Phytochemical Investigation of methanolic extract of *Enhydra fluctuans* (Fam- Asteraceae)

B. PHRM Project

Submitted By
Md. Abdur Rahman
ID No: 2011-3-70-003
Department of Pharmacy
East West University

Submitted to
Professor Dr. Muniruddin Ahmed
Advisor
Department of Pharmacy
East West Pharmacy

June, 2015

EAST WEST UNIVERSITY

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A project Report submitted to the department of Pharmacy, East West University in conformity with the requirements for the Degree of Bachelor of Pharmacy

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In the name of

Almighty Allah,

The most Gracious

&

The most Merciful

This Book

is Dedicated

to

My Parents & Teachers

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I am deeply thankful and grateful to the **Almighty Allah** for helping me in all critical situations to reach at this end stage of my undergraduate research program.

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Md. Abdur Rahman

CERTIFICATE

This research paper is submitted to the Department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy (B.Pharm) was carried out by Md. Abdur Rahman (ID # 2011-3-70-003).

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Dr. Chowdhury Faiz Hossain

Professor and Chairperson

Department of Pharmacy

East West University

Aftabnagar, Dhaka- 1200.

CERTIFICATE

This is to certify that the thesis "Phytochemical Investigation of methanolic extract of *Enhydra fluctuans* (Fam- Asteraceae)" is submitted to the Department Of Pharmacy, East West University, Aftabnagar, Dhaka in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B.Pharm) was carried out by Md. Abdur Rahman (ID # 2011-3-70-003) under my guidance and supervision and that no part of the thesis has been submitted for any other degree. I further certify that all the sources of information and laboratory facilities availed of this connection is duly acknowledged.

……

Professor Dr. Muniruddin Ahmed

Supervisor

Advisor

Department of Pharmacy

East West University

Aftabnagar, Dhaka- 1200.

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Abstract

The objective of this research is to study the phytochemical properties of methanolic extracts of *Enhydra fluctuans*, a plant belonging to the Asteraceae family. The phytochemical evaluation was carried out by performing several test TLCs, preparative TLC, & IR test. *Enhydra fluctuans,*a tropical herb, commonly known as helencha or harkuch,belonging to family Asteraceae, is gaining lot of importance for its therapeutic potentials. This is an edible semi-aquatic herbaceous vegetable plant with serrate leaves, grows in abundant quantiy in Bangladesh. The leaves are slightly bitter, cure inflammation, skin diseases, laxative,bronchitis, nervous affection, leucoderma, biliousness and good in small pox. The plant possesses nutritional value including- _-carotene, saponins, cholesterol, glucoside, enhydrin and so on. It is reported that plant possesses Antioxidant, Hepatoprotective, CNS Depressant, Analgesic and Antidiarrheal activity.Thin-layer chromatography (TLC) is a mature and very established technique, frequently used in many fields of applications ranging from natural product analysis to chemical or pharmaceutical applications. Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. TLC & IR test performed to investigate bioactive natural compounds from *E. fluctuans*and to investigate the crystals obtained from the crude extract of the plant .Some bands were observed through these tests.

Chapter 1: Introduction

1.1 Preamble

The history of plant based health care goes back to antiquity and as old as human civilization. Plants have been primary source of medicines in the traditional healthcare systems around the globe, till recently and even currently in most of the developing countries. The approach to characterization and isolation of active ingredients from plants started in the late 19th century. Consequently chemical substances isolated are currently used as important drugs as such or as their derivative(s) today. From 1983 to 1994, 39% of the New Approved Drugs (NAD) were of natural origin i.e. original natural products, products derived semi synthetically from natural products and synthetic products based on natural molecules (Cragg et al., 1997).

Parallel to synthetic drug demand, the global natural products market is growing exponentially. The global demand for botanical and plant derived drugs is expected to increase from 19.5 billion in 2008 to 32.9 billion USD in 2013, with a compound annual growth rate of 11.0% (Lawson, 2009).

The number of higher plant species on this planet is estimated at 250,000 (Ayensu and DeFilipps, 1978). Of these, only around 6% have been screened for biological activity, and of which only 15% are reported to be phytochemically characterized (Verpoorte, 2000). Asia, especially the southern region shares about 20% of the all known vascular plants in the Globe. This includes 7000-8000 species of medicinal plants. Out of the 17,500 flowering plant species found in India, over 1600 are used in traditional medicine (Pushpangathan, 2004).

According to world health organization (WHO), 65-80% of the global population use plants and plant products for their primary health care (Bagozzi, 2003; Farnsworth et al., 1985). The Investigations on therapeutic applications of plants have led to the discovery of several clinically applicable drugs (e.g., digoxin, digitoxin, morphine, reserpine, taxol,

vinblastine, vincristine etc.). Elucidation of the structure of active principles paved the way for synthesis and derivatization for compounds with higher efficacy and lower adverse effects (e.g., Metformin, nabilone, oxycodon, taxotere, teniposide, verapamil, amiodarone etc) (Daniel et al., 2001).

