Phytochemical Investigation of *Nymphaea pubescens* and Study of its Antimicrobial Activities

A dissertation ubmitted 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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Declaration by the Research candidate

I, Asif Mahmud Tunan, hereby declare that the dissertation entitled "**Bioactivity Guided Isolation and Purification of Active Principles & Pharmacological Evaluation of *Nymphaea pubescens*",** submitted by me to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by me during the period 2011-2012 under the supervision and guidance of **Dr. Chowdhury Faiz Hossain,** Dean, Faculty of Sciences and Engineering and professor, Department of Pharmacy, East West University and Professor, Department of Pharmacy 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.

Place: Dhaka

Date: 30/5/2012

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Thesis Certificate

This is to certify that the thesis entitled "**Bioactivity Guided Isolation and Purification of Active Principles & Pharmacological Evaluation of *Nymphaea pubescens*"**, submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the award of the degree of Bachelor of Pharmacy (B.PHARM) is a complete record of original research work carried out by Asif Mahmud Tunan (ID. 2008-3-70-028) during the period 2011-2012 of his research in the Department of Pharmacy at East West University, under my supervision and guidance and the thesis has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

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Abstract

Nymphaea pubescens Wild (Nymphaeaceae) is a fascinating aquatic plant used in Bangladesh for the treatment of diabetes, liver disorders, urinary problems, menorrhagia, blenorragia; also used as tonic and aphrodisiac. The roots used by the traditional healers of the Tripura, Marma and Murong tribes to treat dysuria, urinary tract infections and leucorrhea. Also, used for indigestion, heart diseases, stomachaches, cancer, and as anti-hemorrhagic. Due to its large traditional use, *N. pubescens* was the plant of choice of our study. Extraction of *Nymphaea pubescens* leaves (700 g) at room temperature by maceration with methanol yielded 122 g (17.43% of dry weight) extracts respectively. The methanol extract of *Nymphaea pubescens* was subjected to Vacuum liquid chromatography and five different fractions were collected using five different solvents; Fractions were- n-hexane Fraction-Fraction -1, DCM fraction-Fraction-2, Ethyl-acetate fraction- Fraction-3, Acetone fraction-Fraction-4 and Methanol fraction- Fraction-5. Their weights were Fraction-1=7.38 gm, Fraction-2=3.28 gm, Fraction-3=34.66 gm, Fraction-4=79.3 gm and Fraction-5=14.27 gm. Among the five fractions n-hexane fraction and Ethyl-acetate fraction were subjected to column chromatography. The n-hexane fraction or Fraction-1 and ethyl acetate fraction or Fraction-3 of *Nymphaea pubescens* were separated by open column chromatography with silica gel and the fractions were collected monitoring the TLC. Fraction-1-40 yielded a colorless crystal, NPH-40. This compound was UV inactive and charring with methanol and H_2SO_4 (9:1) gave characteristic red color indicating the compound is a fatty acid derivative. ^{1}HNMR report of NPH-40 informs that may be this is a mixture of four fatty acids and those are myristic acid, palmitic acid, lauric acid and 4-methyl-4-tetradecenoic acid. Further analysis such as ^{13}CNMR, Mass spectroscopy etc is required for definite result. Fraction-3-22 yielded another colorless crystal, NPE-22. This compound was also UV inactive and charring with methanol and H_2SO_4 (9:1) gave characteristic brown color indicating the compound is a triterpenoid derivative. ^{1}HNMR report of NPE-22 informs that may be this compound is oleanolic acid. Further analysis such as ^{13}CNMR, Mass spectroscopy etc is also required here for definite result. According to the literature review done so far in this research paper, this is the first time that Oleanolic acid has been isolated from *Nymphaea pubescens.*

Key words: Fatty acid, Triterpenoid, Oleanolic acid, VLC, TLC, Open column chromatography.

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Chapter 1

Introduction

1. Introduction

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. [1]

1.1. Medicinal Plant

Medicinal plants are plants which have a recognized medical use. They range from plants which are used in the production of mainstream pharmaceutical products to plants used in herbal medicine preparations. Herbal medicine is one of the oldest forms of medical treatment in human history, and could be considered one of the forerunners of the modern pharmaceutical trade. Medicinal plants can be found growing in numerous settings all over the world.

1.2. Importance and Scope

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other was used for medicinal purposes. It is estimated that world market for plant derived drugs may account for about Rs.2,00,000 crores. It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China, India and Bangladesh, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more to countries such as India, Bangladesh than to rest of the world. These countries provide two third of the plants used in modern system of medicine and the health care system of rural population depend on indigenous systems of medicine. Of the 2, 50,000 higher plant species on earth, more than 80,000 are medicinal. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs which form an important segment of the modern pharmacopoeia. Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants (Eg. diosgenin, solasodine, b-ionone). Not only, that plant-derived drug offers a stable market world wide, but also plants continue to be an important source for new drugs. Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Global estimates indicate that 80% of about 4 billion population can not afford the products of the Western Pharmaceutical Industry and have to rely upon the use of traditional medicines which are mainly derived from plant material. This fact is well documented in the inventory of medicinal plants, listing over 20,000 species. In spite of the overwhelming influences and our dependence on modern medicine and tremendous advances in synthetic drugs, a large segment of the world population still likes

drugs from plants. In many of the developing countries the use of plant drugs is increasing because modern life saving drugs are beyond the reach of three quarters of the third world's population although many such countries spend 40-50% of their total wealth on drugs and health care. As a part of the strategy to reduce the financial burden on developing countries, it is obvious that an increased use of plant drugs will be followed in the future.[2]

1.3. Classification of Medicinal Plants

Of the 2,50,000 higher plant species on earth, more than 80,000 species are reported to have at least some medicinal value and around 5000 species have specific therapeutic value. They are classified according to the part used, habit, habitat, therapeutic value etc, besides the usual botanical classification.

A. Based on part used

i) Whole plant*: Boerhaavia diffusa, Phyllanthus neruri*

ii) Root*: Dasamula*

iii) Stem*:Tinospora cordifolia, Acorus calamus*

iv) Bark*: Saraca asoca*

v) Leaf: *Indigofera tinctoria, Lawsonia inermis, Aloe vera*

vi) Flower*:Biophytum sensityvum, Mimusops elenji*

vii) Fruit*: Solanum species*

viii) Seed*: Datura stramonium*

B. Based on habit

i) Grasses*: Cynodon dactylon*

ii) Sedges*: Cyperus rotundus*

iii) Herbs *: Vernonia cineria*

iv) Shrubs*: Solanum species*

v) Climbers*: Asparagus racemosus*

vi) Trees*: Azadirachta indica*

C. Based on habitat

i) Tropical*: Andrographis paniculata*

ii) Sub-tropical*: Mentha arvensis*

iii) Temperate*: Atropa belladona*

D. Based on therapeutic value

Antimalarial*: Cinchona officinalis, Artemisia annua*

Anticancer*: Catharanthus roseus, Taxus baccata*

Antiulcer*: Azadirachta indica, Glycyrrhiza glabra*

Antidiabetic*: Catharanthus roseus, Momordica charantia*

Anticholesterol*: Allium sativum*

Antiinflammatory*: Curcuma domestica, Desmodium gangeticum*

Antiviral*: Acacia catechu*

Antibacterial*: Plumbago indica*

Antifungal*: Allium sativum*

Antiprotozoal*: Ailanthus sp., Cephaelis ipecacuanha*

Antidiarrhoeal*: Psidium gujava, Curcuma domestica*

Hypotensive*: Coleus forskohlii, Alium sativum*

Tranquilizing*: Rauvolfia serpentina*

Anaesthetic*: Erythroxylum coca*

Spasmolytic*: Atropa belladona, Hyoscyamus niger*

Diuretic*: Phyllanthus niruri, Centella asiatica*

Astringent*: Piper betle, Abrus precatorius*

Anthelmentic*: Quisqualis indica, Punica granatum*

Cardiotonic*: Digitalis sp., Thevetia sp.*

Antiallergic*: Nandina domestica, Scutellaria baicalensis*

Hepatoprotective*: Silybum marianum, Andrographis paniculata*

1.4. Cultivation of Medicinal Plants

Most of medicinal plants, even today, are collected from wild. The continued commercial exploitation of these plants has resulted in receding the population of many species in their natural habitat. Vacuum is likely to occur in the supply of raw plant materials that are used extensively by the pharmaceutical industry as well as the traditional practitioners. Consequently, cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. If timely steps are not taken for their conservation, cultivation and mass propagation, they may be lost from the natural vegetation for ever. In situ conservation of these resources alone cannot meet the ever increasing demand of pharmaceutical industry. It is, therefore, inevitable to develop cultural practices and propagate these plants in suitable agroclimatic regions. Commercial cultivation will put a check on the continued exploitation from wild sources and serve as an effective means to conserve the rare floristic wealth and genetic diversity.[2]

It is necessary to initiate systematic cultivation of medicinal plants in order to conserve biodiversity and protect endangered species. In the pharmaceutical industry, where the active medicinal principle cannot be synthesized economically, the product must be obtained from

the cultivation of plants. Systematic conservation and large scale cultivation of the concerned medicinal plants are thus of great importance. Efforts are also required to suggest appropriate cropping patterns for the incorporation of these plants into the conventional agricultural and forestry cropping systems. Cultivation of this type of plants could only be promoted if there is a continuous demand for the raw materials. It is also necessary to develop genetically superior planting material for assured uniformity and desired quality and resort to organised cultivation to ensure the supply of raw material at growers end. Hence, small scale processing units too have to be established in order that the farmer is assured of the sale of raw material. Thus, cultivation and processing should go hand in hand in rural areas.[2]

In order to initiate systematic cultivation of medicinal and aromatic plants high yielding varieties have to be selected. In the case of wild plants, their demonstration would require careful development work. Sometimes high yielding varieties have also to be developed by selective breeding or clonal micropropagation. The selected propagation materials have to be distributed to the farmer either through nurseries or seed banks. Systematic cultivation needs specific cultural practices and agronomical requirements. These are species specific and are dependent on soil, water and climatic conditions. Hence research and development work has to be done to formulate Good Agricultural Practices (GAP) which should include proper cultivation techniques, harvesting methods, safe use of fertilizers and pestisides and waste disposal.[2]

1.5. Processing and Utilization

Medicinal principles are present in different parts of the plant like root, stem, bark, heartwood, leaf, flower, fruit or plant exudates. These medicinal principles are separated by different processes; the most common being extraction. Extraction is the separation of the required constituents from plant materials using a solvent. In the case of medicinal plants, the extraction procedure falls into two categories.

a) Where it is sufficient to achieve within set limits equilibrium of concentration between drug components and the solution. Eg. Tinctures, decoction, teas, etc.

b) Where it is necessary to extract the drug to exhaustion, ie., until all solvent extractables are removed by the solvent.

Both the methods are employed depending on the requirement although in industry the latter method is mostly used. In all industrial procedures, the raw material is pre-treated with solvent outside the extractor before changing the latter. This prevents sudden bulk volume changes (which are the main cause of channelling during extraction) and facilitates the breaking up of the cell walls to release the extractables. To facilitate the extraction, the

solvent should diffuse inside the cell and the substance must be sufficiently soluble in the solvent. The ideal solvent for complete extraction is one that is most selective, has the best capacity for extraction and is compatible with the properties of the material to be extracted. These parameters are predetermined experimentally. The cost and availability of the solvent are also taken into account. Alcohol, though widely used, because of its great extractive power it is often the least selective, in that it extracts all soluble constituents. Alcohol in various ratios is used to minimise selectivity. The ideal alcohol ratio for woody or bark material is 75%. For leafy material, it is often less than 50% thus avoiding extraction of the chlorophyll which makes purification difficult.

Some materials such as alkaloids being soluble in acids, their extraction is facilitated by adjusting the pH in the acidic range. A number of alkaloids can be extracted easily with hydrocarbons after they have been released from combination with organic acids by grinding with alkali. It is first ground with moist calcium oxide and extracted with chloroform. A large number of alkaloids can be extracted directly with aqueous acids, organic or inorganic acids, and the alkalised extracts counter extracted with hydrocarbons or other apolar solvents.[2]

Experiment used for extraction with solvents usually comprise an extraction vessel with a heating jacket for steam heating or fitted with electrical devices, a condenser in reflux position, a solvent reservoir, a facility to convert to reboiler position or a separate reboiler and a short column for solvent recovery. Some times, sophisticated and costly equipment like the Carousel or the Inoxa extractor is employed.

Technology for the manufacture of standardised extracts and phytochemicals is available and there are many extracts already in the international market as drugs. A drug such as an extract of *Centella asiatica* can be manufactured as an extract containing a standard quantity of asiaticoside. Similarly for senna a standardised extract of which, containing a standard quantity of sennosides a and b could easily be produced with equipment that can be designed and constructed in most developing countries.

The promotion and development of processing of medicinal and aromatic plants have gained momentum recently in many developing countries. Green consumerism and resurgence of interest for plant based products, liberalised and free market economy, increasing awareness about biodiversity conservation and sustainable use of natural resources coupled with poor socio-economic conditions of native populations are ground realities for planning and harnessing the low-cost and purpose oriented process technologies.

UNIDO has developed a Polyvalent Pilot Plant with a view to enabling developing countries to upgrade their technology for the processing of medicinal and aromatic plants. This plant

incorporates all salient features of a low cost, efficient, small capacity factory which can carry out solvent extraction, solvent percolation, concentration of miscella, solvent recovery, steam distillation and oil separation. The design and fabrication of the process equipment need not be over emphasised, as even if a good design is available for adaptation, it must be done to fit the given situation.

