1000ml beaker, Mortar and Pestle, Aluminium foil paper, 5ml vial, Test tube, Test tube holder, Autoclave, Hot air oven, Laminar flow cabinet, Incubator, Refrigerator, Vortex machine, Inoculating loop, Eppendorff tube, Masking tape, Burner, Container, Micropipette, Tips, Pipette, Pipette pump, Pasteur pipette, Dropper, Petridish, Microorganism spreading glass rod, TLC tank, UV lamp, Hot plate, Rotary evaporator, Fume hood, Glass rod, Conical flask (500ml), Funnel, Filter paper. Burette column, Cork borer.

### 5.3. Solvent

Hexane, Ethylacetate, Dichloromethane, Chloroform, Acetone, Methanol, Ethanol, Distilled water, Dimethylsulfoxide (DMSO), Acetic acid, Sulfuric acid.

### 5.4. Plant materials and Extraction

*Nymphoides indica* leaves were collected from Panchagor division of Bangladesh and identified by National Herbarium of Bangladesh, where a voucher specimen was conserved under the reference number DACB Accession Number 35611. The leaves were washed with water and sun dried for 30 days. The leaves were powdered by using Blinder. 250 gm dried powdered leaves were extracted by ethanol by cold extraction process (2litre × 72 hours × 3 times). The extracting solvent was filtered and the filtrate was concentrated by using a rotary evaporator ($45^0$C) yielding crude Ethanol extract (42 gm, yield: 16.8% from dried sample).

**Figure-16:** Rotary Evaporator for extraction

### 5.5. Thin layer Chromatography

A fresh moisture free 25×25 cm TLC plate was taken and cut in appropriate size on the basis of sample numbers to be loaded on the plate with the help of TLC cutter. The TLC plate was marketed 1 cm upper from the bottom and 1 cm lower from the upper side with pencil. Approximately 0.5-2 mg of the dried extract were diluted with 1 ml of the appropriate solvent then, approximately 2-6 µl aliquot of each dilution of the extract was carefully loaded individually onto the baseline of the TLC plates with help of small capillary tube and the sample was allowed to dry. Different solvent systems were used as mobile phases, respectively on the basic of the nature of the compounds. The plate was placed in a closed chamber containing a mobile phase on the bottom. After run of mobile phase up to upper pencil mark, the plate was removed from the TLC tank. The plate was dried with the help of the air of electric fan to evaporate the solvent. Once dried the UV active compounds were observed by using UV lamp, here fluorescence compounds can also be observed. After detection of UV and fluorescence compounds, the plate was sprayed with charring reagent (10% $H_2SO_4$ in methanol). After spraying charring reagents, the plate was completely dried and heated at 100-120$^oC$ for the observation of organic compounds. After detection the individual compounds, the $R_f$ value and color of each compound was noted in the table.

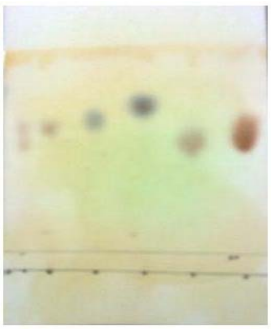


**Figure-17**: Thin layer chromatography

# Chapter-6

# Bioguided Separation and Purification

## 6. Bioguided separation and purification:

### 6.1. Bioguided separation of the Ethanol extract of *Nymphoides indica*

Crude extract (42 g) that is found after the extraction with ethanol was subjected to the vacuum liquid chromatography (VLC).

#### 6.1.1. Vacuum Liquid Chromatography

A Buchner funnel with conical flask was taken. Under the funnel, a filter paper was used. 70 gm of silica gel was taken into the filter as stationary phase. 40 gm of sample from the crude extract was mixed with 20 gm of silica gel in order to make slurry, one day before performing VLC. At the upper portion of the stationary phase, the sampled slurry was drawn into the filter. Upper of the sample covered with a filter paper so that the mobile phase can run uniformly throughout the system. Then the vacuum was started. At the same time mobile phase was drawn through the funnel. At first n-hexane is used. After that dichloromethane, ethyl-acetate, acetone and methanol were consecutively used. 5 litre of the each solvents were used. Due to the affinity of the sample to the solvent, sample were fractionated. 0.28, 0.63, 3.97, 20.2 and 0.74 (gm) were found respectively from n-hexane, dichloromethane, ethyl-acetate, acetone and methanol solvent.

According to the antimicrobial activity, dichloromethane and ethyl-acetate fraction was further separated by running open column chromatography.

**Figure-18:** Vacuum Liquid Chromatography

#### 6.1.2. Open Column Chromatography

For performing the open column of dichloromethane fraction, like VLC, 15 gm of silica gel was taken into a burette as stationary phase and 609 mg of sample was slurred with 6.2 gm of silica gel. After packing of the stationary phase, then slurred sample was taken in the burette.

Then mobile phase was applied. In this case gradient type of mobile phase was used. 50 ml of each eluent was collected in separate beaker. 18 fractions were collected.

Again for the ethyl-acetate fraction, 10 gm silica gel was used as stationary phase and 8 gm of silica gel is used make slurry with sample of 3.13 gm. Like dichloromethane fraction, this was also performed in the burette column and the gradient type od mobile phase is used. 50 ml of each eluent was collected. 35 fractions were collected.

Throughout the whole process precoated TLC was done to monitor the sample status in the each fraction. By doing the process in dichloromethane fraction, a colorless, odorless crystalline pure compound was isolated fraction (3, 4). 1HNMR was done to find out the structure of the compound.

**Figure-19:** Open column Chromatography

# Chapter-7

# Evaluation of Antimicrobial activity

## 7. Evaluation of Antibacterial activity:

### 7.1. Microorganisms

The following microorganisms were used for disk diffusion method: *Staphylococcus aureus, Escherichia coli, Shigella dysenteriae, Salmonella typhi* and *Candida albicans*. The following microorganisms were used for agar well diffusion method: *Staphylococcus aureus, Shigella dysenteriae, Salmonella typhi* and *Candida albicans*. The microbial species were collected from Department of Food and Nutrition, University of Dhaka. They were maintained on agar slant at $4^oC$ and sub-cultured on a fresh appropriate agar plate 24 prior to any antimicrobial test.

### 7.2. Cultured media

Nutrient Agar (NA) was used for the activation of all bacteria and fungi. For the preparation of agar solution, the calculation is 2.8gm nutrient agar is dissolved in 100ml of distilled water. Antibiotic sensitivity test are assessed by different methods. These ares-

### 7.3. Disc Diffusion Method

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

The tested sample will be mixed with MeOH and DMSO in a composition of 80:20 ratios. DMSO used to facilitate the solubilization of the sample and methanol will increase the solubility further. Then the sample will be injected into the disc of 6mm in diameter. Nutrient agar was sterilized by autoclave at $121^oC$ and after the withdrawal from the autoclave, it will be transferred to the petridishes with a diameter of 9 cm. Then the microorganism will be suspended to its isotonic solution of 0.9% NaCl. It will be vortex about 2 minutes to completely disperse the microorganism in the NaCl solution. From this solution 100 µl was transferred to the agar containing petridishes and distributed the solution uniformly. Then the discs containing sample were placed in the agar. Petridishes were incubated at $37^oC$ for 24 hour. On each plate a reference standard (Amoxicillin 20 µg/disc) were used and control (5 µl methanol:DMSO/ disc) were applied in the plate. After the end of the period zone of inhibition is measured in millimeter in the nutrient agar.