Thus plants continue to engage the attention of scientists associated with drug discovery. Evidences accumulated thus clearly show that plants are rich source of bioactive chemical entities. Many phytochemicals are capable of modulating biochemical pathways of higher animals. However phytochemicals can be beneficial or harmful. There is sufficient traditional knowledge to substantiate this but further studies are required to index plants with beneficial and adverse effects. Based on the traditional knowledge, some plants and plant products are documented to be non-toxic and therapeutically potent. This knowledge can be exploited to develop cheaper plant based product/formulation for preventive health and disease management. However scientific evidences need to be created for their efficacy through pharmacological and chemical studies. Great number of plants documented in the Indian traditional medicine yet to be investigated in this line.

The present study proposes to evaluate the *Enhydra fluctuans* whole plant for its phytochemical profile and bioactive potentials. *E. fluctuans* is a tropical plant belongs to the family of Asteraceae. The plant is Grows in swampy ground in Tropical climate. Native to India, Bangladesh, Burma, Sreelankha and several places in south east Asia. Plant is used in ascites, dropsy and anasarca. It is cooked with fish curry and taken to revive appetite after long weakness due to fever or typhoid. Leaves are laxative and antibilious; cure inflammation, leucoderma, bronchitis and biliousness; useful in skin and nervous affections; also useful in tropidity of the liver. Leaf paste is applied over head as a cooling agent and around the inflamed Brest to reduce inflammation (Mpbd.info).

1.1.2 Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers.

Phytochemistry can be considered sub-fields of Botany or Chemistry. Activities can be led in botanical gardens or in the wild with the aid of Ethno botany. The applications of the discipline can be for Pharmacognosy, or the discovery of new drugs, or as an aid for plant physiology studies.

Techniques commonly used in the field of phytochemistry are extraction, isolation and structural elucidation (MS, 1D and 2D NMR) of natural products, as well as various chromatography techniques (MPLC, HPLC, LC-MS).

The list of simple elements of which plants are primarily constructed—carbon, oxygen, hydrogen, calcium, phosphorus, etc.—is not different from similar lists for animals, fungi, or even bacteria. The fundamental atomic components of plants are the same as for all life; only the details of the way in which they are assembled differ.

Phytochemistry is widely used in the field of Chinese medicine especially in the field of herbal medicine.

Phytochemical technique mainly applies to the quality control of Chinese medicine, Ayurvedic medicine (Indian traditional medicine) or herbal medicine of various chemical components, such as saponins, alkaloids, volatile oils, flavonoids and anthraquinones. In the development of rapid and reproducible analytical techniques, the combination of HPLC with different detectors, such as diode array detector (DAD), refractive index detector (RID), evaporative light scattering detector (ELSD) and mass spectrometric detector (MSD), has been widely developed.

In most cases, biologically active compounds in Chinese medicine, Ayurveda, or herbal medicine have not been determined.[citation needed] Therefore, it is important to use the phytochemical methods to screen and analyze bioactive components, not only for the quality control of crude drugs, but also for the elucidation of their therapeutic mechanisms. Modern pharmacological studies indicate that binding to receptors or ion channels on cell membranes is the first step of some drug actions. A new method in phytochemistry called biochromatography has been developed. This method combines human red cell membrane extraction and high performance liquid chromatography to screen potential active components in Chinese medicine. (Wikipedia)

1.2 Traditional healthcare

One of the most notable features of medicine in the later part of the preceding century were vigorous criticisms against traditional systems of healthcare delivery, almost to a point of suffocation. Although most of the issues raised to affirm the seemingly inadequate status of this system are compelling, its absolute undesirability has been difficult to establish. (Nwokocha, 2008)

If irreplaceable genetic resources are lost, traditional medicines and indigenous knowledge will also disappear. To prevent this from happening, prompt action is required at every possible level: local initiatives, support from non-governmental organizations, universities, scientific research and active governmental support for international agreements to protect intellectual property rights. In-situ and ex-situ conservation of medicinal plants must be established in order to highlight the links between people and plants and to show the importance of the plants to human welfare as well as the maintenance of ecological integrity. Future studies should consider ethnobotanical parameters such as known medicinal plant species, specific medicinal uses of plants, species status (wild or cultivated), plant organs used (root, tuber, shoot, exudates, bark, seeds, fruit), and its status of use (used in the past, still in use, used recently). This should

be complemented by a detailed understanding of the concept of traditional medicine and a study of the knowledge transfer systems. (Ndenecho, 2011)

1.2.1 Plant based traditional healthcare: an overview

According to WHO, Traditional Medicine (TM) refers to health practices, approaches, knowledge and belief incorporating plant, animal and mineral based medicine, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illness or wellbeing (Xiaorui Zhang et al., 2002).

Naturopathic, Ayurveda, Reiki, Chinese, Native American medicine, Homeopathic medicine, etc. are some examples of traditional healthcare systems. Fossil records date, use of plants as medicines at least to the middle Paleolithic period, some 60,000 years ago (Solecki and Shanidar, 1975).

One of the oldest records related to the plant based medicine is the Papyrus Eber written nearly 1500 BC and contains information of more than 500 natural ingredients (Ghalioungui, 1969). Similarly, Asia's one of the traditional systems of health care is Chinese medicine that primarily use plants for disease management. Shennong Bencao Jing is the first Chinese herbal book which was compiled during the Han Dynasty but dating back to a much earlier date, possibly 2700 B.C., that lists 365 medicinal plants and their uses. During the middle age, many medical texts have been written. Causes and Cures were written by a 12th century Benedictine nun. `Herbals and medical texts migrated from Asia to middle East and West. The knowledge and applications of herbals greatly increased during the period 15 to 17th century. The books published during that period include Great herbals, 1526; General history off plants, 1597 etc. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices (Narayana et al., 1998).