The polyvalent plant is characterised by simplicity of design, installation, operation, maintenance and repair. Some of its features are

1. Modular construction so as to permit increase in capacity and function by duplicating or adding modules.
2. Simultaneous processing for more than one product, such as extraction at one end, production of solid extract or oleoresins at the other.
3. Standardised or optimised process control and measuring units, pumps and other ancillaries can be easily replaced.
4. All plumbing and electrical wiring are simple and easily accessible.
5. Multipurpose uses. Eg. Solvent/aqueous extraction, continuous extraction, preparation of solid extract and oleoresins, essential oil distillation, fractionation of essential oils and production of absolutes and concretes or even processing of other phytoproducts.[2]

1.6. Formulation and Industrial Utilization

Medicinal plants are used as raw materials for extraction of active constituents in pure form (eg. alkaloids like quinine and quinidine from cinchona bark, emetine from ipecacuanha root, glycosides from digitalis leaves, sennosides from senna leaves), as precursors for synthetic vitamins or steroids, and as preparations for herbal and indigenous medicines. Products such as ginseng, valerian and liquorice roots are part of the herbal and health food market, as well as the food flavours, fragrance and cosmetic industries. Certain plant products are industrially exploited like liquorice in confectionery and tobacco, papaine as meat tenderiser, quinine as soft drink tonic and cinchona as wine flavour. A large quantity of medicinal plant material is used in the preparation of herbal and medicinal teas, eg. chamomile. These herbal and food uses are of great importance, also to the exporters from developing countries. Hundreds of medicinal plants are items of commerce, however relatively small countries are used in formulated herbal remedies.

Several formulations like herbal teas, extracts, decoctions, infusions, tinctures, etc are prepared from medicinal plants.

1. Herbal teas, Herbal remedies: herbal tea or infusion mixtures are mixture of unground or suitably ground medicinal plants to which drug plant extracts, ethereal oils or medicinal substances can be added. Infusion mixtures should be as homogenous as possible.

2. Drug extracts: They are preparations obtained by extracting drugs of a certain particle size with suitable extraction agents (menstrua). The extract obtained after separation of the liquid from the drug residue is called miscella. It may already represent the final liquid dose form eg. as a so called fluid extract, or be used as an intermediary product which is to be further processed as quickly as possible.

3. Aqueous drug extracts: The following degrees of comminution are used for the extract depending on the type of plant parts. Leaves, flowers and herbs shredded (4000mm); woods, barks and roots shredded (2800mm); fruits and seeds (2000mm). Alkaloid containing drugs powdered (700mm).

3.1. Decoctions: The drug in the prescribed comminution is put in to water at a temperature above 90°C. The container is suspended in a water bath and maintained at this temperature for 30 minutes, with repeated stirring. The mixture is then strained while still hot.

3.2. Infusions: One part of the comminuted drug is kneaded several times in a mortar with 3-5 parts of water and left to stand for 15 minutes. The rest of the boiling water is then poured on to the mixture, which is suspended in a container in a water bath and kept for 5 minutes, with repeated stirring at a temperature above 90°C. The mixture is covered and left to stand until cool.

3.3. Macerates: The comminuted drug is left to stand, with occasional stirring, for 30 minutes after the required quantity of water has been poured on to it at room temperature. The extract is then strained and made up to the prescribed weight with rinsings.

3.4. Tinctures: Tinctures are extracts from drug plants prepared with ethanol of varying concentration, ether or mixtures of these, perhaps with certain additives, in such a way that one part of drug is extracted with more than two parts, but at most ten parts, of extraction liquid.

3.5. Fluid extracts**:** Like tinctures, they are liquid preparations, the difference being that they are more concentrated.

3.6. Dry extracts**:** They are usually very hygroscopic and should therefore be ground and mixed under conditions which exclude moisture as much as possible. Intermediate and end product must also be stored under dry conditions. There are also liquid, semisolid, solid and controlled release formulations or preparations. The other dose forms are injections, implants, ocular preparations, inhalations and transdermal systems.[2]

1.7. Selection, Collection, and Identification of Plant Material

1.7.1. Selection and Identification of Plant

As per WHO guidelines (WHO 2003), the plant selected for collection should be taxonomically same as recommended by the national pharmacopoeia or other related documents. If a new plant is being selected for collection then it should be properly identified and documented. The botanical identity, scientific name including genus, species, subspecies or variety and family of the plant should be recorded. If available, the local name should also be verified. Complete taxonomical identification is an important factor during selection as taxonomy of the plant species can play an important role in their biological activity.

In general, the search for the medicinal plants can follow three main routes: random, ethno (including ethnobotanical, ethnomedical and ethnopharmacological) and ecological search. Random search is extremely laborious and the success rate could be very low. Nevertheless, important drugs such as taxol, derivatives of camptothecin and homoharringtonine have been discovered by the National Cancer Institute (NCI) in collaborations with the United States Department of Agriculture (USDA) using this method.

The ethnobotanical, ethnomedical or ethnopharmacological approach uses information obtained from ethnobotanical survey such as the geographical distribution of the plant, its abundance, whether it is threatened or endangered, shrub/fast growing tree, easily cultivable, easily identifiable (with minimum varieties) etc. Information such as the season of collection, parts that are used and whether those parts are seasonal/replenishable and if there is any reported toxicity, are also required. The information can be obtained from traditional medical practitioners and other people such as village elders and local women who are traditional users of medicinal plants.[3]

1.7.2. Collection of Plant

Medicinal plant materials should be collected in the proper season so as to ensure the best possible quality of both the starting material as well as the finished product. Seasonal variations can affect the chemical composition of the plants and thus its biological activity. In most cases, maximum accumulation of chemical constituents occurs at the time of flowering which then declines at the beginning of the fruiting stage. The time of harvest should also depend on the plant part to be used since it is well known that depending on the plant species the level of biologically active constituents can vary in different parts at different stages of the plant growth and development. For example, Kursar et al. (1999) found that younger leaves of tropical rainforest plants contained secondary metabolites that were either present in very little quantities or totally absent in matured leaves. The extracts from these younger

leaves showed better biological activity when tested for anticancer activity or activity against Bacillus subtilis and Artemia salina (brine shrimp). It also applies to other components in the plant material such as the toxic components. Climatic conditions, e.g. light, rainfall, and temperature (including daytime and nighttime temperature differences) also influence the physical, chemical and biological qualities of medicinal plants. The water and temperature stress related increase in the content of active constituents such as the total phenolic compunds was shown by Nacif de Abreu and Mazzafera (2005) in Hypericum brasilience. Hence the best time of collection should be determined according to the levels of the biologically active constituents rather than the vegetative yield.[3]

1.8. Drying and Grinding

After collection the plant material must first be preserved so that the active compound will remain unchanged. The most common method for preserving plant material is drying. Drying also decreases the risk of external attack by moulds. In general, plant materials should be dried at temperature below 30°C to avoid decomposition of thermolabile compound. When air-dried, the plant material has to be spread out with good ventilation to facilitate drying. [4]

Plants can be dried in a number of ways: in the open air (shaded from direct sunlight); placed in thin layers on drying frames, wire-screened rooms, or in buildings; by direct sunlight, if appropriate; in drying ovens/rooms and solar dryers; by indirect fire; baking; lyophilization; microwave; or infrared devices. Where possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents. The method and temperature used for drying may have a considerable impact on the quality of the resulting medicinal plant materials. For example, shade drying is preferred to maintain or minimize loss of color of leaves and flowers; and lower temperatures should be employed in the case of medicinal plant materials containing volatile substances. The drying conditions should be recorded. In the case of natural drying in the open air, medicinal plant materials should be spread out in thin layers on drying frames and stirred or turned frequently. In order to secure adequate air circulation, the drying frames should be located at a sufficient height above the ground. Efforts should be made to achieve uniform drying of medicinal plant materials to avoid mold formation.

Drying medicinal plant material directly on bare ground should be avoided. If a concrete or cement surface is used, the plant materials should be laid on a tarpaulin or other appropriate cloth or sheeting. Insects, rodents, birds and other pests, and livestock and domestic animals should be kept away from drying sites. For indoor drying, the duration of drying, drying temperature, humidity and other conditions should be determined on the basis of the plant

part concerned (root, leaf, stem, bark, flower, etc.) and any volatile natural constituents, such as essential oils. If possible, the source of heat for directs drying (fire) should be limited to butane, propane or natural gas, and temperatures should be kept below 30 °C. If other sources of fire are used, contact between those materials, smoke, and the medicinal plant material should be avoided.

Grinding improves efficiency of extraction by increasing the surface area of plant material. This decreases the amount of solvent needed for extraction as it allows the plant material to pack more densely. Therefore, it is essential to grind samples into finer size for better extraction results.[5]

1.9. Extraction of plant material

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician. The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum. The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modem drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug. General methods of extraction of medicinal plants are-

1.9.1. Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing.

1.9.2. Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

1.9.3. Digestion

This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.

1.9.4. Decoction

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

1.9.5. Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like asava and arista) adopt the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (kasaya), for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. If the fermentation is to be carried out in an earthen vessel, it should not be new: water should first be boiled in the vessel. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels. Some examples of such preparations are karpurasava, kanakasava, dasmularista. In Ayurveda, this method is not yet standardized but, with the extraordinarily high degree of advancement in fermentation technology, it should not be difficult to standardize this technique of extraction for the production of herbal drug extracts.

1.9.6. Ultrasound Extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwoli a root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.[6]

1.10. Fractionation of Crude Extract

Chromatographic procedures are the most diverse and the most widely used techniques in the fractionation of crude extracts. There is no doubt that they have made possible the isolation of many naturally occurring compounds. A crude extract is a mixture of thousands compounds. It is difficult to isolate a single compound from the crude extracts by a single separation technique. Hence the crude extract is initially separated into various discrete fractions containing compounds of similar polarities or molecular sizes. These fractions may be contiguous elute from a chromatography column, e.g. vacuum liquid chromatography (VLC), column chromatography (CC), size-exclusion chromatography (SPE), liquid chromatography (LC), etc.

For initial fractionation of any crude extract, it is advisable that do not generate too many fractions, because it may spread the target compounds overso many fractions. As a result, amount of target compound will be very small that will render the detection. For finer fractionation, often newly discovered detection technique, e.g. ultraviolet (UV), modern preparative, or semi preparative high performance liquid chromatography (HPLC) can be used.[7]

1.11. Isolation

The most important factor that has to be considered before isolation is the nature of the target compounds 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 molecularsize. If isolating a knowm 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 compound 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. The nature of the extract can also be helpful for choosing the right isolation protocol. For example, a MeOH extract or fractions containing polar compounds are better deals 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 experiment. Some of these experiments are summarized below.[8]

1. 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 portioning, usually between water and EtOAc, $CHCl_3$, DCM, or n-hexane, followed by an assay to determine the distribution of compounds in solvent fractions.

2. Acid-base properties: Carrying out partitioning in aqueous solvents at a range of pH values, typically 3, 7, and 10, can help to determine the acid-base properties 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, 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.

3. 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.

4. Heat stability: A typical heat stability test involves incubation of the sample at 90°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 product is carried out at a high temperature, the test for heat stability becomes irrelevant.

5. 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.

6. Chromatographic techniques used in the isolation: The chromatographic techniques used in the isolation of various types of natural products can be broadly classified into two categories:

A. Classical or older chromatographic techniques include-

1. Thin-layer chromatography (TLC)
2. Preparative thin-layer chromatography (PTLC)
3. Open column chromatography (CC)

4. Flash chromatography (FC)

B. Modern chromatographic techniques are-

1. High-performance thin-layer chromatography (HPTLC)
2. Multiflash chromatography (e.g., Biotage)
3. Vaccum liquid chromatography (VLC)
4. Chromatotron
5. Solid-phase extraction
6. Droplet countercurrent chromatography (DCCC)
7. High performance liquid chromatography (HPLC)
8. Hyphenated techniques (e.g., HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR)

1.12. Structure Elucidation

In the early years, structure elucidation posed a big problem to many researchers as the major spectroscopic equipment, i.e. nuclear magnetic resonance (NMR) and mass spectrometer (MS) were not easily available. Many researches had to send samples to institutions of developed countries for these services. The availability of this expensive spectroscopic instrument in many laboratories towards the late nineties facilitated and hastened elucidation of structures. Low fields 1H and 13C NMR (proton noise decoupling, off-resonance technique, DEPT), MS, IR, UV spectra and elemental analysis data were in most cases sufficient to obtain the structures of compounds. The acquisition of new techniques such as two dimensional high field NMR, liquid chromatography–mass spectrometry (LCMS), chemical ionization and fast atom bombardment mass spectrometry (CI and FAB-MS), Fourier Transform Infra Red spectrophotometry (FTIR) and X-ray crystallography in the early nineties, enabled natural products chemists to confidently characterized and identify structures of large and complicated molecules at submilligram quantities.[9]

1.13. Assay

An assay is an investigative (analytic) procedure in laboratory medicine, pharmacology, environmental biology, and molecular biology for qualitatively assessing or quantitatively measuring the presence or amount or the functional activity of a target entity (the analyte) which can be a drug or biochemical substance or a cell in an organism or organic sample. Chemical, biological, or physical assays are necessary to pinpoint the target compounds from a complex natural product extract.

1.13.1. Physical assay

Physical assays may involve the comparison of various chromatographic method and spectroscopic behavior, e.g., HPLC, TLC, LC-MS, LC-NMR, and so on, of the target compounds with a known standard.

1.13.2. Chemical assay

Chemical assay involves in the separation, identification, and quantification of the chemical components of natural and artificial materials. There are two branches in chemical assay: qualitative assay and quantitative assay. Qualitative assay is the determination of those elements and compounds that are present in a sample of unknown material. Quantitative assay is the determination of the amount by weight of each element or compound present.

1.13.3. Bioassay

Bioassays are typically conducted to measure the effects of a substance on a living organism and are essential in the development of new drugs. It involves a procedure by which the potency or the nature of a substance is estimated by studying its effects on living matter. Bioassays could involve the use of in vivo systems, ex vivo systems or in vitro systems.

In vitro refers to studies in experimental biology that are conducted using components of an organism that have been isolated from their usual biological context in order to permit a more detailed or more convenient analysis than can be done with whole organisms. In contrast, the term in vivo refers to work that is conducted with living organisms in their normal, intact state, while ex vivo refers to studies on functional organs that have been removed from the intact organism.