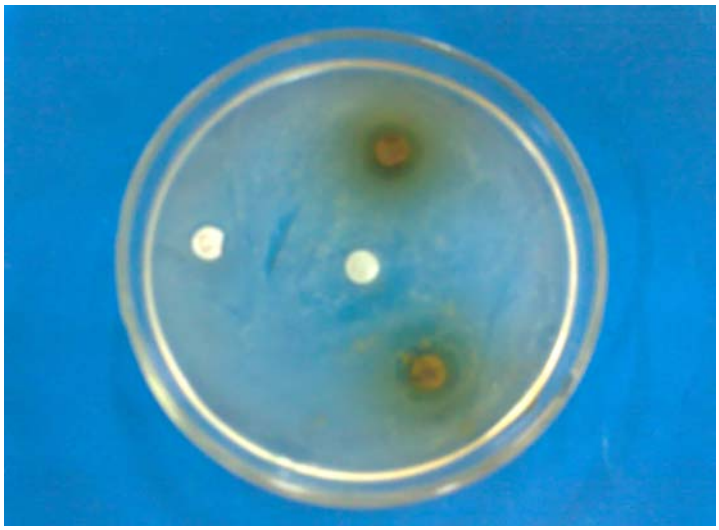


**Figure-20:** Disc diffusion method

### 7.4. Agar Well diffusion Method

The method can be differentiate from disc diffusion method in such a way that in agar well diffusion method, a cork borer is used to make a small well in the nutrient agar and the extract were 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 if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

**Figure-21:** Agar well diffusion method

The antimicrobial activities of the fraction of VLC (Vacuum Liquid Chromatography) were assayed by the standard agar well diffusion method. The tested sample will be mixed with MeOH and DMSO in a composition of 80:20 ratios. DMSO used to facilitate the solubilization of the sample and methanol will increase the solubility further. Nutrient agar was sterilized by autoclave at 121$^{o}$C and after the withdrawal from the autoclave, it will be

transferred to the petridishes with a diameter of 9 cm. Then the microorganism will be suspended to its isotonic solution of 0.9% NaCl. It will be vortex about 2 minutes to completely disperse the microorganism in the NaCl solution. From this solution 100 µl was transferred to the agar containing petridishes and distributed the solution uniformly. Wells of 5mm in diameter were formed on to nutrient agar plates using a sterile cork borer. The wells were filled with the test agents (100 µl each) and the plates were then allowed to stay for 1–2 hour at room temperature. Finally, the plates were incubated at 37$^{o}$C for 18–24 hour and the resulting diameters of zones of inhibition were measured.

# Chapter-8

# Results and Discussion

## 8. Results and Discussion:

### 8.1. Antimicrobial activity of MeOH extract of *Nymphoides indica* using disc diffusion method.

Antimicrobial activity of MeOH extract of *Nymphoides indica* has been evaluated in vitro against four bacterial species and one yeast (Table 5) which are known to cause infections in humans. As summarized in the table, extract showed antimicrobial activity at a dose 1000 µg/disk against tested microorganisms. The highest inhibition zone diameter was 14 mm on *Salmonella typhi*, and the lowest inhibition zone diameter was 10.5 mm on both *Escherichia coli* and *Candida albicans*. The average zone diameter was found in between 10.5 to 14 mm in case of other two bacterial spices, such as *Staphylococcus aureus, Shigella dysenteriae*. The Reference drug amoxicillin showed highest zone of inhibition of 15mm on Escherichia coli and lowest zone of inhibition of null effect on *Candida albicans* at 20µg/disk.

**Table-5**

**In vitro antimicrobial activity of MeOH extract of *Nymphoides indica*.**

| samples | Concent-ration (µg/disc) | Inhibition zone diameter(mm) | | | | |
|---|---|---|---|---|---|---|
| | | Tested microorganisms | | | | |
| | | Escherichia coli | Staphylococcus aureus | Shigella dysenteriae | Salmonella typhi | Candida albicans |
| Extract | 1000 | 10.5 | 12 | 11 | 14 | 10.5 |
| Amoxicillin | 20 | 15 | 14 | 12.5 | 14 | - |
| Contol (MeOH:DMSO) | 5 (µl/disc) | - | - | - | - | - |

- = No inhibition

The results indicate that the extract has significant antimicrobial activity, so further study for isolation and purification of antimicrobial agent from the extract was facilitated.

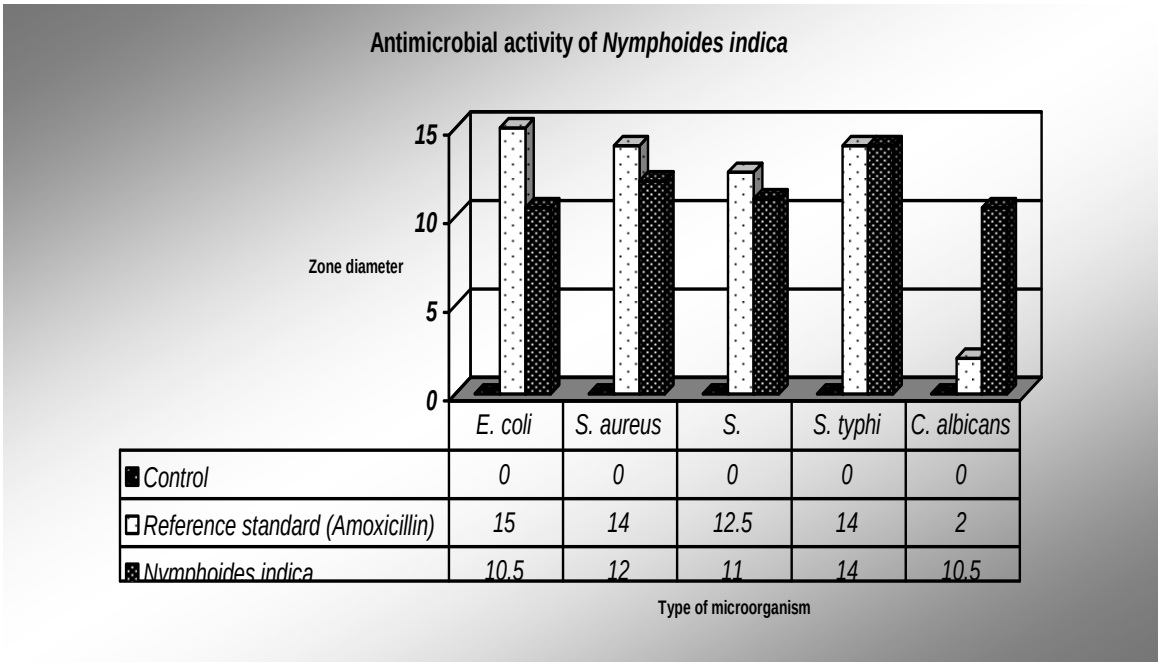



**Figure-22**: Effect of antimicrobial activity of MeOH extract of *Nymphoides indica* compared with a reference standard Amoxicillin

### 8.2. Thin Layer Chromatography

**Table-6**

**Thin layer chromatography of MeOH extract and Ethanol extract of *Nymphoides indica*.**

| Plant Species | Compound | Mobile Phase | Total spot | Spot | $R_f$ Value | Observation Under necked eye | UV Observation | Observation after charring |
|---|---|---|---|---|---|---|---|---|
| Leaves of *Nymphoides indica* | MeOH extract | EtOAc: MeOH (5:1) | 3 | 1 | 0.16 | - | Violet | Black |
| | | | | 2 | 0.78 | - | Black | - |
| | | | | 3 | 0.84 | Dark green | Red | Black |
| | Ethanol extract | EtOAc: MeOH (5:1) | 5 | 1 | 0.11 | - | Violet | Black |
| | | | | 2 | 0.44 | - | Violet | Black |
| | | | | 3 | 0.71 | Light green | Black | Black |
| | | | | 4 | 0.80 | Light green | Purple | Black |
| | | | | 5 | 0.84 | Dark green | Red | Black |

- = No color

### 8.3. Vacuum Liquid Chromatography

After the Ethanolic extraction of *Nymphoides indica*, crude extract was found 42 gm. From this 42 gm crude extract 40 gm was taken to perform VLC in order to fractionate the crude extract.