“Indian traditional medicine “Ayurveda” is a store house of knowledge on traditional healthcare. A most authentic compilation of the teaching of Sushrutha, called "*Sushrutha Samhitha*" contains description about 1120 illness and 700 medicinal plants (Dwivedi and Dwivedi, 2007). Charak Samhita’ and ‘Sushruta Samhita’ described not only the medicinal properties of individual plants but also the poly herbal and herbomineral preparations. These resources, that emphasize the doctrines for life with healthy mind and body, were evolved through day-to-day life experiences as a part of cultural heritage of India. Besides Ayurveda, there are several other complementary and alternative systems of medicine like Homeopathy, Siddha and Unani, which are also practiced and developed with the course of time in India, where plants and plant-based formulations are employed for health care and disease treatments. In "Ayurveda" and almost all other traditional systems of medicine, the predominant raw materials used are plants that they play a major role and constitute the backbone. These systems are based on experience and interaction with nature and natural resources. Variations in geographical landscaping and biodiversities in the Indian subcontinent have helped to develop the use of a variety of plant species and other natural resources for health care and contributed to TM. The inherited cumulative knowledge and experience in Ayurveda as well as other TM may be of interest because of new leads to modern approach for diseases treatment and management.

TM is rapidly growing health care system with great economic importance. In underdeveloped countries majority of the population uses TM to meet their health care needs. In several regions viz. Asia and Latin America, people use TM as a result of their historical background and cultural beliefs. Meanwhile, in many developed countries, TM is becoming more and more popular. In developing countries, broad use of TM is often attributable to its accessibility and affordability and by concern about the adverse effects of chemical drugs used in allopathic. Therefore many people trust the gentleness of TM in managing diseases than allopathic medicine. Moreover together with demand for TM, concerns over the safety, efficacy and quality of TM, products and practices related to it

have also been raised. Since TM has developed within different cultures in different regions, there has been no parallel development of standards and methods. Therefore scientific evidence to prove the rationale of using these formulations in health care is essential to develop and to preserve the cultural heritage. Approaches like high-throughput screening, phytochemical profiling, quality control and standardization of raw materials and formulations, pharmacokinetics, pharmacovigilance and clinical trials of herbal therapeutics will not only help to prove the rationale of using these systems but also to get maximum benefits of the natural resources (Mukherjee, 2005).

1.2.2 Traditional medicines: merits and demerits

In developing countries, 80% of the population still depends on the traditional medicine for their primary health care since these are presumed to be cheaper and affordable. These traditional medicines are developed by practices over centuries by trials and observations. However the practices and remedies drastically vary from one country to another. Though there are variations, there is a consensus that TM generated important knowledge about the therapeutically potent plant(s), plant part, preparation methods, dose etc. but, lack scientific evidences. While some practices seem to offer benefits, others remain questionable. The only remedies to answer the unanswered questions are to do further research based on evidences to address safety, efficacy, and quality. Some traditional practices for several decades have now been evaluated by modern science and found to be harmful to health (e.g. ephedra). Ephedra is used in Chinese traditional medicine to treat short-term respiratory congestion and its long term use is reported to lead to, heart attack and stroke including death (Martinez-Quintana et al., 2010). Safety evaluation of TM products and practices using modern scientific method is also problematic. This is especially true of herbal medicines, the effectiveness and quality of which can be influenced by numerous factors. It is well known that the amount of research work on validation of TM has been inadequate. Further systematic and scientific

studies are required to generate sufficient data for judging the efficacy, and safety. Because of the lack of comprehensive methodologies for scientific validation, developments in drafting regulation and guidelines for TM have slowed. Besides, many TMs have still been practiced and people are claimed to have benefitted. However many herbs that used are untested by scientific methods and their use not monitored. As a result, knowledge of their potential side-effects is limited. This makes identification of the safest and most effective therapies and promotion of their rational use more difficult. Many traditional formulations currently in use are not standardized. Enormous fluctuation is in the phytochemical profiles of raw materials for formulations due to climatic, environmental, soil chemical and genetic could influence their efficacy. If TM is to be promoted as a source of health care, developments of modern methodologies and scientific studies are very much essential.

1.2.3 Scientific approach to plant based health care

Several reviews are published regarding the methods for selecting plants as candidates for drug discovery (Verpoorte, 2000). Random selection followed by biological activity screening is one approach. In this approach, randomly selected plants are subjected to various biological activities and a comprehensive screening of major phytochemicals viz. cardenolides/ bufadenolides, alkaloids, triterpenes, flavonoids, isothiocyanates, iridoids, etc. is performed using various analytical and quantitative tools. Based on the partial chemical analysis, biological activity is attributed to the major phytochemical class (Farnsworth, 1966). This approach has been used in the past and is currently pursued mainly in the developing countries. The tests are simple to perform, but false-positive and false-negative tests often render results difficult to assess (Farnsworth, 1966; Farnsworth et al., 1962; Roper et al., 1965; Segelman et al., 1968).