Chapter 2

Introduction to Plants

2. Introduction to plants

Nymphaea pubescens (Nymphaeaceae) is long-lived aquatic herb, with rootstock rooting in the mud. Leaves are long-stalked and leathery, floating on the surface of water, ovate to almost circular, prominently toothed, slightly peltate, 12 to 15 centimeters across, with the base deeply heart-shaped, and densely hairy beneath. Petioles are long, slender and submerged. Flowers are fragrant, white or red, about 8 cm in diameter, borne on long peduncles. Petals are linear-oblong to lanceolate. Fruits are globular, with longigtudinally numerous, striated seeds. *Nymphaea pubescens* is widely distributed in Bangladesh especially in the winter season. The plant is locally khown as shada shapla. It is marketed as vegetable in Dhaka city to all other market in Bangladesh. It is a national flower and icon of Bangladesh and is a delicious item of food for Bangladeshi people. Bangladeshi people purchase it as vegetables because the whole plant especially flower can be eaten as vegetable after cooking.

Figure 1: *Nymphaea pubescens* Willd

2.1. Taxonomy

Domain: *Eukaryota*

Kingdom**:** *Plantae*

Subkingdom: *Viridaeplantae*

Phylum: *Tracheophyta*

Subphylum: *Euphyllophytina*

Infraphylum: *Radiatopses*

Class: *Magnoliopsida*

Subclass: *Nymphaeidae*

Superorder: *Nymphaeanae*

Order: *Nymphaeales*

Family: *Nymphaeaceae*

Subfamily: *Nymphaeoideae*

Tribe: *Nymphaeeae*

Genus: *Nymphaea*

Botanical name: *Nymphaea pubescens* Willd.

2.2. Distribution

- In shallow lakes and ponds, often locally abundant, from nothern Luzon to Mindanao.
- Usually cultivated for its attractive flowers.
- Also occurs in tropical Asia to Malaya.

2.3. Properties

- The juice is bitter and astringent has some narcotic properties.
- Flowers are astringent and cardiotonic.
- Juice is bitter.

2.4. Parts utilized

Rhizomes, flowers, seeds, leaves.

2.5. Uses

Decoction of rhizome of red flowered plant is given for blood dysentery. Rhizome juice is prescribed against leucorrhoea. Powdered rhizome with honey is given for piles, dysentery and dyspepsia. Root juice is drunk to keep stomach cool and to get relief from burning sensation during urination. Root paste of the red flowered plant is given for treating menorrhagia. Paste of root of the plant with flowers of *Hibiscus rosa-sinensis,* bark of *Ficus religiosa* and seeds of *Sesamum indicum* (Rasi) is taken for abortion.[14]

2.6. Quantitative screening of biomolecules and phytochemicals

Quantitative screening of biomolecules revealed that the plant has total chlorophyll 5.54 mg/g, carotenoids 3.11 mg/g, proteins 348 mg/g, carbohydrates 165 mg/g, lipids 100 mg/g, and total phenols 850 mg/g.

The phytochemical screening of ethanol extract of *Nymphaea pubescens* tuber revealed the presence of alkaloids, flavonoids, glycosides,terpenoids, tannins, phenols, saponins and steroids.[14]

2.7. Nymphaeaceae Family

Nymphaeaceae is a family of flowering plants. Members of this family are commonly called water lilies and live in freshwater areas in temperate and tropical climates around the world. The family contains eight genera. There are about 70 species of water lilies around the world. Water lilies are rooted in soil in bodies of water, with leaves and flowers floating on the water surface. The leaves are round, with a radial notch in Nymphaea and Nuphar, but fully circular in Victoria.

Water lilies are divided into two main categories: hardy and tropical. Hardy water lilies bloom only during the day, but tropical water lilies can bloom either during the day or at night, and are the only group to contain blue-flowered plants.

The Nymphaeaceae are aquatic, rhizomatous herbs. The family is further characterized by scattered vascular bundles in the stems, and frequent presence of latex, usually with distinct, stellate-branched sclereids projecting into the air canals. Hairs are simple, usually producing mucilage (slime). Leaves are alternate and spiral, opposite or occasionally whorled, simple, peltate or nearly so, entire to toothed or dissected, short to long Petiole (botanyiolate), with blade submerged, floating or emergent, with palmate to pinnate venation. Stipules are either present or absent. Flowers are solitary, bisexual, radial, with a long pedicel and usually floating or raised above the surface of the water, with girdling vascular bundles in receptacle. Tepals are 4-12, distinct to connate, imbricate, and often petal-like. Petals lacks or 8 to numerous, inconspicuous to showy, often intergrading with stamens. Stamens are 3 to numerous, the innermost sometimes represented by staminodes. Filaments are distinct, free or adnate to petaloid staminodes, slender and well differentiated from anthers to laminar and poorly differentiated from anthers; pollen grains usually monosulcate or lacking apertures. Carpels are 3 to numerous, distinct or connate. Fruit is an aggregate of nuts, a berry, or an irregularly dehiscent fleshy capsule. Seeds are often arillate, more or less lacking endosperm.[10]

Chapter 3

Literature Review

3. Literature Review

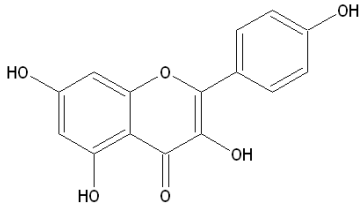
Isolation and purification of compounds from the plants of nymphaeaceae family were not done in a great extend. However hypoglycemic, antioxidant, antibacterial and chemical tests had been performed widely for various plants of nymphaeaceae family. For example hypoglycemic activity of *Nymphaea stellata* leaves, antibacterial activity of *Nymphaea lotus* leaves, anxiolytic activity of *Nymphaea alba*, and many other tests had been already reported.

3.1. Name of some plants of Nymphaeaceae family and their constituents and uses

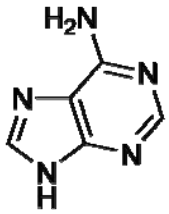
A. *Nelumbo nucifera* Gaertn. Or Padma

Constituents: Bioassay-guided fractionation and repeated chromatography of *Nelumbo nuciferahas* led to the isolation and identification of rutin, (+)-catechin, hyperoside, isoquercitrin, quercetin and astragalin. Thirteen flavonoids: kaempferol and seven of its glycosides, and two isorhamnetin glycosides are isolated from N. nucifera, as well as four non-flavonoid compounds: adenine, myo-inositol, arbutin and β-sitosterol glucopyranoside.[11]

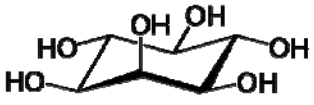
In sharp contrast, the fruit wall plus seed coat of Nelumbo is believed to be composed of a complex of polysaccharides, based on primarily galactose and mannose units, and insoluble tannins, which are suggested to play the same structural role as the lignin-cellulose in the sclerotic seed coat.[12]



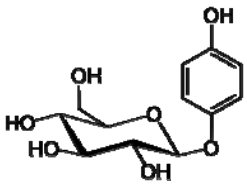
Kaempferol



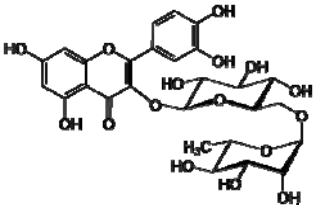
Adenine



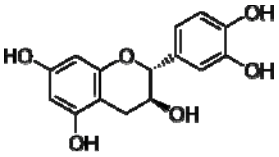
Myo-inositol



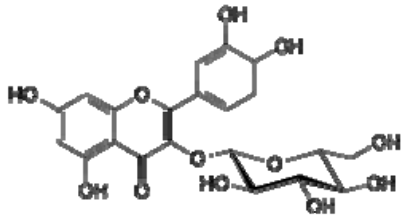
Arbutin



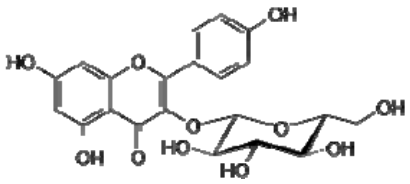
Rutin



(+)-Catechin



Isoquercitrin



Astragalin

Uses: (a) Paste of young leaf along with fruits of Emblica myrobalan (*Phyllanthus emblica*) is applied on forehead to get relief from headache.

(b) Flower petal decoction is given against diarrhoea.

(c) Young flower paste is prescribed as cardiac tonic and also in fever and liver ailments.

(d) Dried seed powder is taken along with fresh cow milk against headache.

(e) Young seed paste is used externally as a cooling medicine for skin disease.

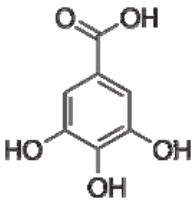
(f) Powdered root is taken for expelling ringworms.

(g) Root paste kept in a fine cloth and rolled to a thread (*salita*), and dipped in cow ghee is inserted inside the nostril of the unconscious patient suffering from fits and kept till the patient become conscious.

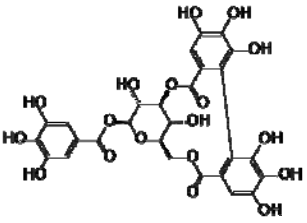
(h) Root paste in lemon juice is taken for the treatment of piles

B. *Nymphaea nouchali* Burm. Or Red water lily:

Constituents: Different solvent extracts of the entire plant have shown the presence of sterols, alkaloids, saponins, tannins, and flavonoids. Nymphayol (25,26-dinorcholest-5-en-3b-ol), a new sterol has been isolated from the successive chloroform extract of the flower. Protein, pentosan, mucilage, and tannins are reported in the seeds. Astragalin, corilagin, gallic acid, gallic acid methyl ester, isokaempferide, kaempferol, quercetin-3-methyl ether, quercetin, 2,3,4,6-tetra-o-galloyl dextroglucose, and 3-o-methylquercetin-3'-o-beta dextroxylopyranoside have been identified in the flowers. The HPTLC method for quantitative determination of gallic acid from hydroalcoholic dried flower extract has been reported. The leaves and shoots of *N. nouchali* (Red water lily) have been studied for their chemical composition. The proximate analysis showed dry matter -8.4%, crude protein-16.8, ash-18.7, crude fat-2.8, crude fiber-26.3, and nitrogen free extract-35.4 for *N. nouchali*. Mineral content showed sodium-1.19, potassium-2.23, calcium-0.52, phosphorus-0.32, and calcium / phosphorus ratio 1.63 for N. nouchali.[13]



Gallic acid



Corilagin

Uses: (a) Rhizome along with refined form of gur (Nabata) and roots of Lawsonia inermis grinded in rice washed water is taken to cure diabetes.

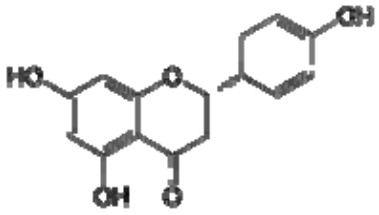
(b) Flowers are socked in water overnight; decanted water is drunk for various cardiac problems.

(c) Seed decoction soaked in cloth is applied for the treatment of skin infection.

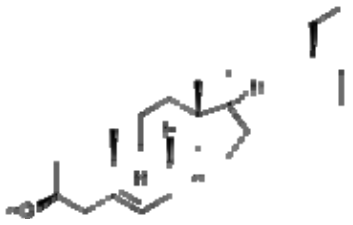
(d) Raw rhizome is the best medicine for dysentery.

C. *Nymphaea caerulea* or Blue Egyptian water lily

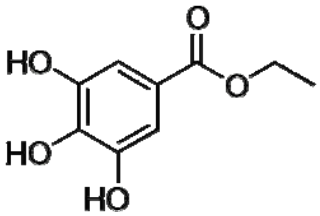
Constituents: As part of an ongoing search for antioxidants from medicinal plants, 20 constituents were isolated from the *Nymphaea caerulea* flowers, including two 2S,3S,4S-trihydroxypentanoic acid, and myricetin 3-O-(3′′-O-acetyl)-α-l-rhamnoside, along with the known myricetin 3-O-α-l-rhamnoside, myricetin 3-O-β-d-glucoside, quercetin 3-O-(3′′-O-acetyl)-α-l-rhamnoside, quercetin 3-O-α-l-rhamnoside, quercetin 3-O-β-d-glucoside, kaempferol 3-O-(3′′-O-acetyl)-α-l-rhamnoside, kaempferol 3-O-β-d-glucoside, naringenin, (S)-naringenin 5-O-β-d-glucoside, isosalipurposide, β-sitosterol, β-sitosterol palmitate, 24-methylenecholesterol palmitate, 4α-methyl-5α-ergosta-7,24(28)-diene-3β,4β-diol, ethyl gallate , gallic acid, p-coumaric acid, and 4-methoxybenzoic acid.[14,15]



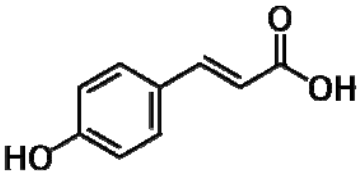
Naringenin



β-Sitosterol



Ethyl gallate



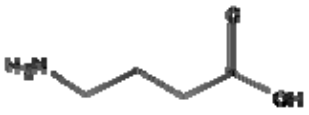
Coumaric acid

Uses: (a) Recent studies have shown Nymphaea caerulea to have mild psycho-active properties.

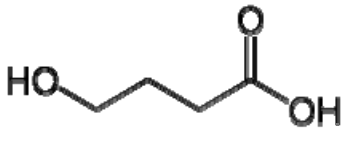
(b) It can act as a mild sedative.

D. *Nymphaea lotus* or White Lotus:

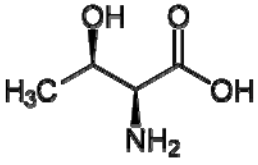
Constituents: Compounds isolated from *N. lotus* are amino butanoic acid, threonine. 4-hydroxy butanoic acid, tyrosine, threonine, arginine, valine, leucine, D and L isoleucine, aspartic acid, phenyl alanine.[16]



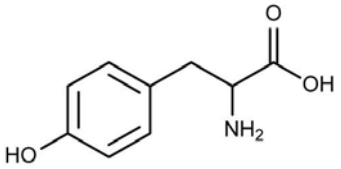
4- Amino butanoic acid



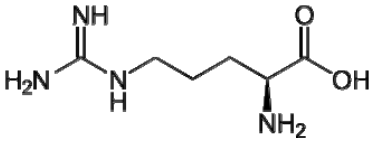
4-hydroxy butanoic acid



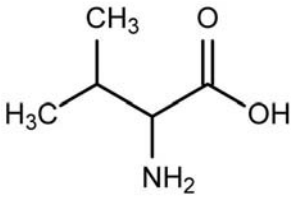
Threonine



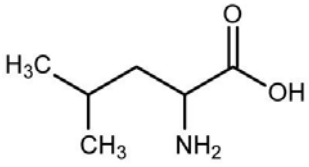
Tyrosine



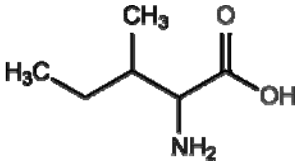
Arginine



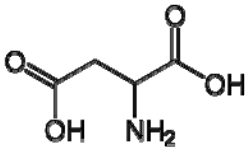
Valine



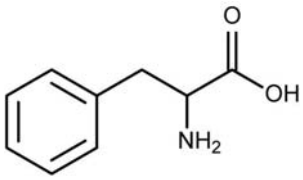
Leucin



Isoleucine



Aspartic acid



Phenyl alanine

Uses: (a) White lotus was used in ancient Egypt, as a key to good health, sex and re-birth.