**Table-7**

**Quantitation of the amount of crude Ethanolic extract of *Nymphoides indica* after VLC.**

| Serial | Mobile Phase | Volume applied (L) | Quantity (gm) |
|---|---|---|---|
| Fraction-1 | n-Hexane | 5 | 0.28 |
| Fraction-2 | Dichloromethane (DCM) | 5 | 0.63 |
| Fraction-3 | Ethyl-acetate | 5 | 3.97 |
| Fraction-4 | Acetone | 5 | 20.2 |
| Fraction-5 | Methanol | 5 | 0.74 |

### 8.4. Thin Layer Chromatography

After the operation of VLC, TLC was done for each fraction except n-Hexane fraction.

**Table-8**

**TLC of the each fraction of VLC**

| Plant Species | Fraction | Mobile Phase | Total spot | Spot | $R_f$ Value | Observation Under necked eye | UV Observation | Observation after charring |
|---|---|---|---|---|---|---|---|---|
| Leaves of *Nymphoides indica* | DCM fraction | Hexane : EtOAc: Acetic acid (5:1) | 3 | 1 | 0.51 | - | Purple | Black |
| | | | | 2 | 0.72 | - | Purple | - |
| | | | | 3 | 0.89 | Light green | Red | Black |
| | Ethyl-acetate fraction | Hexane : EtOAc: Acetic acid (5:1) | 6 | 1 | 0.17 | - | Purple | Black |
| | | | | 2 | 0.38 | Green | Black | Black |
| | | | | 3 | 0.49 | Green | Black | Black |
| | | | | 4 | 0.62 | - | Dark yellow | - |
| | | | | 5 | 0.72 | - | Dark yellow | - |
| | | | | 6 | 0.87 | Light green | Red | Black |
| | Acetone fraction | Chloroform: MeOH (3:1) | 6 | 1 | 0.15 | Yellow | Brown | - |
| | | | | 2 | 0.19 | - | Black | Black |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 3 | 0.34 | - | Fluorescence | - |
| | | | | 4 | 0.47 | Light yellow | Brown | Black |
| | | | | 5 | 0.64 | Yellow | Black | Black |
| | | | | 6 | 0.81 | Yellow | Red | Black |
| | MeOH fraction | Chloroform: MeOH (3:1) | 3 | 1 | 0.09 | Light yellow | Light Purple | Black |
| | | | | 2 | 0.21 | - | Light Purple | - |
| | | | | 3 | 0.83 | Light yellow | Light Purple | - |

- = No color

**8.5. Antimicrobial activity of the VLC fractions of the crude extract of *Nymphoides indica* using agar well diffusion method.**

Antimicrobial activity of VLC fraction of the Ethanol extract of *Nymphoides indica* has been evaluated in vitro against three bacterial species and one yeast (Table 9) which are known to cause infections in humans. As summarized in the table, extract showed antimicrobial activity at a dose 100 µg/disk against tested microorganisms. The highest inhibition zone diameter was 18 mm on *Staphylococcus aureus* in the ethyl-acetate fraction, and the lowest inhibition zone diameter was 0 mm on *Candida albicans* in the n-Hexane fraction. The average zone diameter was found in between 7 to 18 mm in case of other two bacterial spices, such as *Salmonella typhi, Shigella dysenteriae*. There were no zone of inhibition in case of acetone and MeOH fractions. In this method, 5 mm of well was produced in each case in which the drug was placed.

**Table-9**

**In vitro antimicrobial activity of VLC fractions of *Nymphoides indica* extract.**

| Fraction | Concent-ration µg/well (9 mm) | Inhibition zone diameter(mm) | | | |
|---|---|---|---|---|---|
| | | Tested microorganisms | | | |
| | | Staphylococcus aureus | Shigella dysenteriae | Salmonella typhi | Candida albicans |
| n-Hexane fraction | 100 | 9 | 9 | 12 | - |
| Dichloromethane fraction | 100 | 10.5 | 12 | 11 | 7 |
| Ethyl-acetate fraction | 100 | 18 | 14.5 | 15 | 9 |
| Acetone fraction | 100 | - | - | - | - |
| MeOH fraction | 100 | - | - | - | - |

- = No inhibition

The results indicate that the extract has significant antimicrobial activity, so further study for isolation and purification of antimicrobial agent from the extract was facilitated in case of Dichloromethane and Ethyl-acetate fraction. Further purification was not possible in case of n-Hexane fraction due to the trace amount present in the extract.

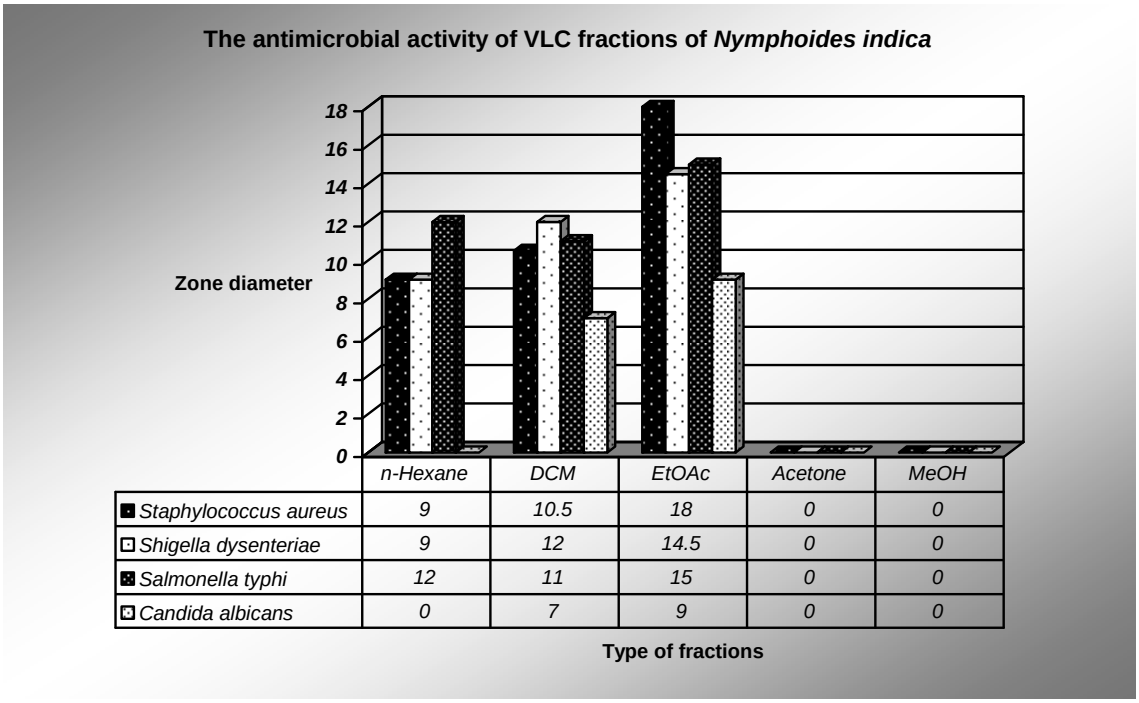



**Figure-23**: Effect of antimicrobial activity of VLC fractions of *Nymphoides indica*

**8.6. Open Column Chromatography of Dichloromethane fraction.**

Open column chromatography was done for Dichloromethane fraction. 18 sub-fractions were collected from DCM fraction. By performing TLC, the fractions that contain same constituents were mixed together. In sub-fraction 3, 4 a colorless crystal compound (18 mg) is isolated.