More importantly, it is frequently impossible to relate one class of phytochemicals to a specific biologic activity; for example, the alkaloids or flavonoids produce a vast array of

biologic effects that are usually not predictable in advance. Another approach is the random selection of plant material against a targeted disease followed by one or more biologic assays. Crude extracts prepared from a spectrum of plants / plant materials are subjected to in vitro and in vivo analyses, and based on the promising result, more experiments are conducted to identify the active principle. National Cancer Institute (NCI) adopted this approach for anticancer drug screening using experimental animals (Douros and M., 1980; Douros and Suffness, 1981; Farnsworth et al., 1966). Taxol and camptothecin (Wall and Wani, 1996) were discovered in this program aswell they have reported several other compounds that were unsuccessful in human studies. In 1986 the NCI program abandoned this approach and continued to collect and screen plants using a battery of 60 human tumor cell lines and also initiated a screening of plants for anti-HIV activity in vitro. Calanolide A, currently in Phase I clinical trials, was developed from this program (Anonymous, 2000; Kashman et al., 1992).

Approaches based on prior arts utilize knowledge of traditionally practiced medicinal plants and plant products. Several types of ethnomedical information are available about the plants used in organized traditional medical systems viz. Ayurveda, Unani, Kampo, traditional Chinese medicine etc. against several ailments (Bannerman and Chen, 1983).

Based on the knowledge about the ethno medicine (plant species, anatomical part used, targeted disease etc.), taxonomic relations (similar therapeutical potency in related species) and current scientific data (chemical composition, limited in vitro and in vivo study results, etc.), new scientific investigations have to be redesigned in order to identify and characterize active principles. If scientific information about phytochemical composition and activity of each of the chemical entities in a particular plant species are known, standardized health care product formulation based on the said plant species would be relatively easy. However complete phytochemical characterization of plant species is the practical difficulty associate with this method. Though a complete chemical characterization is done, if knowledge about the biological functions of the characterized chemical entities is not available, further attributions of biological activity require wide

array of in vitro and in vivo screening. Scientific approach in plant based healthcare mainly focus on the selection of standardized methods, use of plants/plant products, evaluation of biological activity, identification of active principle, toxicological and pharmacological study, dose optimization etc. Fig. 1-1 is a conceptual scheme for modern approach in development of plant based healthcare products. According to this scheme the plant(s)/plant product is selected based on any one or all of the following criteria viz.; ethno medicinal knowledge, taxonomical relation to well-studied therapeutically important plants and the current scientific knowledge available about a plant species. After selection, a thorough bioactivity evaluation/validation has to be done by differential analysis and bioassay guided fractionation. Finally identification of active fraction/principle and chemical characterization including structure elucidation has also to be done.

After establishing the chemistry of active principle, a standard drug screening protocol may be followed to validate the results that include in vivo animal studies and various clinical trials. On successful completion of these scientific methodologies, a standardized plant based healthcare product can be formulated for disease prevention, cure or management.