(b) The plant is an aphrodisiac for both men and women and a general remedy for all kind of illnesses.

(c) Continued use of tiger lotus enhances sexual vigor and general good health.

(d) It is a tonic richer than ginseng, pain reliever richer than arnica, circulation stimulant richer than ginkgo biloba and sexual stimulant richer than Viagra.

(e) The flowers of white lotus are used for preparing tea that creates a warm, euphoric glow.

(f) The dried flowers are smoked by themselves or mixed with other herbs to add flavor to smoking mixtures.

(g) The effects of tiger lotus are enhanced when soaked in wine or other alcohol.

(h) The plant is effectively used to increase memory and create a feeling of euphoria and ecstasy, without the use of narcotics.

(i) Its rhizomes are cooling, sweet, bitter and tonic and used in diarrhea, dysentery, dyspepsia and general debility.

(j) White lotus is used internally in treatment of gastrointestinal disorders and jaundice.

(k) The leaves are used in cutaneous, subcutaneous parasitic infection, eye treatments and pregnancy.

(l) The seeds are used in sauces, condiments, spices and flavorings.

E. *Nymphaea odorata* or White water lily

Constituents: Assay-guided fractionation of the ethanol extract of Nymphaea odorata resulted in the identification of kaempferol 3-O-α-l-rhamnopyranoside, quercetin 3-O-α-l-rhamnopyranoside, myricetin 3-O-α-l-rhamnopyranoside, quercetin 3-O-(6‘‘-O-acetyl)-β-d-galactopyranoside, myricetin 3-O-β-d-galactopyranoside, and myricetin 3-O-(6‘‘-O-acetyl)-β-d-galactopyranoside.[17]

Uses: (a) The root is alterative, anodyne, antiseptic, astringent and demulcent.

(b) A tea made from the roots is used in the treatment of TB, chronic bronchial complaints, diarrhoea, dysentery, gastrointestinal inflammation, gonorrhoea, vaginal discharge, inflamed glands, mouth sores and to stop bleeding.

(c) A poultice made from the roots is used in the treatment of swellings, boils, tumours, inflamed skin, vaginitis etc.

(d) The roots are harvested in the autumn once the plant has died down, and are dried for later use.

(e) A complete cure of uterine cancer by a decoction and uterine injection has been recorded.

F. *Nymphaea alba* or White Lotus Waterlilly

Constituents: Proximate analysis revealed that the plant has dry matter below 10%. Crude protein content of *Nymphaea* is 20.28%, Ash content varies from 8%, Calcium content is 1.10% and Fiber content is about 21%.[18]

Uses: (a) White Lotus is considered to be an astringent, antiseptic, anesthetic, anaphrodisiac and sedative.

(b) It can be used in treatment of bronchial congestion, and it can disperse the stagnated mucus from the chest.

(c) A decoction made from the root is beneficial in treatment of diarrhea caused by irritable bowel syndrome, and also in treatment of dysentery.

(d) White Lotus flowers are considered to reduce sexual drive and to have a soothing and sedative effect on the nervous system. Therefore, they can be used in treatments of insomnia and different sorts of anxieties.

G. *Nymphaea pubescens* or Hairy Water Lily

Constituents: The leaves and rhizomes contain an abundant amount of tannic acid, an alkaloid resembling nupharin, glucose, metaarabic acid, starch, fat; and ash. The leaves contain myricitin, saccharose and phytosterin.

Uses: (a) The seeds, long stalk of flowers and rhizomes are edible; eaten boiled or roasted. (b) Flowers stalks considered an excellent source of iron and a fair source of calcium. (c) Juice is astringent; decoction of the juice used as injection for gonorrhea. (d) Plant juice considered mildly narcotic, rubbed on the forehead and temples to induce sleep.

(e) Powdered roots used as demulcent for piles; also for dysentery and dyspepsia. (f) Powdered root prescribed for piles as demulcent; also for dysentery and dyspepsia. (g) Flowers used as astringent and cardiotonic. (h) In Bangladesh, the roots used by the traditional healers of the Tripura, Marma and Murong tribes to treat dysuria, urinary tract infections and leucorrhea. Also, used for indigestion, heart diseases, stomachaches, cancer, and as anti-hemorrhagic. (i) In Nepal and India flowers used in treatment of diabetes.

(j) In Ayurveda and Siddha systems of medicines, used for diabetes, liver disorders, urinary problems, menorrhagia, blenorragia; also used as tonic and aphrodisiac.

3.2. *Nymphaea pubescens*

The phytochemical screening of ethanol extract of *Nymphaea pubescens* revealed the presence of alkaloids, flavonoids, glycosides, terpenoids, tannins, phenols, saponins and steroids. Acute toxicity study revealed the non-toxic nature of the ethanol extract *Nymphaea pubescens* tuber. The hypoglycaemic effect of *Nymphaea pubescens* tuber was found to be inducing insulin release from pancreatic cells of diabetic. *Nymphaea pubescens* tuber extract can reduce the levels of serum urea and creatinine and confirms the protection of vital tissues (Kidney and liver) including the pancreas, thereby reducing the causation of diabetes in the experimental animals. Ethanol extract of *Nymphaea pubescens* tuber offers a promising therapeutic value in prevention of diabetes. These effects could be mainly attributed to its antioxidant properties as shown by significant quenching impact on the extract of lipid peroxidation along with, enhancement of antioxidant defense systems in pancreatic tissue. The antioxidative property of *Nymphaea pubescens* extract certainly is due to its chemical constituents. Phytochemical investigations of *Nymphaea pubescens* have demonstrated the presence of flavonoids and phenolic compounds as main active ingredients having potent antioxidant activities. Further studies will be needed in future to determine the main active ingredient having the beneficial antidiabetic, hypolipidaemic and antioxidant effects.[20]

Chapter 4

Materials and Methods

4. Materials and Methods

4.1. Drugs and Chemicals

Acetic acid was obtained from Sigma-Aldrich Laborchemikalien Gmbh, Germany. Sodium chloride for normal saline was obtained from Merck, Germany. NaOH was obtained from BDH chemicals, UK. Micrometer screw gauge was obtained from BMA Bhaban. Organic solvents were obtained from Merck, Germany. Dimethyl Sulfoxide (DMSO) obtained from Sigma-Aldrich Laborchemikalein Gmbh, Germany.

Penicillin, Amoxicillin were obtained from Benex limited, USA. Nutrient agar (NA) was obtained from Techno Pharmchem, Bahdurgarh, India. TLC were run on Merck TLC plates precoated with $Si_{60}\,F_{254}$ with visualization by spraying with 1:9 $H_2\,SO_4$ in MeOH and heating. Open column chromatography was done by using Silica gel 60 (0.063-0.020mm), Merck, Germany. The UV Visible spectrum was obtained by using an SHIMADZU UV-1700 Pharma Spec UV-VIS Spectrophotometer. The proton NMR spectra were recorded on an Ultra shield Bruker DPX 400 spectrometer. The NMR spectra were recorded running gradients and using residual solvent peaks as internal reference.

4.2. Equipments

Spatula, Small beaker, Distilled water, Analytical balance, 1000ml beaker, Mortar and Pestle, Aluminum foils paper, 5ml vial, Distilled water, Autoclave, Hot air oven, Laminar flow cabinet, Loop, Burner, Micropipettes, Micropipettes tip, Petri dishes, Microorganism spreading glass rod, TLC tank, UV lamp, Hot plate, Rotary evaporator, Vacuum pump.

4.3. Solvents

Hexanes, Ethyl acetate, Dichloromethane, Acetone, Methanol, Ethanol, Distilled water, Dimethylsulfoxide (DMSO), Acetic acid, Sulfuric acid.

4.4. Plant materials and Extraction

Nymphaea pubescens leaves were collected from Panchagar in 17th August, 2011 and identified by National herbarium of Bangladesh, where a voucher specimen was conserved under the reference number DACB Accession No. 35362. The leaves were washed with water, cut into pieces, sun dried for 15 days. The leaves were powdered by using Blinder. 700gm dried powder were extracted by methanol by cold extraction process (2800ml-72hrs-4 times). Solvent from each sample was filtered, and evaporated off under reduced pressure in a rotary evaporator to obtain 122gm of crude extract. A yield of 17.43 of extract was obtained.

4.5. Thin layer chromatography (TLC)

A fresh moisture free 25×25cm 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 1cm upper from the bottom and 1cm 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 dilution of the extract was carefully loaded onto the baseline of the TLC plates with the help of small capillary tube and the sample was allowed to dry. The solvent system was ethyl acetate and methanol in 5:1 ratio used as mobile phases. 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 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°C for the observation of organic compounds. After detection individual compounds, the Rf value and color of each compound was noted in the table.

Table 1

Thin layer chromatography (TLC)

Plant Species	Compound	Mobile Phase	Total spot	Spot	Rf Value	Observation Under necked eye	UV Observation	Observation after charring	Quantity
Leaf of *Nymphaea pubescens*	MeOH Extract	EtAc:MeOH (5:1)	3	1	0.83	Red	Red	Black	***
				2	0.33	–	Light violet	–	*
				3	0.16	–	Yellow	–	**

– =No color, *** = Huge amount, ** =Moderate amount. * =small amount.

4.6. According to antimicrobial activity separation of the MeOH extract by Vacuum Liquid Chromatography (VLC)

The technique can be considered as preparative TLC run as a column, with a vacuum provided to speed up eluent flow rates. It differs from flash chromatography in that the column is allowed to run dry after each fraction is collected. This is similar to preparative TLC because plates can be dried after a run and then re-eluted.

A short column or a Buchner filter funnel fitted with glass frit (10-20 μm, porosity D or porosity 2) is dry packed with sorbent (10-40 μm of TLC grade, e.g., Merck 60H or 60G Silica gel). The sorbent is allowed to settle by gentle tapping under gravity. Then vacuum is applied and the sorbent compressed to a hard layer by pressing with a rubber stopper and tapping. 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. Alternatively the sample is preadsorbed on silica gel. The column is developed with appropriate solvents mixtures, starting with solvent of low polarity and gradually increasing the polarity, pulling the column dry between each fraction collected.

Figure 2: Vaccum Liquid Chromatography

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

For VLC, 115.7gm of crude extract was mixed with 30gm silica gel to form dry slurry, which was then loaded onto a VLC column filled with silica gel as stationary phase.

The column was eluted stepwise under vacuum with solvents. The solvents used were n-Hexane, dichloromethane (DCM), ethyl acetate, acetone, and methanol. The solvents were eluted until it ran clear of the column. Eluant fractions of 5000ml each were collected and numbered from 1 to 5. The mass of the eluted fractions was determined after the solvents were evaporated in a rotary evaporator.

Table 2

Fractions of VLC

Fraction	1	2	3	4	5
Solvent	n-hexane	DCM	Ethyl Acetate	Acetone	Methanol
Amount (gm)	7.38	3.28	34.66	79.3	14.27

4.6.1. TLC on VLC fractions

The fractions obtained from the VLC were analyzed by TLC. A fresh moisture free 25×25cm 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 1cm upper from the bottom and 1cm lower from the upper side with pencil. Approximately 0.5-2 mg of the extract from each fractions were diluted with 1 ml of the appropriate solvent then, approximately 2-6 (µl) aliquot of dilution of the extract was carefully loaded onto the baseline of the TLC plates with the help of small capillary tube and the sample was allowed to dry. The solvent system was ethyl acetate and n-Hexane in 8:2 ratio used as mobile phases. 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 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°C for the observation of organic compounds. After detection individual compounds, the Rf value and color of each compound was noted in the table.

Table 3

TLC on VLC fractions

Fraction	Total spot	Spot	RF value	Observation under necked eye	UV observation	Observation after charring	Quantity
1	3	1	0.11	Brown		-	*
		2	0.77	-	Red	-	*
		3	0.91	-	Red	-	*
2	3	1	0.11	Brown	-	-	*
		2	0.77	Light green	-	-	*
		3	0.88	Red	-	Red dark	***
3	6	1	0.11	Brown	-	-	***
		2	0.22	-	Dark	-	*
		3	0.44	-	Black	-	*
		4	0.66	-	-	Dark Black	*
		5	0.77	-	Black	-	*
		6	0.93	-	Dark	-	*
4	2	1	0.11	Brown	-	-	**
		2	0.93	-	Red	-	*
5	1	1	0.11	Brown	-	-	*

– =No color, *** = Huge amount, ** =Moderate amount. * =small amount

4.7. According to antimicrobial activity separation of the fraction extract obtained from Vacuum Liquid Chromatography (VLC) by Column Chromatography

Column chromatography is suitable for the physical separation of gram quantities of material. A solvent acts as the mobile phase while a finely divided solid surface acts as the stationary phase. The stationary phase will adsorb the components of the mixture to varying degrees. As the solution containing the mixture passes over the adsorbent, the components are distributed between the solvent and adsorbent surface. This process may be described by three-way equilibrium between the sample, the solvent and the adsorbent.

The solvent and sample compete for positions on the solid adsorbent, the solvent displacing the sample reversibly and continuously in the direction of the solvent flow. Consequently, a weakly adsorbed compound will spend more time in the solvent, and will therefore be eluted first.