**Table-10**

**Quantitation of the amount of Dichloromethane sub-fractions after column chromatography.**

| Fraction | Mobile phase composition | Volume collected (ml) | Quantity (mg) |
|---|---|---|---|
| Fr-DCM-1 | 10% EtOAc in n-Hexane | 50 | 165 |
| Fr-DCM-2 | 10% EtOAc in n-Hexane | 50 | 75 |
| Fr-DCM-3, 4 | 10% EtOAc in n-Hexane | 100 | 18 (crystal) |
| Fr-DCM-5 | 10% EtOAc in n-Hexane | 50 | 11 |

| | | | |
|---|---|---|---|
| Fr-DCM-6 | 10% EtOAc in n-Hexane | 50 | 17 |
| Fr-DCM-7 | 10% EtOAc in n-Hexane | 50 | 22 |
| Fr-DCM-8 | 10% EtOAc in n-Hexane | 50 | 8 |
| Fr-DCM-9, 10 | 10% EtOAc in n-Hexane | 100 | 45 |
| Fr-DCM-11 | 30% EtOAc in n-Hexane | 50 | 134 |
| Fr-DCM-12 | 30% EtOAc in n-Hexane | 50 | 8 |
| Fr-DCM-13 | 30% EtOAc in n-Hexane | 50 | 8 |
| Fr-DCM-14, 15 | 30% EtOAc in n-Hexane | 100 | 7 |
| Fr-DCM-16, 17 | 30% EtOAc in n-Hexane<br>50% EtOAc in n-Hexane | 100 | 90 |
| Fr-DCM-18 | 10% EtOAc in n-Hexane | 50 | - |

- = Amount was not measured

**8.7. Thin Layer Chromatography**

TLC was done in case of all the fractions of Dichloromethane.

**Table-11**

**TLC of the fractions of DCM.**

| VLC fraction | Fraction | Mobile Phase | Total spot | Spot | $R_f$ Value | Observation Under necked eye | UV Observation | Observation after charring |
|---|---|---|---|---|---|---|---|---|
| DCM | Fr-DCM-1 | Hexane : EtOAc (7:3) | 1 | 1 | 0.78 | Yellow | Dark Yellow | Black |
| | Fr-DCM-2 | Hexane : EtOAc (7:3) | 1 | 1 | 0.84 | Yellow | Dark Yellow | Black |
| | Fr-DCM-3 | Hexane : EtOAc (7:3) | 2 | 1 | 0.59 | - | Black | Black |
| | | | | 2 | 0.81 | - | - | Black |
| | Fr-DCM-4 | Hexane : EtOAc (7:3) | 2 | 1 | 0.59 | - | Black | Black |
| | | | | 2 | 0.81 | - | - | Black |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fr-DCM-5 | Hexane : EtOAc (7:3) | 2 | 1 | 0.59 | - | Black | Black |
| | | | | 2 | 0.76 | Light green | Redish Black | Black |
| | Fr-DCM-6 | Hexane : EtOAc (7:3) | 2 | 1 | 0.59 | - | Light black | - |
| | | | | 2 | 0.75 | Dark green | Redish Black | Black |
| | Fr-DCM-7 | Hexane : EtOAc (7:3) | 2 | 1 | 0.59 | - | Light black | - |
| | | | | 2 | 0.75 | Dark green | Redish Black | Black |
| | Fr-DCM-8 | Hexane : EtOAc (7:3) | 3 | 1 | 0.38 | - | Black | - |
| | | | | 2 | 0.51 | - | Black | - |
| | | | | 3 | 0.75 | Dark green | Redish Black | Black |
| | Fr-DCM-9 | Hexane : EtOAc (7:3) | 4 | 1 | 0.38 | - | Black | - |
| | | | | 2 | 0.51 | - | Black | - |
| | | | | 3 | 0.68 | Dark green | Redish Black | Black |
| | | | | 4 | 0.78 | - | Red | - |
| | Fr-DCM-10 | Hexane : EtOAc (7:3) | 4 | 1 | 0.38 | - | Black | - |
| | | | | 2 | 0.51 | - | Black | - |
| | | | | 3 | 0.68 | Dark green | Redish Black | Black |
| | | | | 4 | 0.78 | - | Red | - |
| | Fr-DCM-11 | Hexane : EtOAc (1:1) | 1 | 1 | 0.91 | Dark green | Redish Black | Black |
| | Fr-DCM-12 | Hexane : EtOAc (1:1) | 5 | 1 | 0.65 | - | Dark | - |
| | | | | 2 | 0.71 | - | Black | - |
| | | | | 3 | 0.76 | - | Red | Black |
| | | | | 4 | 0.82 | - | Black | Black |
| | | | | 5 | 0.91 | - | Red | Black |
| | Fr-DCM- | Hexane : EtOAc (1:1) | 2 | 1 | 0.62 | - | Dark | Black |
| | | | | 2 | 0.71 | - | Red | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 13 | | | | | | | |
| | Fr-DCM-14 | Hexane : EtOAc (1:1) | 2 | 1 | 0.41 | - | Dark | Black |
| | | | | 2 | 0.68 | - | Red | - |
| | Fr-DCM-15 | Hexane : EtOAc (1:1) | 3 | 1 | 0.35 | - | Fluorescence | - |
| | | | | 2 | 0.41 | - | Dark | Black |
| | | | | 3 | 0.62 | - | Red | - |
| | Fr-DCM-16 | Hexane : EtOAc (1:1) | 1 | 1 | 0.35 | - | Fluorescence | - |
| | Fr-DCM-17 | Hexane : EtOAc (1:1) | 1 | 1 | 0.35 | - | Fluorescence | - |
| | Fr-DCM-18 | Hexane : EtOAc (1:1) | 1 | 1 | 0.18 | - | Red | - |

- = No color

**8.8. Open Column Chromatography**

Open column chromatography was done for Ethyl-acetate fraction. 35 sub-fractions were collected from DCM fraction. By performing TLC, the fractions that contain same constituents were mixed together. No crystal compound were isolated may be due to the higher over loading of sample in the column (burette).

**Table-12**

**Quantitation of the amount of Ethyl-acetate sub-fractions after column chromatography.**

| Fraction | Mobile phase composition | Volume collected (ml) | Quantity (mg) |
|---|---|---|---|
| Fr-EtOAc-1 | 20% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-2 | 20% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-3 | 20% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-4 | 20% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-5, 6, 7, 8 | 20% EtOAc in n-Hexane | 200 | |

| | | | |
|---|---|---|---|
| Fr-EtOAc-9, 10 | 20% EtOAc in n-Hexane | 100 | 157 |
| Fr-EtOAc-11, 12 | 40% EtOAc in n-Hexane | 100 | 255 |
| Fr-EtOAc-13 | 40% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-14 | 40% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-15 | 40% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-16 | 40% EtOAc in n-Hexane | 50 | 179.5 |
| Fr-EtOAc-17, 18, 19 | 40% EtOAc in n-Hexane<br>60% EtOAc in n-Hexane | 150 | 285 |
| Fr-EtOAc-20 | 60% EtOAc in n-Hexane | 50 | |
| Fr-EtOAc-21, 22<br>Fr-EtOAc-23, 24, 25, 26, 27 | 60% EtOAc in n-Hexane<br>80% EtOAc in n-Hexane | 350 | |
| Fr-EtOAc-28, 29, 30 | 80% EtOAc in n-Hexane<br>10% MeOH in EtOAc | 50<br>50<br>50 | |
| Fr-EtOAc-31 | 10% MeOH in EtOAc | 50 | |
| Fr-EtOAc-32 | 10% MeOH in EtOAc | 50 | |
| Fr-EtOAc-33 | 10% MeOH in EtOAc | 50 | |
| Fr-EtOAc-34 | 10% MeOH in EtOAc | 50 | |
| Fr-EtOAc-35 | 10% MeOH in EtOAc | 50 | |

**8.9. Thin Layer Chromatography**

TLC was done in case of all the fractions of Ethyl-acetate.