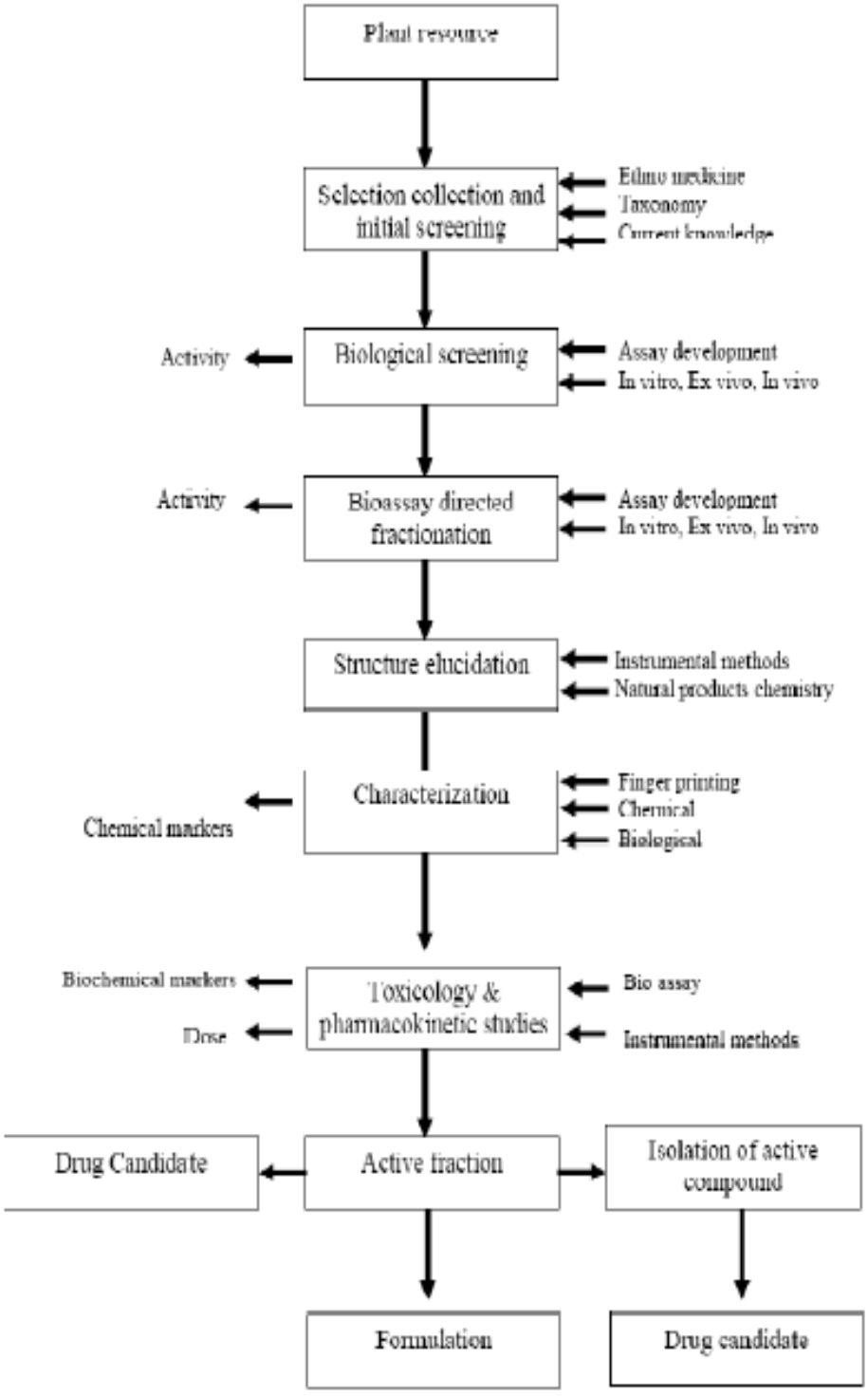


Fig. 1-1 Conceptual scheme for modern approach to plant based health care

1.3 Plants as source of drugs

Drug is a substance that alters body function (Cohen, 2010). Drug discovery is a lengthy, expensive difficult and often an inefficient process with low success rate (Blake et al., 2009).

Though there are several advancements in medical treatments, including gene therapy, immunotherapy, DNA vaccines, regenerative therapy etc., for acute diseases, effective medical treatment would be based on small organic molecules. The process of finding a new drug against a chosen target disease usually involves high throughput screening (HTS), wherein large libraries of chemicals are tested for their ability to modify the target. Despite the rise of combinatorial chemistry as an integral part of lead discovery process, natural products still play a major role as starting material for drug discovery (Feher and Schmidt, 2003).

From 1981 to 2006, of the identified bioactive chemical entities, 63% were natural derived or partially synthesized based on the chemical backbone from natural products (Newman and Cragg, 2007). As it is well known, vast crude drugs used in almost all traditional healthcare systems are plant based extracts and hence plants could be an important sources of starting material for novel chemical entities for modern drug discovery (Lewinsohn and Gijzen, 2009; Nussbaum et al., 2006).

Several drugs have been isolated from plants and are practiced today as antimicrobial agent (e.g. glaucaroubin) (Efren et al., 1956), analgesic (e.g. morphine and codeine) (Benyhe, 1994; Bosch et al., 1981), antihypertensive (e.g. romitoxin) (Liu et al., 2003), and anti-inflammatory drugs (e.g. aescin, reserpine etc.) (Bonaccorsi, 1968; Sirtori, 2001). Moreover, plant derived antitumor agents (e.g. taxol, etoposide) (Aspandiar et al., 1987; Guchelaar et al., 1994) as well as cardioprotective agents (e.g. digitalis, acetyldigox in, digoxin etc.) (Charles et al., 1953) are also being used now. Table 1-1 shows some important phytochemicals that are now used as drugs.

1.4 Literature survey

It showed that the 122 bioactive phytochemicals identified in 1985 were all derived from only 94 plants species (Farnsworth et al., 1985) out of estimated 250000 flowering plants on the planet. Therefore there should be abundance of drugs remaining to be discovered. This is a representation of the volume of phytochemical resources available and how much has been identified.

Therefore HTS of plants is essential to explore the hidden therapeutically potent phytochemicals.

Table 1-1 Few phytochemicals with therapeutic properties

Drug	Action or clinical use	Plant source
Trichosanthin	Abortifacient	*Thymus vulgaris*
Glaucaroubin	Amoebicide	*Simarouba glauca*
Emetine	Amoebicide; emetic	*Cephaelis ipecacuanha*
Picrotoxin	Analeptic	*Anamirta cocculus*
Morphine	Analgesic	*Papaver somniferum*
Codeine	Analgesic; antitussive	*Papaver somniferum*
Rotundine	Analgesic; sedative	*Stephania sinica*
Agrimophol	Anthelmintic	*Agrimonia eupatoria*
Atropine	Anticholinergic	*Atropa belladonna*
Protoveratrines	Antihypertensive	*Veratrum album*
Reserpine	Antihypertensive	*Rauvolfia serpentina*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Quinine	Antimalarial	*Cinchona ledgeriana*
Monocrotaline	Antitumor agent	*Crotolaria sessiliflora*
Colchicine	Antitumor agent; antigout	*Colchicum autumnale*

Etoposide	Antitumour agent	*Podophyllum peltatum*
Noscapine	Antitussive	*Papaver somniferum*
Kainic Acid	Ascaricide	*Digenea simplex*
Santonin	Ascaricide	*Artemisia maritima*
Andrographolide	Bacillary dysentery	*Andrographis paniculata*
Khellin	Bronchodilator	*Ammi visnaga*
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Deslanoside	Cardiotonic	*Digitalis lanata*
Digitalin	Cardiotonic	*Digitalis purpurea*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis lanata*
Gitalin	Cardiotonic	*Digitalis purpurea*
Scillarin A	Cardiotonic	*Urginea maritim*
Vincamine	Cerebral stimulant	*Vinca mino*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum*
Caffeine	CNS stimulant	*Camellia sinensis*
Podophyllotoxin	Condylomata acuminata	*Podophyllum peltatum*
Theobromine	Diuretic; bronchodilator	*Theobroma cacao*
(+)-Catechin	Haemostatic	*Potentilla fragaroides*
Danthron	Laxative	*Cassia spp*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Gossypol	Male contraceptive	*Gossypium spp.*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Valepotriates	Sedative	*Valeriana officinalis*