Figure 3: Column Chromatography

The best conditions for running the separation may be determined by experimental TLC. This will indicate the best adsorbent and the best solvent for the separation. The most important experimental consideration is the preparation of the packed column. The packing should be homogeneous and should not contain trapped air or vapor bubbles. The trapped gasses lead to channeling through the adsorbent, and a loss of resolution.

4.7.1. Column 1 (NP-CC-1)

Column 1 was run using the n-hexane soluble component obtained from VLC fraction 1. 6.5831gm sample was taken for column. 115gm silica (Silica gel 60, 0.063-0.200 mm, 70-230 mesh ASTM) was taken to make stationary phase. Column length was 46cm and diameter was 2.6cm. The initial mobile phase was 100% n-hexane (non-polar). After collecting 30 fractions, the gradient mobile phase was used and the mobile phase was n-hexane and ethyl acetate in a ratio of 9.5:0.5. After collecting next 10 fractions again the composition of the mobile phase was changed in a ratio of 9:1 of n-hexane and ethyl acetate. Then collecting next 10 fractions the mobile phase composition was 8:2. Further collection of 10 fractions was done and changed the mobile phase composition in 6:4 ratios. Next 20 fractions were collected in a composition of 5:5. Final 10 fractions were collected using a mobile phase of 100% ethyl acetate as the eluent was incrementally lowered. Finally methanol was used as mobile phase to clear the column. So in total 100 fractions were collected from column 1. All the eluents were stored and labeled in vial. Crystal was found in 40 no fraction and the amount was 17mg.

Fractions of Column 1 (NP-CC-1)

Fraction	Mobile phase	Amount of collection (ml)
1	n-hexane (100%)	100
2	n-hexane (100%)	100
3	n-hexane (100%)	100
4	n-hexane (100%)	100
5	n-hexane (100%)	100
6	n-hexane (100%)	100
7	n-hexane (100%)	100
8	n-hexane (100%)	100
9	n-hexane (100%)	100
10	n-hexane (100%)	100
11	n-hexane (100%)	100
12	n-hexane (100%)	100
13	n-hexane (100%)	100
14	n-hexane (100%)	100
15	n-hexane (100%)	100
16	n-hexane (100%)	100
17	n-hexane (100%)	100
18	n-hexane (100%)	100
19	n-hexane (100%)	100
20	n-hexane (100%)	100
21	n-hexane (100%)	100
22	n-hexane (100%)	100
23	n-hexane (100%)	100
24	n-hexane (100%)	100
25	n-hexane (100%)	100
26	n-hexane (100%)	100
27	n-hexane (100%)	100
28	n-hexane (100%)	100
29	n-hexane (100%)	100

30	n-hexane (100%)	100
31	n-hexane: Ethyl acetate (9.5:0.5)	100
32	n-hexane: Ethyl acetate (9.5:0.5)	100
33	n-hexane: Ethyl acetate (9.5:0.5)	100
34	n-hexane: Ethyl acetate (9.5:0.5)	100
35	n-hexane: Ethyl acetate (9.5:0.5)	100
36	n-hexane: Ethyl acetate (9.5:0.5)	100
37	n-hexane: Ethyl acetate (9.5:0.5)	100
38	n-hexane: Ethyl acetate (9.5:0.5)	100
39	n-hexane: Ethyl acetate (9.5:0.5)	100
40	n-hexane: Ethyl acetate (9.5:0.5)	100
41	n-hexane: Ethyl acetate (9:1)	100
42	n-hexane: Ethyl acetate (9:1)	100
43	n-hexane: Ethyl acetate (9:1)	100
44	n-hexane: Ethyl acetate (9:1)	100
45	n-hexane: Ethyl acetate (9:1)	100
46	n-hexane: Ethyl acetate (9:1)	100

47	n-hexane: Ethyl acetate (9:1)	100
48	n-hexane: Ethyl acetate (9:1)	100
49	n-hexane: Ethyl acetate (9:1)	100
50	n-hexane: Ethyl acetate (9:1)	100
51	n-hexane: Ethyl acetate (8:2)	100
52	n-hexane: Ethyl acetate (8:2)	100
53	n-hexane: Ethyl acetate (8:2)	100
54	n-hexane: Ethyl acetate (8:2)	100
55	n-hexane: Ethyl acetate (8:2)	100
56	n-hexane: Ethyl acetate (8:2)	100
57	n-hexane: Ethyl acetate (8:2)	100
58	n-hexane: Ethyl acetate (8:2)	100
59	n-hexane: Ethyl acetate (8:2)	100
60	n-hexane: Ethyl acetate (8:2)	100
61	n-hexane: Ethyl acetate (6:4)	100
62	n-hexane: Ethyl acetate (6:4)	100
63	n-hexane: Ethyl	100

	acetate (6:4)	
64	n-hexane: Ethyl acetate (6:4)	100
65	n-hexane: Ethyl acetate (6:4)	100
66	n-hexane: Ethyl acetate (6:4)	100
67	n-hexane: Ethyl acetate (6:4)	100
68	n-hexane: Ethyl acetate (6:4)	100
69	n-hexane: Ethyl acetate (6:4)	100
70	n-hexane: Ethyl acetate (6:4)	100
71	n-hexane: Ethyl acetate (5:5)	100
72	n-hexane: Ethyl acetate (5:5)	100
73	n-hexane: Ethyl acetate (5:5)	100
74	n-hexane: Ethyl acetate (5:5)	100
75	n-hexane: Ethyl acetate (5:5)	100
76	n-hexane: Ethyl acetate (5:5)	100
77	n-hexane: Ethyl acetate (5:5)	100
78	n-hexane: Ethyl acetate (5:5)	100
79	n-hexane: Ethyl acetate (5:5)	100

80	n-hexane: Ethyl acetate (5:5)	100
81	n-hexane: Ethyl acetate (5:5)	100
82	n-hexane: Ethyl acetate (5:5)	100
83	n-hexane: Ethyl acetate (5:5)	100
84	n-hexane: Ethyl acetate (5:5)	100
85	n-hexane: Ethyl acetate (5:5)	100
86	n-hexane: Ethyl acetate (5:5)	100
87	n-hexane: Ethyl acetate (5:5)	100
88	n-hexane: Ethyl acetate (5:5)	100
89	n-hexane: Ethyl acetate (5:5)	100
90	n-hexane: Ethyl acetate (5:5)	100
91	Ethyl acetate (100%)	100
92	Ethyl acetate (100%)	100
93	Ethyl acetate (100%)	100
94	Ethyl acetate (100%)	100
95	Ethyl acetate (100%)	100
96	Ethyl acetate	100

	(100%)	
97	Ethyl acetate (100%)	100
98	Ethyl acetate (100%)	100
99	Ethyl acetate (100%)	100
100	Ethyl acetate (100%)	100

4.7.2. Column 2 (NP-CC-2)

Column 2 was run using the ethyl acetate soluble component obtained from VLC fraction 3. 30.72gm sample was taken for column. 85gm silica (Silica gel 60, 0.063-0.200 mm, 70-230 mesh ASTM) was taken to make stationary phase. Column length was 35cm and diameter was 2.8cm. The initial mobile phase was 100% n-hexane (non-polar). After collecting 40 fractions, the gradient mobile phase was used and the mobile phase was n-hexane and ethyl acetate in a ratio of 9.5:0.5. After collecting next 15 fractions again the composition of the mobile phase was changed in a ratio of 9:1 of n-hexane and ethyl acetate. Then collecting next 15 fractions the mobile phase composition was 8:2. Further collection of 10 fractions was done and changed the mobile phase composition in 6:4 ratios. Next 20 fractions were collected in a composition of 5:5. 60% ethyl acetate and 40% n-hexane mobile phase composition was used for the next 25 fractions. Final 20 fractions were collected using a mobile phase of 100% ethyl acetate as the eluent was incrementally lowered. Finally methanol was used as mobile phase to clear the column. So in total 160 fractions were collected from column 2. All the eluents were stored and labeled in vial. Crystal was found in 22 no fraction and the amount was 23mg.

Fractions of column 2 (NP-CC-2)

Fraction	Mobile phase	Amount of collection (ml)
1	n-hexane (100%)	100
2	n-hexane (100%)	100
3	n-hexane (100%)	100
4	n-hexane (100%)	100

5	n-hexane (100%)	100
6	n-hexane (100%)	100
7	n-hexane (100%)	100
8	n-hexane (100%)	100
9	n-hexane (100%)	100
10	n-hexane (100%)	100
11	n-hexane (100%)	100
12	n-hexane (100%)	100
13	n-hexane (100%)	100
14	n-hexane (100%)	100
15	n-hexane (100%)	100
16	n-hexane (100%)	100
17	n-hexane (100%)	100
18	n-hexane (100%)	100
19	n-hexane (100%)	100
20	n-hexane (100%)	100
21	n-hexane (100%)	100
22	n-hexane (100%)	100
23	n-hexane (100%)	100
24	n-hexane (100%)	100
25	n-hexane (100%)	100
26	n-hexane (100%)	100
27	n-hexane (100%)	100
28	n-hexane (100%)	100
29	n-hexane (100%)	100
30	n-hexane (100%)	100
31	n-hexane (100%)	100
32	n-hexane (100%)	100
33	n-hexane (100%)	100
34	n-hexane (100%)	100
35	n-hexane (100%)	100
36	n-hexane (100%)	100

37	n-hexane (100%)	100
38	n-hexane (100%)	100
39	n-hexane (100%)	100
40	n-hexane (100%)	100
41	n-hexane: Ethyl acetate (9.5:0.5)	100
42	n-hexane: Ethyl acetate (9.5:0.5)	100
43	n-hexane: Ethyl acetate (9.5:0.5)	100
44	n-hexane: Ethyl acetate (9.5:0.5)	100
45	n-hexane: Ethyl acetate (9.5:0.5)	100
46	n-hexane: Ethyl acetate (9.5:0.5)	100
47	n-hexane: Ethyl acetate (9.5:0.5)	100
48	n-hexane: Ethyl acetate (9.5:0.5)	100
49	n-hexane: Ethyl acetate (9.5:0.5)	100
50	n-hexane: Ethyl acetate (9.5:0.5)	100
51	n-hexane: Ethyl acetate (9.5:0.5)	100
52	n-hexane: Ethyl acetate (9.5:0.5)	100
53	n-hexane: Ethyl acetate (9.5:0.5)	100
54	n-hexane: Ethyl acetate (9.5:0.5)	100
55	n-hexane: Ethyl	100

	acetate (9.5:0.5)	
56	n-hexane: Ethyl acetate (9:1)	100
57	n-hexane: Ethyl acetate (9:1)	100
58	n-hexane: Ethyl acetate (9:1)	100
59	n-hexane: Ethyl acetate (9:1)	100
60	n-hexane: Ethyl acetate (9:1)	100
61	n-hexane: Ethyl acetate (9:1)	100
62	n-hexane: Ethyl acetate (9:1)	100
63	n-hexane: Ethyl acetate (9:1)	100
64	n-hexane: Ethyl acetate (9:1)	100
65	n-hexane: Ethyl acetate (9:1)	100
66	n-hexane: Ethyl acetate (9:1)	100
67	n-hexane: Ethyl acetate (9:1)	100
68	n-hexane: Ethyl acetate (9:1)	100
69	n-hexane: Ethyl acetate (9:1)	100
70	n-hexane: Ethyl acetate (9:1)	100
71	n-hexane: Ethyl acetate (8:2)	100

72	n-hexane: Ethyl acetate (8:2)	100
73	n-hexane: Ethyl acetate (8:2)	100
74	n-hexane: Ethyl acetate (8:2)	100
75	n-hexane: Ethyl acetate (8:2)	100
76	n-hexane: Ethyl acetate (8:2)	100
77	n-hexane: Ethyl acetate (8:2)	100
78	n-hexane: Ethyl acetate (8:2)	100
79	n-hexane: Ethyl acetate (8:2)	100
80	n-hexane: Ethyl acetate (8:2)	100
81	n-hexane: Ethyl acetate (6:4)	100
82	n-hexane: Ethyl acetate (6:4)	100
83	n-hexane: Ethyl acetate (6:4)	100
84	n-hexane: Ethyl acetate (6:4)	100
85	n-hexane: Ethyl acetate (6:4)	100
86	n-hexane: Ethyl acetate (6:4)	100
87	n-hexane: Ethyl acetate (6:4)	100
88	n-hexane: Ethyl	100

	acetate (6:4)	
89	n-hexane: Ethyl acetate (6:4)	100
90	n-hexane: Ethyl acetate (6:4)	100
91	n-hexane: Ethyl acetate (5:5)	100
92	n-hexane: Ethyl acetate (5:5)	100
93	n-hexane: Ethyl acetate (5:5)	100
94	n-hexane: Ethyl acetate (5:5)	100
95	n-hexane: Ethyl acetate (5:5)	100
96	n-hexane: Ethyl acetate (5:5)	100
97	n-hexane: Ethyl acetate (5:5)	100
98	n-hexane: Ethyl acetate (5:5)	100
99	n-hexane: Ethyl acetate (5:5)	100
100	n-hexane: Ethyl acetate (5:5)	100
101	n-hexane: Ethyl acetate (5:5)	100
102	n-hexane: Ethyl acetate (5:5)	100
103	n-hexane: Ethyl acetate (5:5)	100
104	n-hexane: Ethyl acetate (5:5)	100