**Table-13**

**TLC of the fractions of EtOAc.**

| VLC fraction | Fraction | Mobile Phase | Total spot | Spot | $R_f$ Value | Observation Under necked eye | UV Observation | Observation after charring |
|---|---|---|---|---|---|---|---|---|
| EtOAc | Fr- | Hexane : | 2 | 1 | 0.45 | - | Black | Black |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | EtOAc-1 | EtOAc (1:1) | | 2 | 0.76 | Dark green | Dark Red | Black |
| | Fr-EtOAc-2 | Hexane : EtOAc (1:1) | 2 | 1 | 0.45 | Dark green | Red | Black |
| | | | | 2 | 0.67 | - | Red | Black |
| | Fr-EtOAc-3 | Hexane : EtOAc (1:1) | 4 | 1 | 0.09 | - | Fluorescence | Black |
| | | | | 2 | 0.45 | Dark green | Red | Black |
| | | | | 3 | 0.61 | Green | Red | Black |
| | | | | 4 | 0.73 | - | Red | Black |
| | Fr-EtOAc-4 | Hexane : EtOAc (1:1) | 4 | 1 | 0.09 | - | Fluorescence | Black |
| | | | | 2 | 0.45 | Light green | Red | Black |
| | | | | 3 | 0.61 | Green | Red | Black |
| | | | | 4 | 0.73 | - | Red | Black |
| | Fr-EtOAc-5 | Hexane : EtOAc (1:1) | 3 | 1 | 0.21 | - | Black | Black |
| | | | | 2 | 0.48 | - | Red | - |
| | | | | 3 | 0.73 | - | Red | - |
| | Fr-EtOAc-6 | Hexane : EtOAc (1:1) | 4 | 1 | 0.21 | - | Black | Black |
| | | | | 2 | 0.45 | Light green | Red | - |
| | | | | 3 | 0.61 | Light green | Red | - |
| | | | | 4 | 0.73 | - | Red | - |
| | Fr-EtOAc-7 | Hexane : EtOAc (1:1) | 4 | 1 | 0.21 | - | Black | Black |
| | | | | 2 | 0.45 | Light green | Red | - |
| | | | | 3 | 0.61 | Light green | Red | - |
| | | | | 4 | 0.73 | - | Red | - |
| | Fr-EtOAc-8 | Hexane : EtOAc (1:1) | 3 | 1 | 0.21 | - | Black | Black |
| | | | | 2 | 0.45 | Light green | Red | - |
| | | | | 3 | 0.61 | - | Red | - |
| | Fr-EtOAc-9 | Hexane : EtOAc (1:1) | 3 | 1 | 0.31 | - | Fluorescence | - |
| | | | | 2 | 0.41 | - | Light red | - |
| | | | | 3 | 0.69 | - | Light red | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fr-EtOAc-10 | Hexane : EtOAc (1:1) | 3 | 1 | 0.31 | - | Fluorescence | - |
| | | | | 2 | 0.41 | - | Light red | - |
| | | | | 3 | 0.69 | - | Light red | - |
| | Fr-EtOAc-11 | Hexane : EtOAc (1:1) | 3 | 1 | 0.14 | - | Light red | Black |
| | | | | 2 | 0.28 | - | Fluorescence | Black |
| | | | | 3 | 0.45 | - | Red | Black |
| | Fr-EtOAc-12 | Hexane : EtOAc (1:1) | 4 | 1 | 0.14 | - | Red | Black |
| | | | | 2 | 0.28 | - | Fluorescence | Black |
| | | | | 3 | 0.45 | - | Red | Black |
| | | | | 4 | 0.72 | - | Light red | - |
| | Fr-EtOAc-13 | Hexane : EtOAc (1:1) | 4 | 1 | 0.14 | - | Red | Black |
| | | | | 2 | 0.28 | - | Fluorescence | Black |
| | | | | 3 | 0.45 | - | Red | Black |
| | | | | 4 | 0.72 | - | Light red | - |
| | Fr-EtOAc-14 | Hexane : EtOAc (1:1) | 1 | 1 | 0.24 | - | Red | Black |
| | Fr-EtOAc-15 | Hexane : EtOAc (1:1) | 3 | 1 | 0.17 | - | Red | Black |
| | | | | 2 | 0.31 | - | Red | - |
| | | | | 3 | 0.52 | - | Light red | - |
| | Fr-EtOAc-16 | Hexane : EtOAc (1:1) | 1 | 1 | 0.17 | - | Red | Black |
| | Fr-EtOAc-17 | Hexane : EtOAc (1:1) | 1 | 1 | 0.17 | - | Red | Black |
| | Fr-EtOAc-18 | Hexane : EtOAc (1:1) | 1 | 1 | 0.17 | - | Red | Black |
| | Fr-EtOAc-19 | Hexane : EtOAc (1:1) | 1 | 1 | 0.17 | - | Red | Black |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fr-EtOAc-20 | DCM : MeOH (9.5 : 0.5) | 5 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.46 | Dark green | Red | Black |
| | | | | 3 | 0.58 | Dark green | Red | Black |
| | | | | 4 | 0.73 | - | Light red | Black |
| | | | | 5 | 0.92 | - | Light red | - |
| | Fr-EtOAc-21 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.46 | Dark green | Red | Black |
| | | | | 3 | 0.58 | Light green | Red | Black |
| | Fr-EtOAc-22 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.42 | - | Purple | Black |
| | | | | 3 | 0.58 | Dark green | Red | Black |
| | Fr-EtOAc-23 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.42 | - | Purple | Black |
| | | | | 3 | 0.58 | Light green | Red | Black |
| | Fr-EtOAc-24 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.42 | - | Purple | Black |
| | | | | 3 | 0.58 | - | Red | Black |
| | Fr-EtOAc-25 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.42 | - | Red | Black |
| | | | | 3 | 0.58 | - | Red | Black |
| | Fr-EtOAc-26 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.23 | - | Purple | Black |
| | | | | 2 | 0.42 | - | Red | Black |
| | | | | 3 | 0.58 | - | Red | Black |
| | Fr-EtOAc-27 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.19 | - | Purple | Black |
| | | | | 2 | 0.31 | - | Light red | Black |
| | | | | 3 | 0.50 | - | Light red | - |
| | Fr-EtOAc-28 | DCM : MeOH (9.5 : 0.5) | 3 | 1 | 0.19 | - | Purple | Black |
| | | | | 2 | 0.31 | - | Light red | Black |
| | | | | 3 | 0.50 | - | Light red | - |
| | Fr-EtOAc-29 | DCM : MeOH (9.5 : 0.5) | 2 | 1 | 0.30 | - | Red | Black |
| | | | | 2 | 0.56 | - | Light red | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fr-EtOAc-30 | DCM : MeOH (9.5 : 0.5) | 2 | 1 | 0.30 | - | Red | Black |
| | | | | 2 | 0.56 | - | Light red | - |
| | Fr-EtOAc-31 | DCM : MeOH (8:2) | 3 | 1 | 0.29 | - | Light red | - |
| | | | | 2 | 0.71 | - | Red | Black |
| | | | | 3 | 0.94 | - | Red | Black |
| | Fr-EtOAc-35 | DCM : MeOH (8:2) | 2 | 1 | 0.37 | - | Red | Black |
| | | | | 2 | 0.83 | - | Light red | - |

- = No color

**8.10. Thin Layer Chromatography**

TLC was performed in case of fraction – DCM – 3,4. In this fraction, a colorless, odorless white crystalline solid was found. The compound was easily soluble in Dicholoromethane.