1.5 Plant based prophylactic agents

Plant based drugs are organic compounds derived from plants that alter normal body functions. Plant drugs are distinct from plant based nutrients. Apart from nutrients and molecules that have direct effect on human body, there are several non-nutritive organic compounds, in plants, having no apparent detrimental action on human body when consumed with food in the natural concentration. Many compounds belong to this group act as prophylactic agent (Prophylaxis: treatment given or action taken to prevent disease

(Simpson and Weiner, 2010)) in the system and help to protect the body from several degenerative diseases caused, especially by oxidative stress in consequent to metabolism.

A cross cultural population comparison study showed that plasma levels of antioxidant molecules viz. vitamin C, β carotene, vitamin E and selenium were significantly higher in men, 40-49 aged, from Switzerland and Italy compared to their counterpart in Finland and Scotland (Gey, 1987). In that study, the author highlighted that there is an antioxidant index with respect to antioxidant molecule level in each subjects. The antioxidant molecule's levels in such subjects correlates with a positive antioxidant index and this relation was inversely related to mortality rates by ischemic heart diseases (IHD). The antioxidant hypothesis evolved from this study with the proposal that high intake of dietary antioxidants prevent oxidation of plasma and thereby oxidative stress (Gey, 1987).

In the last decade, considerable worldwide attention has been given to plant phenolic antioxidants including flavonoids and various other phytochemicals found in many fruits and vegetables, red wine, etc. for their protective effect against the damage from oxidative stress. The literature on antioxidants has expanded tremendously because of accumulation of evidence that they may contribute to the recognized extra nutritional benefits of food and beverages containing phenolic compounds (Frankel and German, 2006; Kroon and Williamson, 2005). Prophylactic activities of phytochemicals are attributed to their antioxidant/radical scavenging activities to retard oxidative stress caused by free radicals in vivo.

Free radicals are highly reactive chemical entities normally produced but often overproduced in all higher organisms. When free radicals are excessively produced, it can damage biomolecules viz. fatty acid, protein and DNA and can be one among many reasons for the early incidence of degenerative disease.

1.6 Etiology of chronic degenerative diseases; special attention to free radicals

With increase in life expectancy, chronic degenerative diseases have become by far the principal cause of death world over. The highest mortality (26.3%) was attributed to cardiovascular diseases (CVD). The next highest mortality was due to malignant neoplasms (24.1%). Other causes were; chronic lower respiratory diseases (4.9%), Diabetes mellitus (3%), and Alzheimer's disease (1.8 %) followed by others (Heron, 2010).

In most cases, the etiology is highly complicated and multi-factorial such as genetic, environmental, occupational, dietary habit, lifestyle etc. In all above said factors, free radical production is a common phenomenon that speeds up the onset and progression of degenerative diseases. Free radical theory of aging argues that oxygen free radicals produced during normal respiration would cause cumulative damage which would eventually lead to organism's loss of functionality and ultimately death (Harman, 1972; Harman, 1992). However evidences to substantiate this hypothesis lack at present. During the last 3 decades huge body of literatures have been published that correlate free radicals and onset of cancer, CVD, diabetes, cataract etc. It can be presumed that free radicals along with several other risk factors may accelerate the onset and progression of several degenerative diseases. One of the approaches thus could be to use free radical scavengers / antioxidants to prevent or retard onset and progression.

1.6.1 Free radical mediated oxidation of biomolecules

The biomolecules are more prone to oxidative stress. These undergo structural and subsequent functional changes when exposed to oxidative stress. The changes lead to the abnormalities in the physiological homeostasis and leads to many pathological conditions.

Lipid peroxidation: Lipid is probably the most studied substrate for oxidation by free radicals in biological system. Oxidation of lipid in non-enzymatic mode is mainly by free radicals and ROS. The free radicals that induce lipid oxidation are superoxide radical, peroxide radical and nitric oxide. Superoxide does not abstract hydrogen atom even from very reactive bis-allylic methylene groups (Afanas'ev, 1989; Bielski et al., 1983), although it's conjugated acid HOO• is more active in hydrogen atom abstraction and probably capable of initiating the lipid peroxidation (Bielski et al., 1983). Though the superoxide is too inert to initiate lipid peroxidation, it can initiate lipid oxidation through other ways such as by reduction of ferric to ferrous ion that catalyzes the Fenton's reaction. Subsequent studies showed that formation of hydroxyl radicals, even if it take place during lipid peroxidation, are of no real importance and that have been reported by several authors (Bast and Steeghs, 1986; Beloqui and Cederbaum, 1986; Gutteridge, 1982; Vile and Winterbourn, 1987).