105	n-hexane: Ethyl acetate (5:5)	100
106	n-hexane: Ethyl acetate (5:5)	100
107	n-hexane: Ethyl acetate (5:5)	100
108	n-hexane: Ethyl acetate (5:5)	100
109	n-hexane: Ethyl acetate (5:5)	100
110	n-hexane: Ethyl acetate (5:5)	100
111	n-hexane: Ethyl acetate (4:6)	100
112	n-hexane: Ethyl acetate (4:6)	100
113	n-hexane: Ethyl acetate (4:6)	100
114	n-hexane: Ethyl acetate (4:6)	100
115	n-hexane: Ethyl acetate (4:6)	100
116	n-hexane: Ethyl acetate (4:6)	100
117	n-hexane: Ethyl acetate (4:6)	100
118	n-hexane: Ethyl acetate (4:6)	100
119	n-hexane: Ethyl acetate (4:6)	100
120	n-hexane: Ethyl acetate (4:6)	100
121	n-hexane: Ethyl	100

	acetate (4:6)	
122	n-hexane: Ethyl acetate (4:6)	100
123	n-hexane: Ethyl acetate (4:6)	100
124	n-hexane: Ethyl acetate (4:6)	100
125	n-hexane: Ethyl acetate (4:6)	100
126	n-hexane: Ethyl acetate (4:6)	100
127	n-hexane: Ethyl acetate (4:6)	100
128	n-hexane: Ethyl acetate (4:6)	100
129	n-hexane: Ethyl acetate (4:6)	100
130	n-hexane: Ethyl acetate (4:6)	100
131	n-hexane: Ethyl acetate (4:6)	100
132	n-hexane: Ethyl acetate (4:6)	100
133	n-hexane: Ethyl acetate (4:6)	100
134	n-hexane: Ethyl acetate (4:6)	100
135	n-hexane: Ethyl acetate (4:6)	100
136	Ethyl acetate (100%)	100
137	Ethyl acetate (100%)	100

138	Ethyl acetate (100%)	100
139	Ethyl acetate (100%)	100
140	Ethyl acetate (100%)	100
141	Ethyl acetate (100%)	100
142	Ethyl acetate (100%)	100
143	Ethyl acetate (100%)	100
144	Ethyl acetate (100%)	100
145	Ethyl acetate (100%)	100
146	Ethyl acetate (100%)	100
147	Ethyl acetate (100%)	100
148	Ethyl acetate (100%)	100
149	Ethyl acetate (100%)	100
150	Ethyl acetate (100%)	100
151	Ethyl acetate (100%)	100
152	Ethyl acetate (100%)	100
153	Ethyl acetate (100%)	100
154	Ethyl acetate	100

	(100%)	
155	Ethyl acetate (100%)	100
156	Methanol (100%)	100
157	Methanol (100%)	100
158	Methanol (100%)	100
159	Methanol (100%)	100
160	Methanol (100%)	100

4.8. Evaluation of Antibacterial activity:

4.8.1. Microorganisms

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

4.8.2. Culture media

Nutrient Agar (NA) was used for the activation of all bacteria and fungi.

4.8.3. Disc diffusion method

The antimicrobial activity of the tested samples (extracts, isolated compound and reference drugs) was assayed by the standard disc diffusion method. Tested samples were dissolved in methanol and injected into sterilized discs of 6mm in diameter. Nutrient Agar(NA) sterilized at 121°C by using autoclave and cooled to 60–70 °C were distributed to sterilized petri dishes with a diameter of 9 cm (15 ml). After preparation of the suspensions of test organisms (C.F.U 1.6×10 organisms per mL), 100µL of suspension was added to each petri dishes and distributed homogeneously. Dishes injected with tested materials were placed on the solid agar medium. Petri dishes were incubated at 37 ◦C for 24 h. On each plate an appropriate reference antibiotic (Amoxicillin 30µg/disc and penicillin 10µg/disc) disc and control (5µl methanol/disk) disk were applied. At the end of the period, inhibition zones formed on the NA were evaluated in millimeters. Studies were performed in duplicate, and the developing inhibition zones were compared with those of reference disks.

4.8. ^{1}H NMR of n-hexane and Ethyl Acetate Fraction

Column chromatography of n-hexane and ethyl acetate fraction was performed. Crystal was found on the 40th subfraction of hexane and 22nd subfraction of ethyl acetate. Then ^{1}H NMR was performed to determine the structure of the crystals.

Nuclear magnetic resonance (NMR) spectroscopy is a useful tool in elucidating chemical structures. ^{1}H spectra were obtained from Bangladesh Council of Scientific & Industrial Research (BCSIR) on a 400 MHz Varian NMR machine (Bucker Instrument).

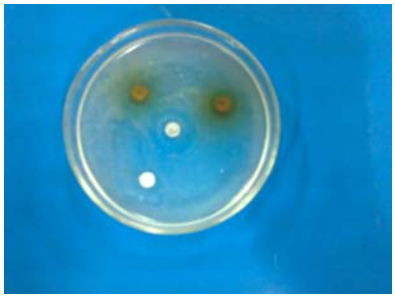
Chapter 5

Results and Discussion

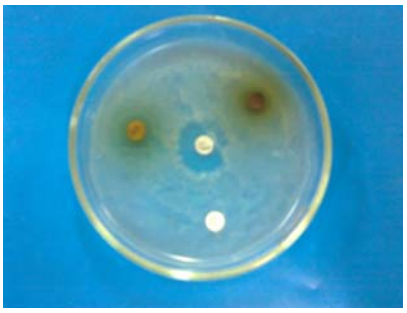
5. Results and Discussion

5.1. Antimicrobial activity

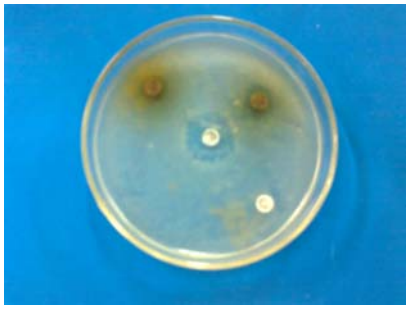
Antimicrobial activity of extract has been evaluated in vitro against four bacterial species and one fungus which are known to cause infections in humans. As summarized in the table, extract showed antimicrobial activity at a dose 1000 µg/disc against tested microorganisms. The highest inhibition zone diameter was 12 mm on *Salmonella typhi*, at a dose of 1000 µg/disk, and the lowest inhibition zone diameter was 8.5 on *Shigella dysenteriae* at a dose of 1000 µg/disk. The Reference drug amoxicillin showed highest zone of inhibition of 15mm on *Pseudomonas aeruginosa*, and no zone of inhibition on *candidas albicans* at 20µg/disk.



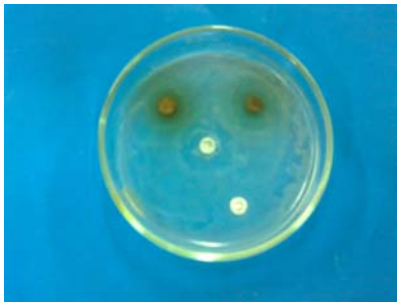
Escherichia coli



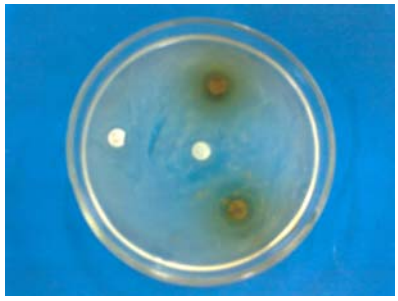
Staphylococcus aureus



Shigella dysenteriae



Salmonella typhi



Candida albicans

Figure 5: Antimicrobial activity of *Nymphaea pubescens*

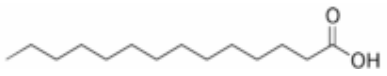
samples	Concentration (µg/disc)	Inhibition zone diameter(mm)				
		Tested microorganisms				
		Escher-ichia coli	*Staphylococ-cus aureus*	*Shigella dysenteri-ae*	*Salmone-lla typhi*	*Candida albicans*
Extract	1000	11	9.5	8.5	12	9
Amoxicillin	20	15	14	12.5	14	□

□ = ***No Inhibition***

5.2. ^{1}H NMR data

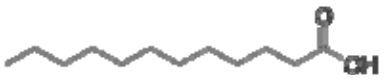
5.2.1. ^{1}H NMR data for 40th subfraction of n-hexane fraction (NPH-40)

^{1}H-NMR of NPH 40 showed characteristic triplet of terminal methyl group at δ 0.86 (t, J= 6.2) and a big methelene envelop at δ 1.25 (m). The integration of the signal of terminal methyl group indicated the presence of four terminal methyl groups. This analysis clearly revealed that NPH-40 is a mixture of 4 fatty acid derivatives. Integration of methelene signal at δ 1.25 is 98 which indicated the number of CH_2 group is 48 in those fatty acids. ^{1}H-NMR of NPH 40 also showed a signal of an olefinic proton at δ 5.34 (s) which indicates the presence of a double bond in one of the fatty acids derivatives. The unsaturated fatty acid contains a methyl group at the double bond which was indicated by the signal at δ 1.61 (s). Thus we propose NPH 40 is a mixture of myristic acid, palmitic acid, lauric acid and 4-methyl-4-tetradecenoic acid.