**Table-14**

**TLC of Fraction-DCM-3,4 (crystal compound).**

| VLC fraction | Fraction | Mobile Phase | Total spot | Spot | $R_f$ Value | Observation Under necked eye | UV Observation | Observation after charring |
|---|---|---|---|---|---|---|---|---|
| DCM | Fr-DCM-3,4 | n-Hexane: EtOAc (7:3) | 2 | 1 | 0.13 | - | Light black | - |
| | | | | 2 | 0.18 | - | - | Black |

- = No color

**8.11. Structure Elucidation**

The crystalline compound that was found from the DCM fraction of 3, 4 was performed 1HNMR. The data of 1HNMR was given below-

1HNMR (δ, $CDCl_3$, 400 MHz): 0.54 (3H,s, H-23 ) 0.79 (3H, s, H-22), 1.02 (1H, 2H, d, J = 5.84 Hz, 5-$H_1$, 1-$H_2$), 1.25 (3H, s, H-25, 26, 27), 1.39 (3H, s, H-29), 1.50 (2H, d, J = 19.03 Hz, H-7, 15, 21), 1.63 (1H, s, H-9), 1.78 (2H, d, J = 15.6 Hz, H-2, 6, 16, 20), 2.00 (1H, 2H, t, d, J = 13.74 Hz, 11-$H_2$, 18-$H_1$, 19-$H_1$), 2.34 (1H, s, H-3), 3.59 (1H, s, H-3a), 5.04 (1H, d, J = 7.81 Hz, H-12), 5.15 (2H, s, H-30).

In the $^1H$ NMR spectral analysis of NIH-3 showed seven methyl singlet signals at δ 0.54 ($CH_3$), 0.79 ($CH_3 \times 3$), 1.24 ($CH_3 \times 2$) and 1.78 ($CH_3$). Two exomethylene protons showed peak at δ 5.15 and a characteristic oxymethyne peak at δ 3.59. This spectra shows close

similarity with structure of lupeol. Additionally the $^1$H NMR spectra of NIH-3 also showed an olefenic proton peak at δ 5.04, which indicated NIH-3 containing an additional double bond. The position of double bond was proposed to be at $C_{12}$ and $C_{13}$ which is very rational and common according to the biosynthetic pathway of this type of triterpenes.

It was not completely possible to elucidate the accurate structure of the isolated compound by performing 1HNMR only. To know the exact structure of this compound we have to perform 13CNMR, NOE, HMBC, HSBC, HSQC, NOSY, QOSY.

So by performing 1HNMR and TLC the structure may be a triterpenoid (molecular formulae is $C_{30}H_{45}O$). So the possible structure of the isolated compound may be-

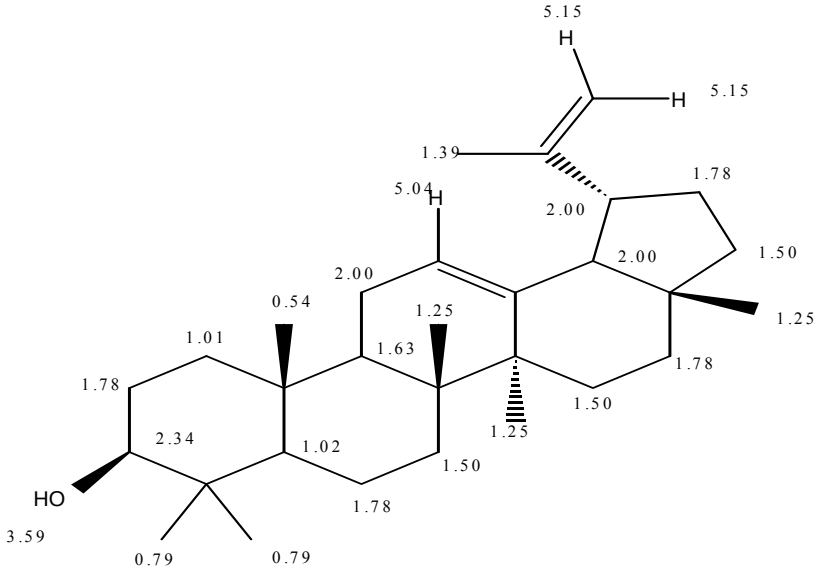



**Figure-24**: NIH-3

The structure of lupeol is-

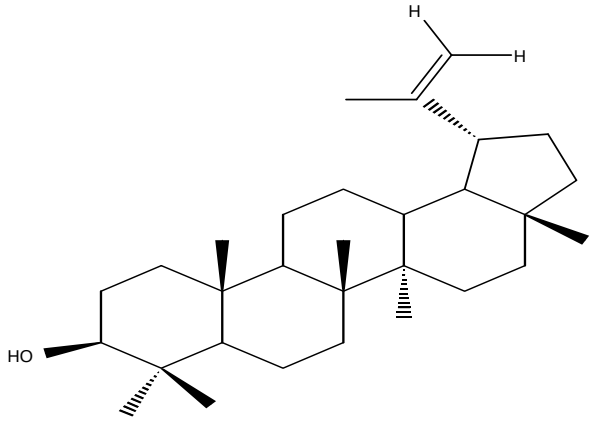
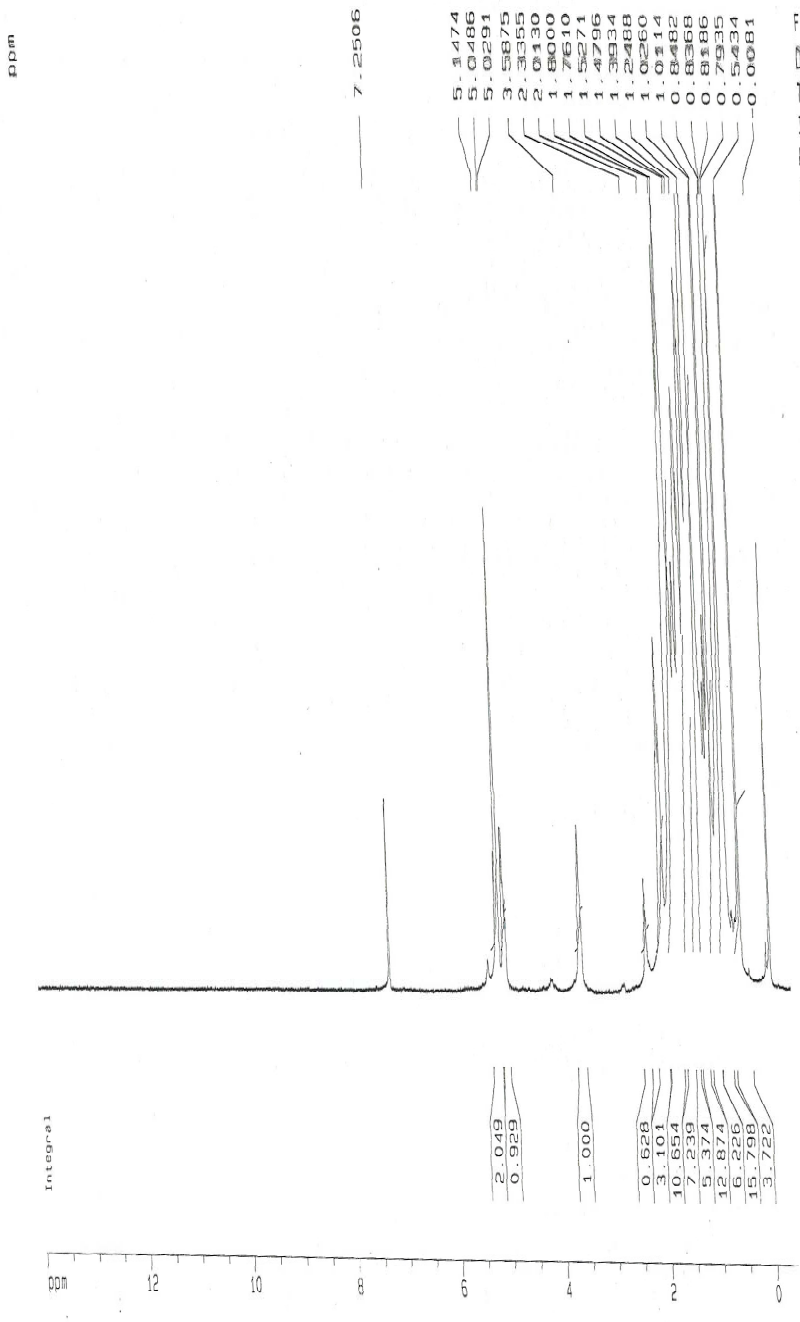