The possibility of hydroxyl radical dependent lipid peroxidation was studied earlier and reported that hydroxyl radicals are involved in the NADPH dependent microsomal lipid peroxidation (Lai and Piette, 1977). Peroxy radicals especially neutral, positively and negatively charged alkyl peroxyl radicals are more efficient initiators of LDL oxidation compared to that of superoxide (Bedard et al., 2001). NO is also incapable of abstracting hydrogen atom from unsaturated substrates similar to superoxide, but forms various other reactive species capable of initiating lipid oxidation. The pro-oxidant effect of NO depends on the relative concentration of NO and oxygen, the direct interaction of NO with free radicals formed in the lipid peroxidation and conversion of NO into peroxy nitrites or other reactive NO metabolites (Bloodsworth et al., 2000; O'Donnell et al., 1997; Rubbo et al., 1994).

Amino acid oxidation: Amino acids are also more sensitive to free radicals and ROS damage. Oxidation of amino acid moieties of functional and structural protein leads to the inactivation of enzymes, receptors, hormones, loss of structural integrity etc. Free radicals cause fragmentation of protein and cross linking of amino acids by hydroxyl

radicals in the absence of dioxygen. (Dean et al., 1986). Alpha-position of the simple aliphatic amino acid or amino acid residue in the polypeptide chain is more prone to the hydroxyl radical-mediated abstraction of hydrogen atom. As the number of carbon atoms in an amino acid increase, that amino acid would be more prone to free radical mediated cross link with other aliphatic amino acids. In the case of aromatic amino acids, the ring is the primary site of attack leading to ring scission, and in the case of tyrosine, to the formation of Tyr-Tyr cross-linked dimmers (Stadtman, 1993).

Davies et al. reported that superoxide radical alone does not damage amino acids but, in the presence of hydroxyl radicals, it causes several fold damage to the protein compared to that of the damage caused by hydroxyl radical alone (Davies, 1987; Davies et al., 1987). ROS mediated oxidation of amino acids can also lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and to conversion of some amino acid residues to carbonyl derivatives (Stadtman and Levine, 2003)

DNA oxidation: DNA is another target for ROS, RNS, free radicals, transition metals, etc. Reactions of hydroxyl radicals with DNA have been thoroughly studied based on the effect of ionizing radiations on DNA. One important attribute of free radical mediated damage to DNA molecule is the multiple ways of free radical attack. Mostly the DNA damage happens through base modification (Aruoma et al., 1989; Halliwell and Aruoma, 1991). Another important mode of DNA damage is the oxidative stress induced strand breaks.

Hydroxyl radicals can easily abstract hydrogen atom from the ribose of sugarphosphate backbone and causes single strand break (Breen and Murphy, 1995).

Though the single strand breaks are not very harmful, double strand breaks cancause cell death. The double strand breaks are formed because of multi plehydroxyl radical attack (Ward, 1985). Mitochondrial DNA is more sensitive to ROS compared to nDNA. Both

xanthine oxidase and menadione generated oxygen radicals caused severe damage to mtDNA with no significant effect on nDNA (Grishko et al., 2001).

This theory is supported by the evidence that the lack of histone proteins in the mtDNA makes this more susceptible to ROS induced damage (Ballinger et al., 2000; Yakes and Houten, 1997). Reactive nitrogen species is also known to have the capacity to induce damage to DNA.

Amount of nitric oxides generated by interleukin-1-B-induced nitric oxide synthase is sufficient to cause damage to DNA in many cell line in vitro (Delaney et al., 1993).

1.6.2 Consequences of free radicals and ROS mediated oxidation of biomolecules

Free radicals, ROS and RNS cause irreversible damage to biomolecules such as fatty acid, amino acid, and DNA. Oxidative modification / damage of these molecules lead to the onset of several degenerative diseases such as cancer, vascular diseases, diabetes, inflammation etc.

Cancer: The possible role of free radicals in cancer has been discussed based on the discovery of excess production of free radicals in tumor cells (Saprin et al., 1965). Further, the discovery of superoxide in biological system and superoxide dismutase attracted much attention towards the association of free radicals and carcinogenesis. Studies in expression level of SOD gene in normal and cancer cells pointed out this association because, the tumor cells express low levels of SOD (Oberley, 1982). In addition, Mn SOD is not expressed at all in cancer cells even at elevated level of superoxide (Oberley and Oberley, 1988). Consequently, elevated superoxide cause DNA damage and thus initiate carcinogenesis (Nakamura et al., 1988**).** During initiation process, the involvement of free radicals were emphasized based on the fact that organic peroxides promotes carcinogenesis (Floyd, 1990). Further, ROS and RNS react with

guanine and forms 8-OHdG. The role of 8-OHdG in the process of carcinogenesis is well established (Floyd, 1990;) by its potential to mutate a few cancer related genes and transformation of proto-oncogenes to oncogenes (Cerutti, 1994). Another study reported that enhanced production of hydroxyl radicals can initiate carcinogenesis, in particular, cologenic hydroxyl radical and other ROS generation is considered as one important factor for the cause of colorectal carcinoma (Babbs, 1990). The involvement of inflammation, especially inflammatory phagocytes, on the cancer promotion has been understood very long time back. During inflammation, stimulated macrophage induce DNA damage supposedly through the generation of free radicals (Chong et al., 1985). The ROS, RNS, and various other carcinogens together may change the normal cellular genome to neoplastic one at the onset of cancer.