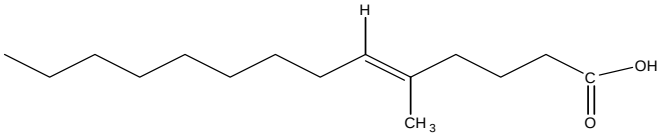


Myristic acid

Palmitic acid

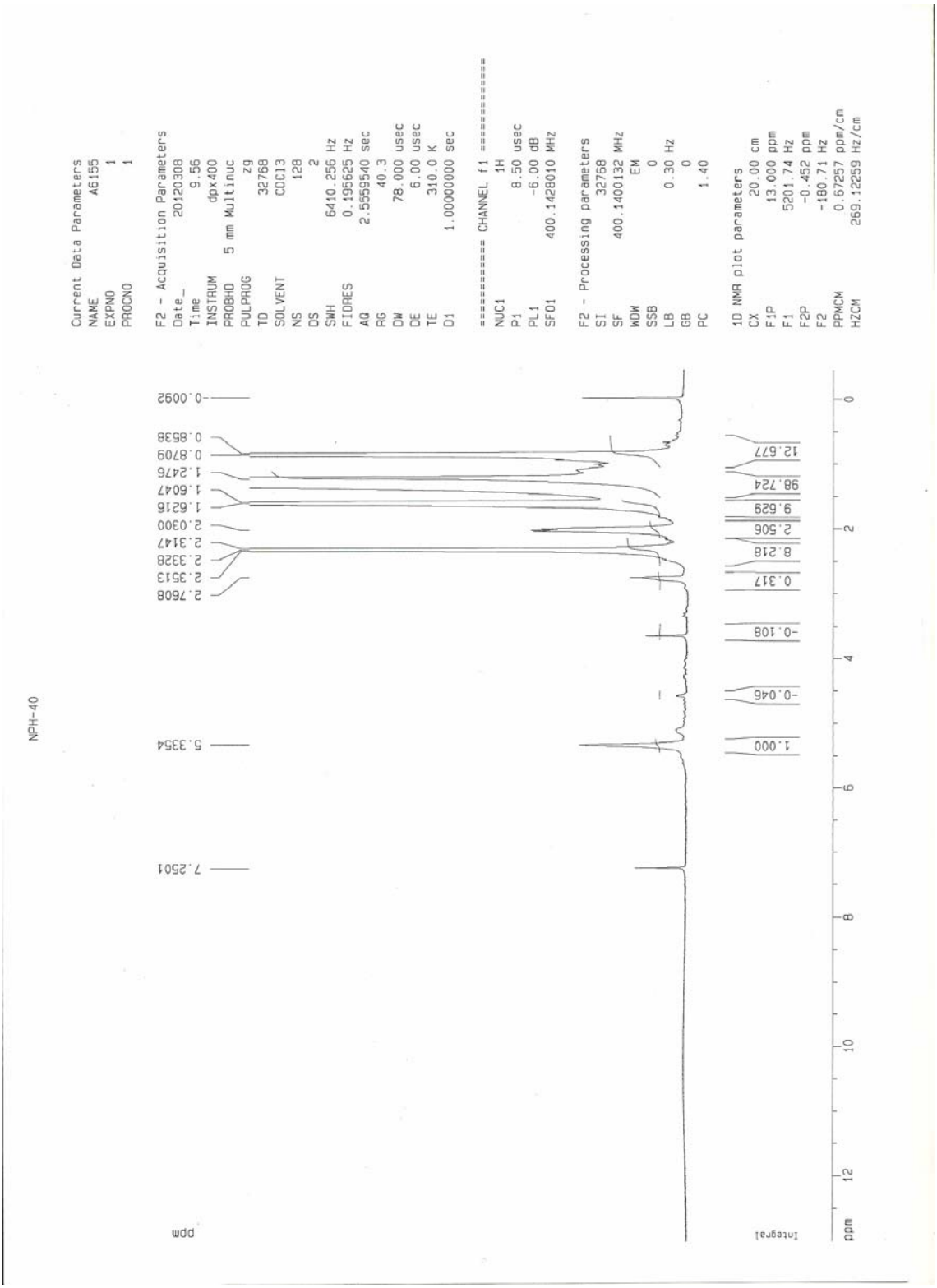


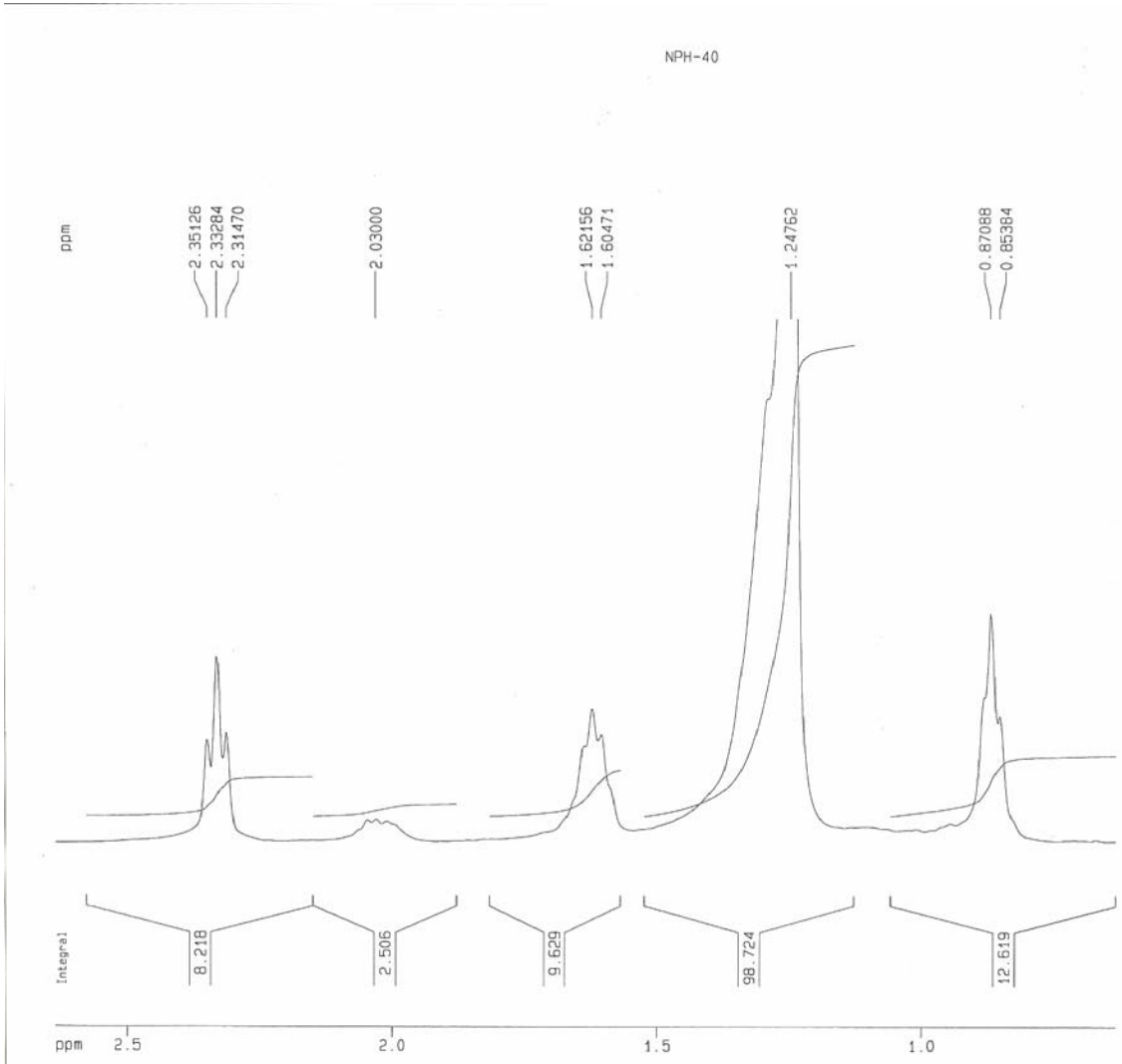
Lauric acid



4-methyl-4-tetradecenoic acid.

The result of nuclear magnetic resonance spectroscopy experiment for the 40th subfraction of n-hexane fraction is presented in figure 6.





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PROCNO	1

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PULPROG	zg
TD	32768
SOLVENT	CDCl3
NS	128
DS	2
SWH	6410.256 Hz
FIDRES	0.195625 Hz
AQ	2.5559540 sec
RG	40.3
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
SF01	400.1428010 MHz

F2 - Processing parameters

SI	32768
SF	400.1400132 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	20.00 cm
F1P	2.636 ppm
F1	1054.61 Hz
F2P	0.633 ppm
F2	253.29 Hz
PPMCM	0.10013 ppm/cm
HZCM	40.06624 Hz/cm

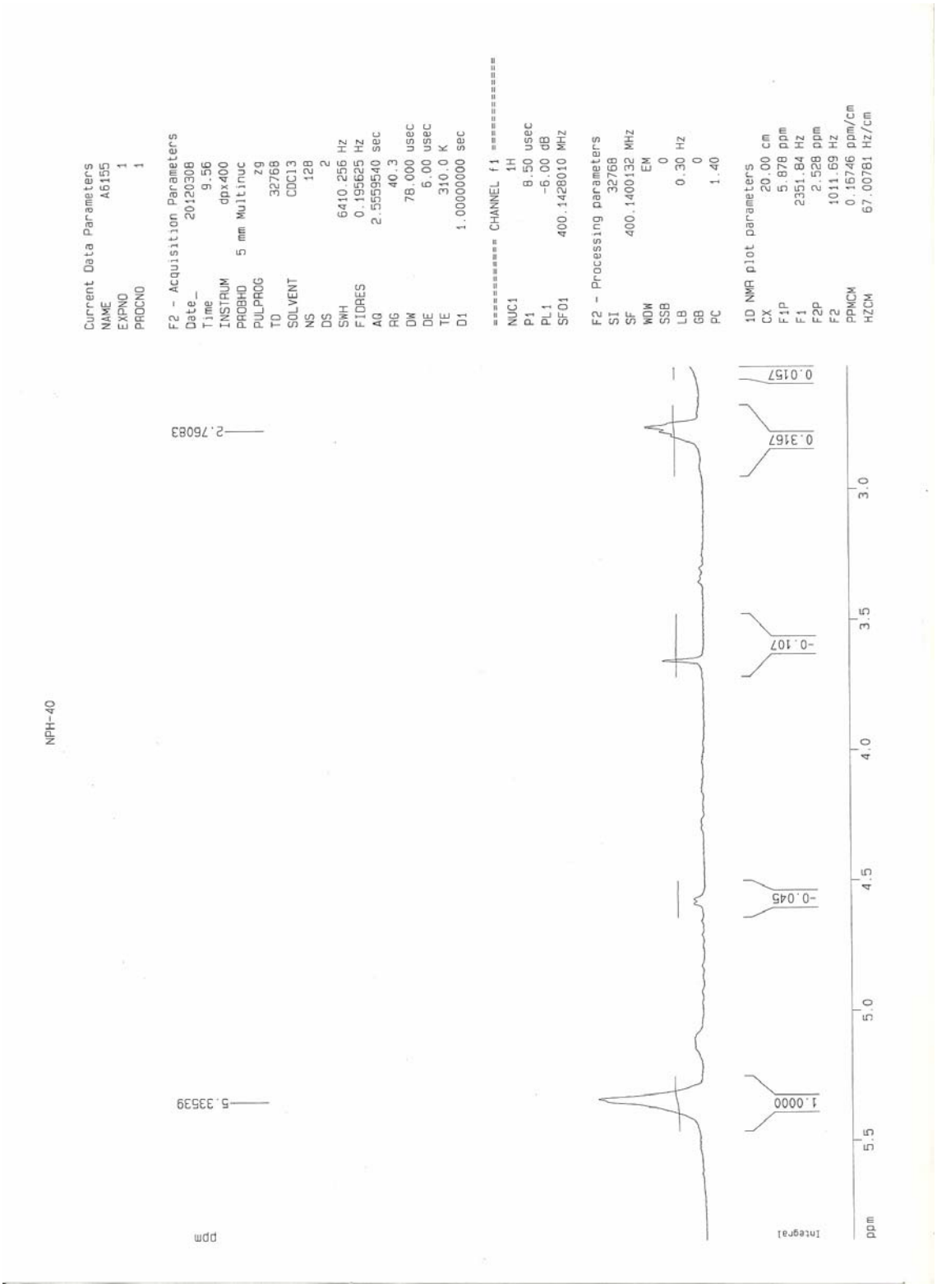
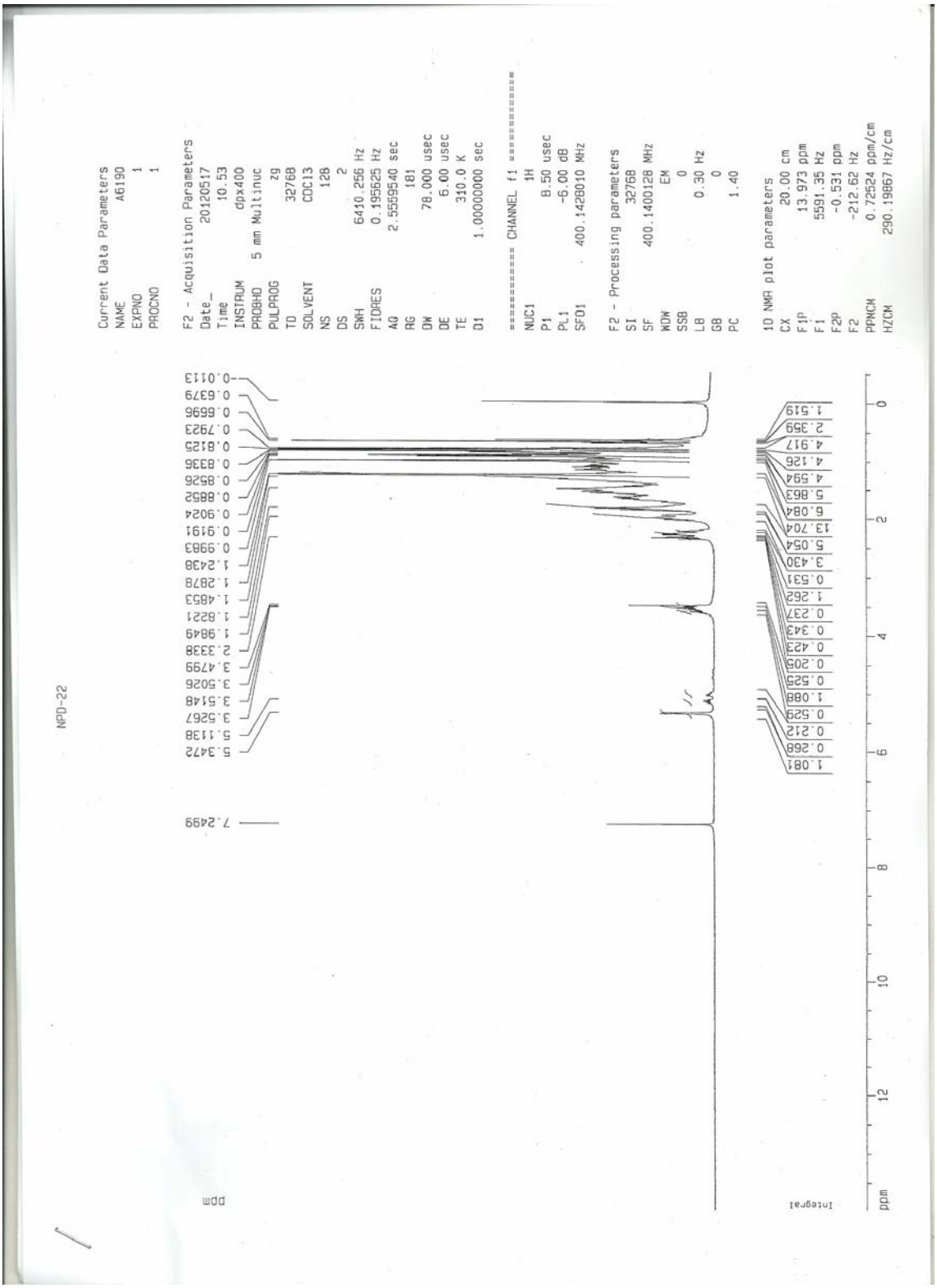
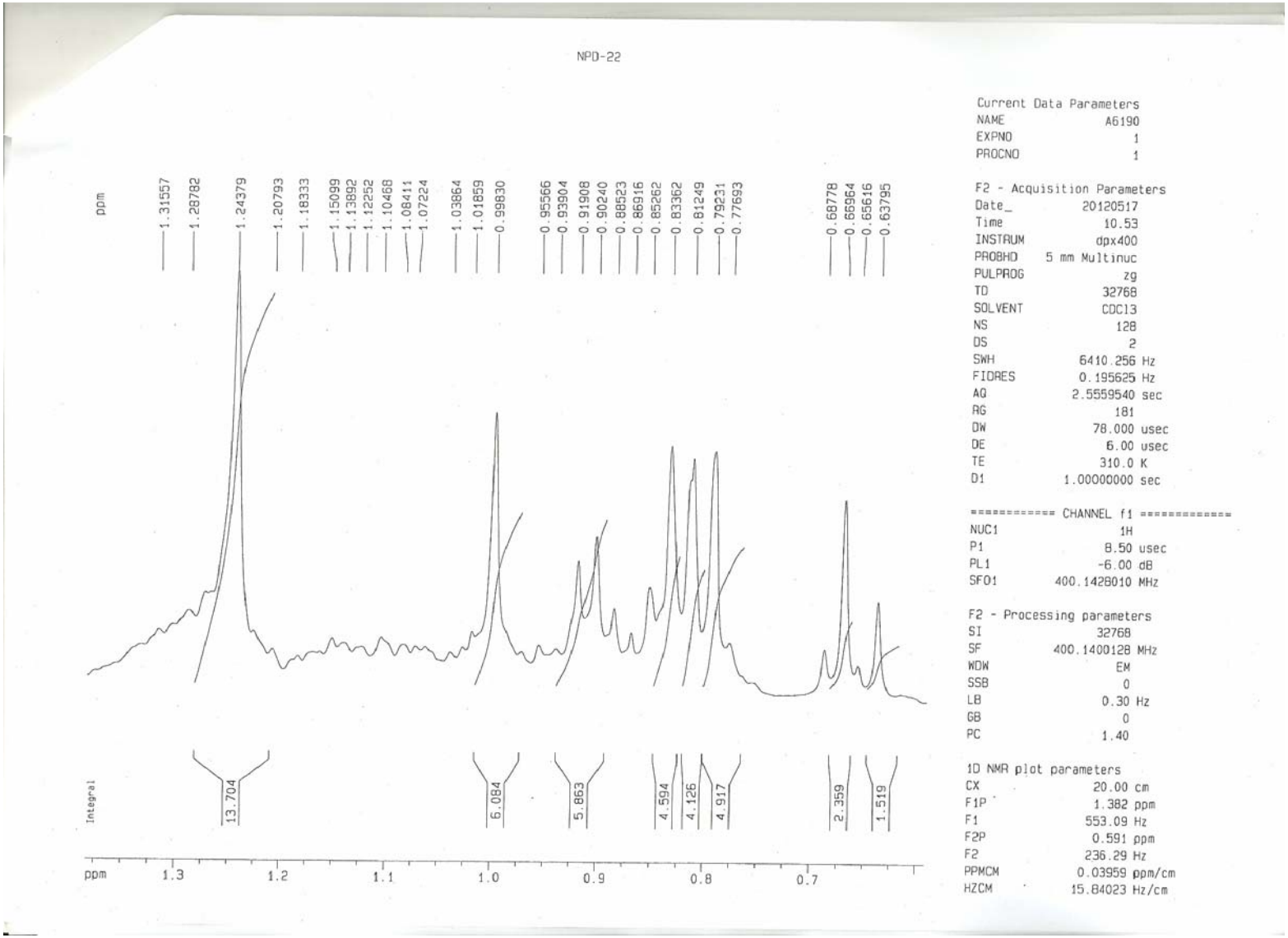


Figure 6: 1^{H} NMR spectrum for the 40^{th} subfraction of n-hexane fraction

5.2.2. ^{1}H NMR data for 22nd subfraction of ethyl acetate fraction (NPE-22)

The result of nuclear magnetic resonance spectroscopy experiment for the 22nd subfraction of ethyl acetate fraction is presented in figure 7.