**Figure-25**: Lupeol

The 1HNMR spectrum of NIH-3 is given below-

NIH-3 in CDCl3, 1H Spectrum




Current Data Parameters

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| NAME | A6152 |
| EXPNO | 1 |
| PROCNO | 1 |

F2 - Acquisition Parameters

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| Time | 9.27 |
| INSTRUM | dpx400 |
| PROBHD | 5 mm Multinuc |
| PULPROG | zg |
| TD | 32768 |
| SOLVENT | CDCl3 |
| NS | 118 |
| DS | 2 |
| SWH | 6410.256 Hz |
| FIDRES | 0.195625 Hz |
| AQ | 2.5559540 sec |
| RG | 181 |
| DW | 78.000 usec |
| DE | 6.00 usec |
| TE | 310.0 K |
| D1 | 1.00000000 sec |

========== CHANNEL f1 ==========

| | |
|---|---|
| NUC1 | 1H |
| P1 | 8.50 usec |
| PL1 | -6.00 dB |
| SFO1 | 400.1429010 MHz |

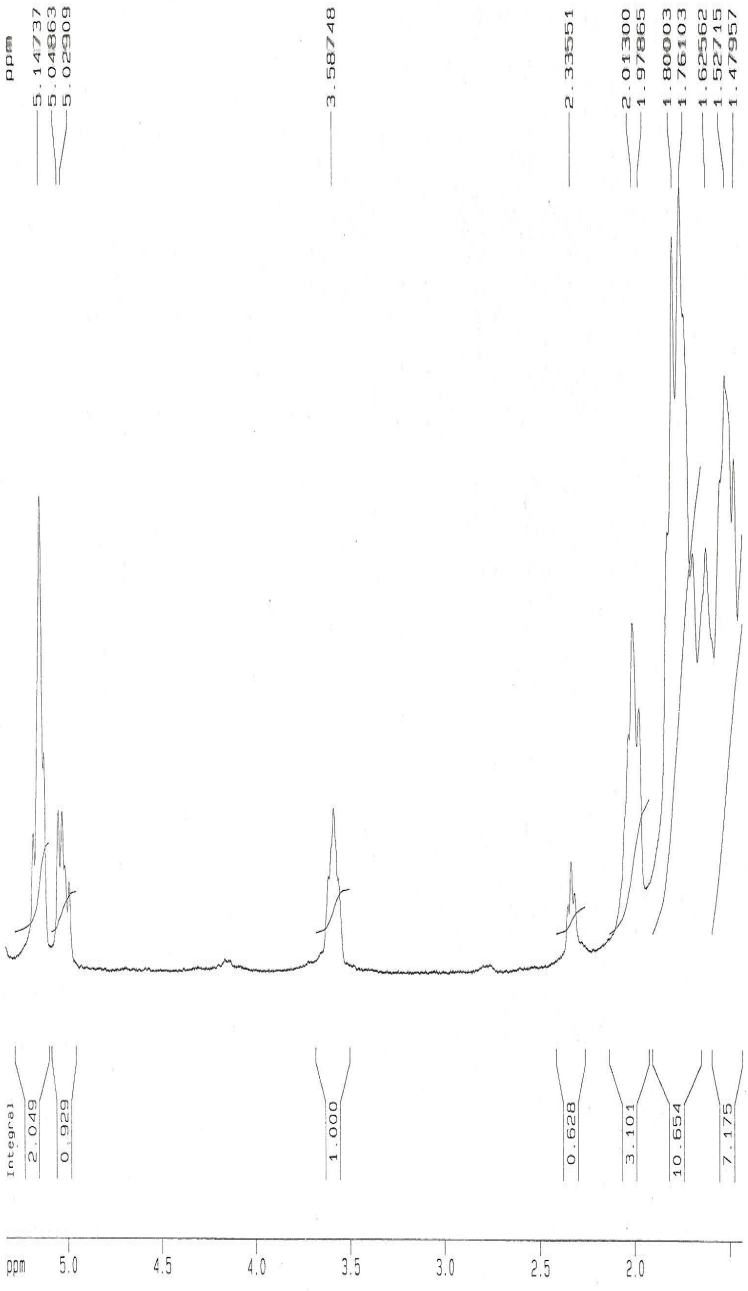
F2 - Processing parameters

| | |
|---|---|
| SI | 32768 |
| SF | 400.1400128 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.40 |

1D NMR plot parameters

| | |
|---|---|
| CX | 20.00 cm |
| F1P | 14.015 ppm |
| F1 | 5607.84 Hz |
| F2P | -0.383 ppm |
| F2 | -153.28 Hz |
| PPMCM | 0.71999 ppm/cm |
| HZCM | 288.05582 Hz/cm |

NIH-3 in CDCl3, 1H Spectrum

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F2 - Acquisition Parameters

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| PULPROG | zg |
| TD | 32768 |
| SOLVENT | CDCl3 |
| NS | 118 |
| DS | 2 |
| SWH | 6410.256 Hz |
| FIDRES | 0.195625 Hz |
| AQ | 2.5559540 sec |
| RG | 181 |
| DW | 78.000 usec |
| DE | 6.00 usec |
| TE | 310.0 K |
| D1 | 1.00000000 sec |

========== CHANNEL f1 ==========

| | |
|---|---|
| NUC1 | 1H |
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| PL1 | -6.00 dB |
| SFO1 | 400.1428010 MHz |

F2 - Processing parameters

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| SF | 400.1400128 MHz |
| WDW | EM |
| SSB | 0 |
| LB | 0.30 Hz |
| GB | 0 |
| PC | 1.40 |

1D NMR plot parameters

| | |
|---|---|
| CX | 20.00 cm |
| F1P | 5.332 ppm |
| F1 | 2133.62 Hz |
| F2P | 1.437 ppm |
| F2 | 574.93 Hz |
| PPMCM | 0.19477 ppm/cm |
| HZCM | 77.93430 Hz/cm |

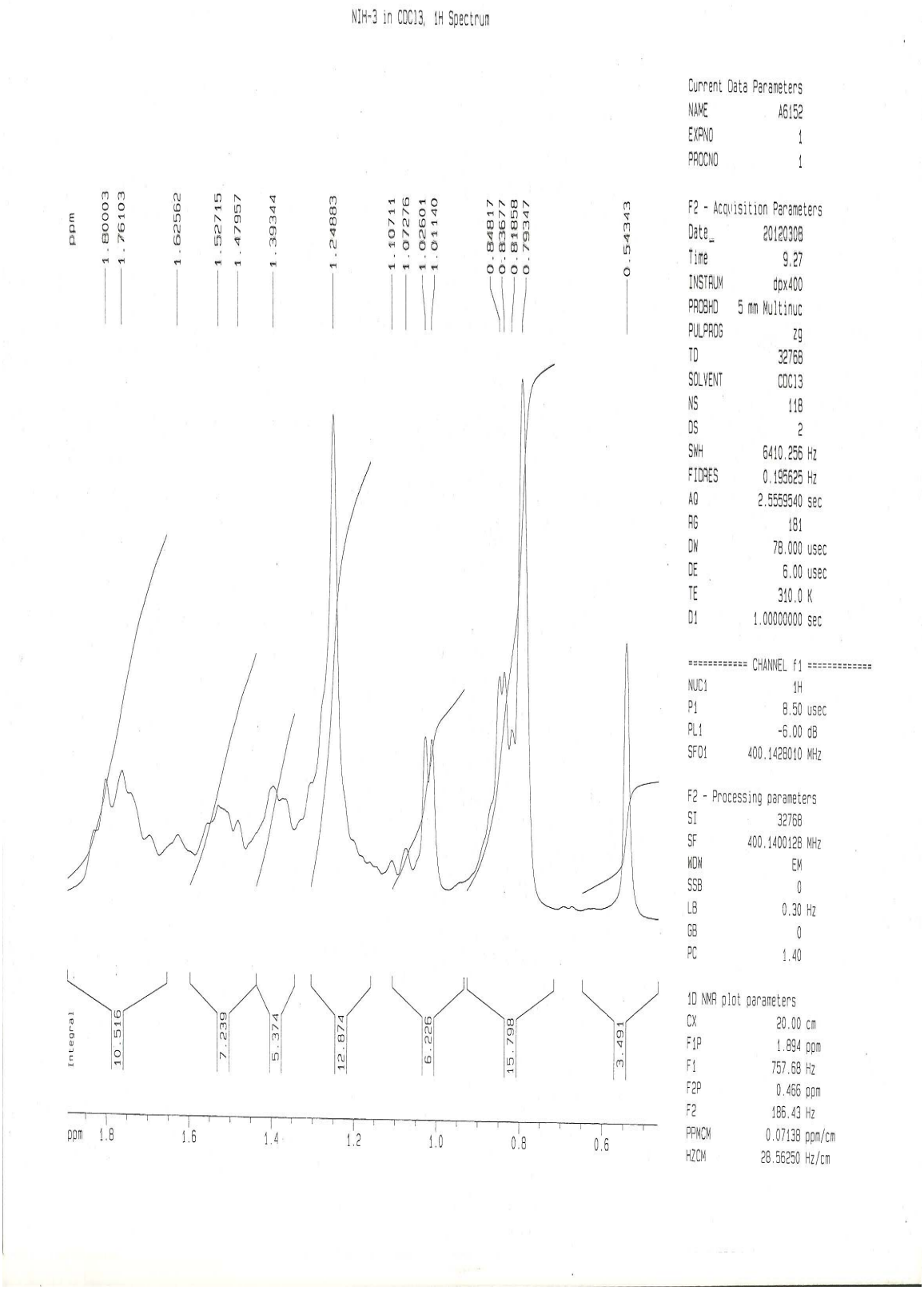


**Figure-26:** $^{1}$H NMR spectrum of NIH-3

# Chapter-9

# Conclusion

**9. Conclusion:**

Natural products spans an extremely large and diverse range of chemical compounds derived and isolated from biological sources. Compounds and extracts derived from the biosphere have found uses in medicine. *Nymphoides indica* is a rooted floating plants mainly found in Asia and the sub-continental region. . Due to the potential effect of *Nymphoides indica* against microorganisms reconfirmed by performing antimicrobial susceptibility test from its methanolic extract, the plant was collected to isolate the active principle that gives the antimicrobial effect. The highest anibacterial effect was observed in *Salmonella typhi* which is 14 mm diameter of zone of inhibition in a dose of 1000 μg/disc. For the continuation of the research work large scale *Nymphoides indica* was collected from panchagarh. 250 gm of dried crashed powder was obtained and extracted by macerating the powder in ethanol, yeilded 42 gm (16.8 % dry weight) of crude extract. The extract was further subjected to VLC (vacuum liquid chromatography) and fractionated into diffferent solvents. Fractions were- Fraction-1 (0.28 gm) eluted with 5000 ml n-hexane, Fraction-2 (0.63 gm) eluted 2000 ml dichloromethane, Fraction-3 (3.97 gm) eluted with 5000 ml ethyl acetate, Fraction-4 (20.2 gm) eluted with 5000 ml acetone and Fraction-5 (0.74 gm) eluted with 5000 ml methanol. The antimicrobial effect was further tested by using the VLC fractions. The effect was found in the fractions of n-hexane, dichloromethane and ethyl acetate fraction. Highest effect was found with EtOAc fraction that is 18 mm diameter of zone of inhibition in *Staphylococcus aureus* in a dose of 100 μg/disc. The Fr-2 was further fractionated by using open column chromatography with silica gel which gave and the fractions were collected by monitoring the thin layer chromatography (TLC). 18 fractions were collected from the fraction. Fr-2-3 and Fr-2-4 gave a colorless crystalline compound (NIH-3) which is UV inactive and after charring gave dark black color.

Fr-3 (ethyl acetate) fraction was subjected to the open column chromatography under the same condition like dichloromethane fraction. 35 fractions were collected form the fraction. No pure compound was isolated form the Fr-3 (ethyl acetate) fraction. This occurred may be due to the over loading of extract to the column. For this reason a mixture of color band was shown in the column. Further chemical separation and testing biological activities of these fractions are yet to be done due to the time limitation.

The results suggest the presence of either good antimicrobial potency of the extract or of a high concentration of an active principle in the extracts of *Nymphoides indica*. It should be supposed that the isolated compound NIH-3 which is a triterpenoid might be responsible for

this activity. From the NIH-3 it should be possible to find a potent antimicrobial drug which might be act as a broader spectrum against a wide range of microorganisms.

The structure elucidation of the NIH-3 was done by only $^{1}H$ NMR. But to confirm about the exact structure of NIH-3 we have to carry out $^{13}C$ NMR, NOE, HMBC, HSBC, HSQC, NOSY, QOSY which will give gross information that what type of functional group or structural skeleton is responsible for therapeutic activity that is finding out the pharmacophore of the compound.

NIH-3 may also be the active constituent that is responsible for the effect that is also shown on *Nymphoides indica*. The greater extent of antioxidant, antidiarrhoeal, analgesic and anti-inflammatory effect was also found in *Nymphoides indica* methanolic extract. But any separation and purification was not performed to isolate the active principle from *Nymphoides indica* in past. For identifying the active constituent the research work was carried out. So it might be possible to develop a new drug not only in the field of microbial but also in the field of antioxidant, antidiarrhoeal, analgesic and anti-inflammatory category in which the active principle might be NIH-3.

So further work on *Nymphoides indica* or performing different biological test on NIH-3 as well as other pure compounds that will be isolated from the *Nymphoides indica* may give a new or novel compound in the world of pharmacy.

A common misperception has been that natural product research has not kept pace with other drug discovery techniques and, as a consequence, become uncompetitive for lead discovery. However, improvements in instrumentation, robotics, and bioassay technology have increased the speed of bioassay guided isolation and structure elucidation of natural products considerably, and these improvements have allowed natural product research to be more competitive with synthetic compound screening. The natural product-derived drugs also contribute considerably to the profitability of many pharma and biotech companies. An inadequate number of lead compounds in many therapeutic areas and the unique chemical space occupied by natural products, have led to a renewed interest in natural product research. However, this renewed interest can be sustained only if natural product research can continue to be competitive with other drug discovery techniques. Key factors to remaining competitive include continual improvements in the speed of dereplication, isolation, structure elucidation, and compound supply processes and prudent selection of drug targets for the screening of natural product libraries.

# Chapter-10

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