Current Data Parameters

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PROCNO	1

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PULPROG	zg
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SOLVENT	CDCl3
NS	128
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	400.1428010 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	1.382 ppm
F1	553.09 Hz
F2P	0.591 ppm
F2	236.29 Hz
PPMCM	0.03959 ppm/cm
HZCM	15.84023 Hz/cm

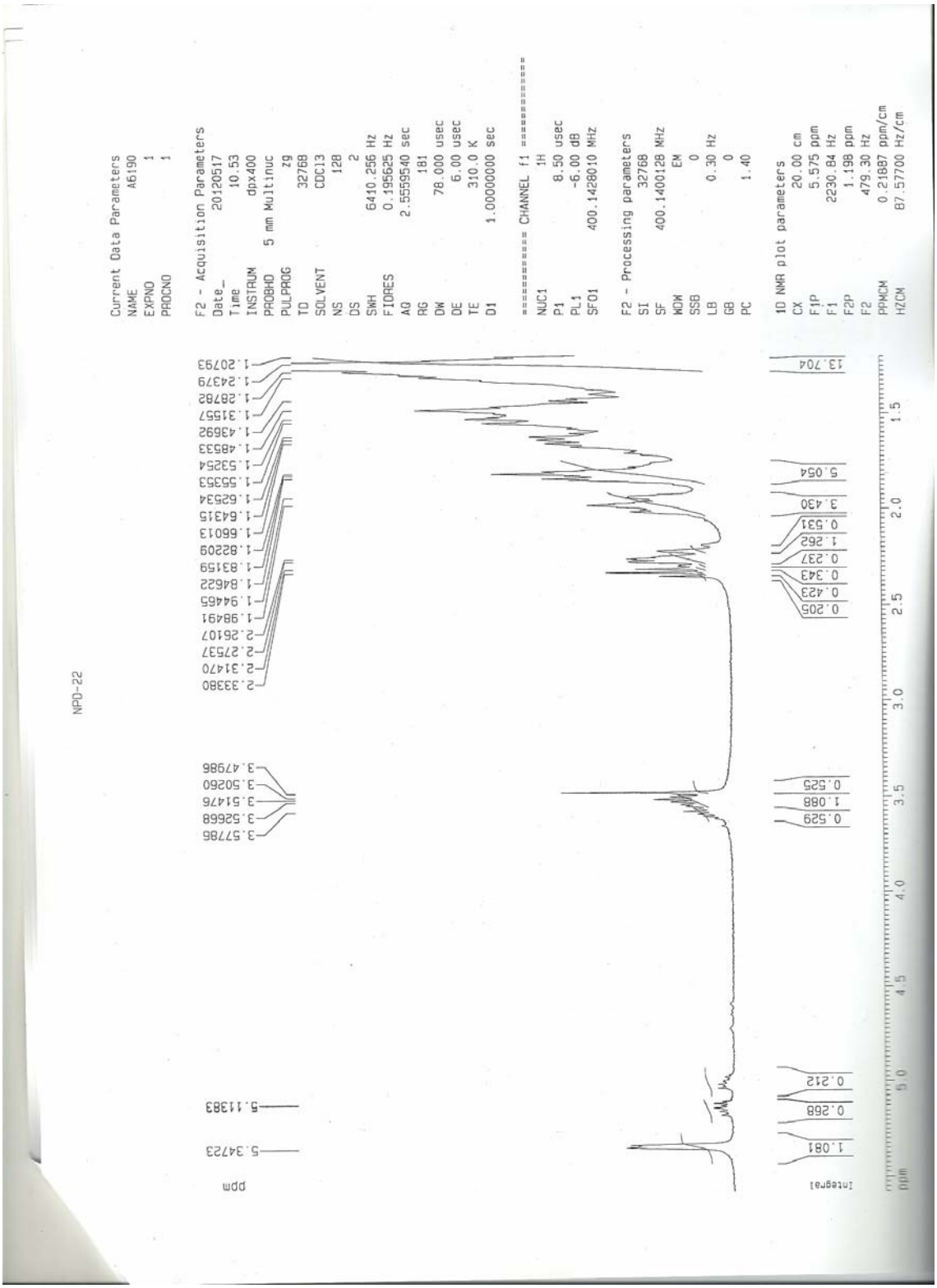
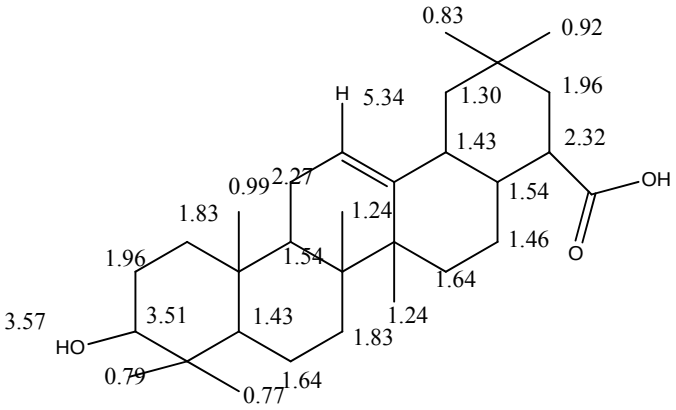


Figure 7: ^{1}H NMR spectrum for the 22nd subfraction of ethyl acetate fraction

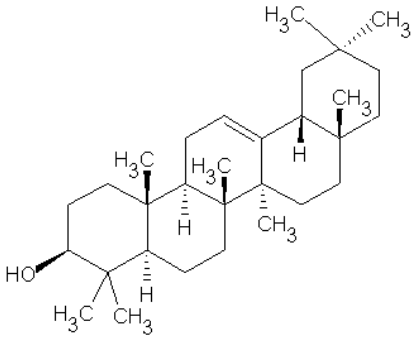
^{1}H NMR spectra of NPE 22 revealed the presence of seven methyl groups by the singlet resonance signal at δ 0.79(3H), 0.81(3H), 0.84(3H), 0.90(3H), 0.96(3H) and 1.00(6H). This singlet methyl signals are characters to triterpene derivatives. NPE 22 also showed a signal of an olifenic proton doublet at δ 5.34 (d). This analysis indicated NPE 22 is very similar to β-amyrine. But NPE 22 has one methyl group less than β-amyrine. NPE 22 was eluted with polar solvent and good TLC was obtained after using AcOH. Thus structure of NPE 22 is proposed as oleanolic acid.



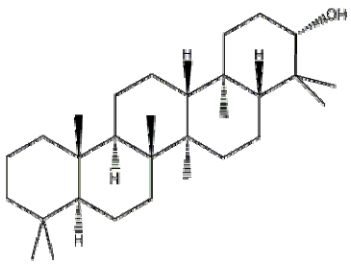
Oleanolic acid

^{1}H-NMR: 0.77(3H, s, H-23), 0.79(3H, s, H-24), 0.83(3H, s, H-29), 0.92 (3H, s, H-30), 0.99 (3H, s,H-25), 1.24 (3H, s, H-26, H-27), 1.30 (2H, d, J= 11.1, H-19), 1.43 (1H, s, d, J= 19, H-5,18), 1.46(2H, s, d, J= 19 , H-16), 1.54(1H, d, J=8.4, H-9,17), 1.64(2H, t, J= 8, H-6,15), 1.83(2H, td, J=5.8,3.8, H-1,7), 1.96(2H, t, J=16.1, H-2, 21), 2.27(2H, d, j=5.7, H-11), 2.32(1H, d, J= 7.6, H -22), 3.57 (1H, s, H-2_b) 3.51(1H, t, J=4.8, H=3_a), 5.34(1H, s, H-12).

Other possible structures assumed from the spectrum are-

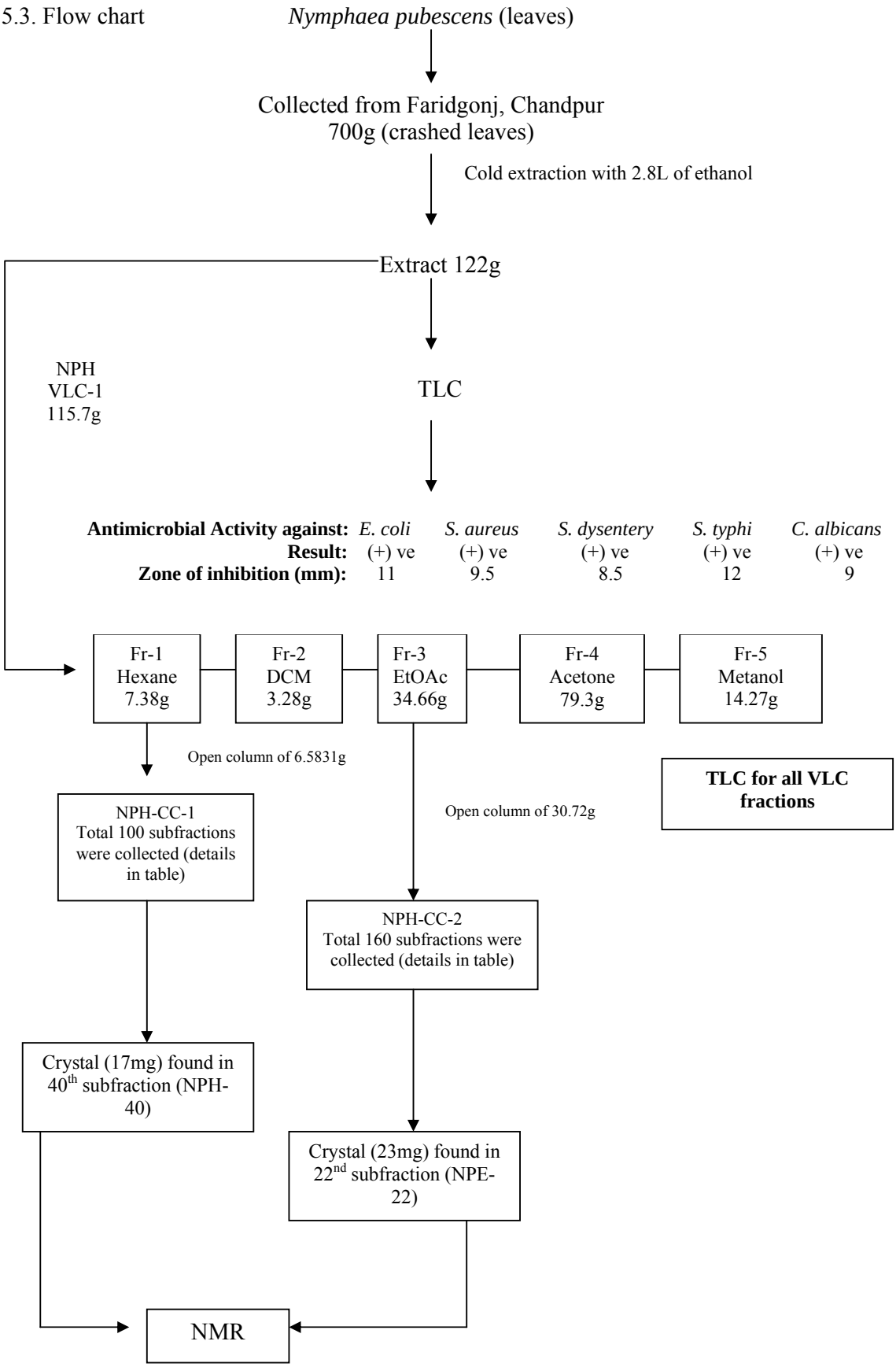


β-amyrin



Tetrahymanol

5.3. Flow chart



Chapter 6

Conclusion

6. Conclusion

Methanol extract of *Nymphaea pubescens* was analyzed by TLC to get primary idea about its content. As crude extract is a mixture of thousand compounds, initially separating the extract into various discrete fractions will help to isolate pure compounds easily. Thus the crude methanol extract of *Nymphaea pubescens* was subjected to Vacuum liquid Chromatography and five different fractions were collected using five different solvents, Fractions were- n-hexane fraction-Fraction-1, DCM fraction-Fraction-2, Ethyl-acetate fraction-Fraction-3, Acetone fraction- Fraction-4 and Methanol fraction- Fraction-5. Their weights were Fraction-1=7.38 gm, Fraction-2=3.28 gm, Fraction-3=34.66 gm, Fraction-4=79.3 gm and Fraction-5=14.27 gm. Among the five fractions n-hexane fraction and Ethyl-acetate fraction were subjected to column chromatography. From n-hexane fraction, 100 column fractions were isolated. From Fraction-1-40 crystal was obtained (NPH-40). The physical appearance of the crystal was pure white. Analysis by TLC revealed the compound is UV inactive and shows red color upon charring. ^{1}HNMR report of NPH-40 informs may be this is a mixture of four fatty acids. Further analysis such ^{13}CNMR, Mass spectroscopy etc is required for definite result. All the other fractions are needed to be analyzed for structure elucidation.

The Ethyl acetate fraction or Fraction-3 was then subjected to open Colum chromatography and 160 fractions were separated according to different color bands in the column. From Fraction-3-22 crystal was obtained (NPE-22). The physical appearance of the crystal was pure white. Analysis by TLC revealed the compound is UV inactive and shows brown color upon charring. ^{1}HNMR report of NPE-22 informs may be this is oleanolic acid. Further analysis such ^{13}CNMR, Mass spectroscopy etc is required for definite result. All the other fractions are needed to be analyzed for structure elucidation.

Bioactivities of these fractions are also need to be tested. Moreover, further studies are warranted to clearly understand the underlying mechanism of the observed bioactivities and to isolate the active phytochemical constituent (s) responsible for such activities in animal models.

Chapter 7

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