Diabetes: The involvement of free radicals in the development of diabetes is a core research area in the epidemiological studies of diabetes. Much study has been done on the free radicals in biology and diabetes independently. However, the number of comprehensive studies to understand the involvement of free radicals in the etiology of diabetes is very few. Type 1 diabetes is caused by destruction of pancreatic beta cells responsible for the production of insulin. In human the diabetogenic process is caused by immune destruction of beta cells; part of this process is apparently by white cell production of ROS. Wellestablished evidence is the experimental diabetic inducing agents; alloxan and streptozotocin. Though the mechanism of action of these two compounds are different, both results in the production of ROS. The presence of ROS scavengers effectively inhibited the development of diabetes in these compound induced diabetic models (Oberley, 1988). Josefsen et al. demonstrated that the circulating monocytes in newly diagnosed type-1 diabetes were activated which could play a very important role in the destruction of β-cells of pancreas .These monocytes were reported to produce excess superoxide in patients with early hypertriglyceridemia and diabetes. In type-2 DM also the plasma redox balance is disturbed and oxidative stress is observed. This is evidenced by several fold reduced plasma superoxide dismutase level and other

endogenous antioxidants in type-2 DM patients compared to that of nondiabetic control (Collier et al., 1990). Thus the oxidative stress caused at the onset of type-2 DM may promote the progression of pancreatic cell damage as well as leads to higher prevalence to mortality from CVD (MacRury et al., 1993).

Vascular diseases: Vascular diseases such as atherosclerosis, peripheral artery disease, hypertension, peripheral vascular disease etc. are caused by xenobiotics, physical inactivity, unhealthy diet etc. However the free radicals and ROS in the vascular system promote the onset as well as progression. The role of free radicals in the etiology of a few vascular diseases are presented in the following sections

*Ischemic reperfusion injury**:*** Hypoxia and reoxygenation generally causes injury to cells. The major cause of circulatory shook, myocardial ischemia, and stroke are believed to be reoxygenation. During reoxygenation, large amount of ROS especially superoxide and hydroxyl radicals (Werns et al., 1985) are formed and recognized as the cause of reoxygenation injury. Formation of ROS under this pathological conditions were established by several studies (Das et al., 1986; Hess et al., 1982; Werns et al., 1985).

Atherosclerosis: A large number of reports emphasize that excess superoxide play an important role in the onset of atherosclerosis and hence promote endothelial dysfunction. Moreover the oxidized proteins, lipids, LDL and nucleic acids as a result of plasma oxidative stress also promote the progression of vascular tissue damage (Beckman and Koppenol, 1996) and atherosclerotic plaque formation. Promotion of atherosclerosis as a result of reduced expression of extracellular SOD and mutation in endothelial is an important evidence for the role of ROS in vascular diseases (Faraci and Didion, 2004; Fukai et al., 2002)**.**

Hypertension: The possible role of free radicals in the pathogenesis of atherosclerosis and hypertension has been suspected for long time. This was evidenced by low serum antioxidant capacity and hypertension (Salonen et al., 1988), high serum antioxidant

capacity and low level of atherogenic protein. correlation between antioxidant supplement and normotension (Salonen et al., 1994) etc.

Inflammation: Under chronic inflammatory condition, a large number of ROS are produced. Superoxide thus produced stimulate the release of IL-1 from blood monocytes. IL-1 act as feedback booster and in turn increases the formation of excess ROS in the vicinity (Babior et al., 1973). The excess ROS thus produced oxidize lipoprotein, lipids, protein etc. and accelerate atherogenic processes in the vascular system**,** induce carcinogenesis (Coussens and Werb, 2002), neurodegeneration (Akiyama et al., 2000) etc.

Life expectancy: Aging is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age. Among the several ageing theories proposed, the "free radical theory of aging" (Harman, 1956; Harman, 1992) has gained universal acceptance and is supported by the fact that the sum of the deleterious free radical reactions going on continuously throughout the cells and tissues constitutes the aging process (Sohal and Weindruch, 1996). The free radial theory is supported by the "rate of living" hypothesis, which links metabolic rate and subsequent free radical production with the short lifespan of organisms (Ku et al., 1993). Under vigorous metabolism, free radicals and ROS are produced and can damage proteins, DNA and lipids and this oxidation process accelerate aging process (Barja and Herrero, 2000; Sohal et al., 1995; Sohal and Weindruch, 1996). Caloric restriction and thereby reducing free radical production and oxidative stress has been shown to increase lifespan in animal studies (Agarwal and Rao, 1998;

Sohal and Weindruch, 1996). The inverse relation between free radicals and oxidative stress versus longevity is reported by several studies (Buchan and Sohal, 1981; Merry, 2004; Yan and Sohal, 2000). Other studies also supported this relation indirectly such as low cellular superoxide and hydrogen peroxide production, as a result of high antioxidant

enzymes level, and maximum life span (Barja, 1998; Ku et al., 1993). Genetic studies viz. over expression of superoxide dismutase in transgenic flies, catalase enzyme in *C. elegans*, mitochondrial catalase enzyme in mice etc. support the relation between free radicals and longevity (Melov et al., 1995; Tower, 2000).

1.6.3 Scavenging of free radicals and other ROS

In a physiological system, free radicals are formed as a part of normal metabolism and by exogenous factors, and the antioxidant defense system continuously scavenges the excess oxidants, ROS and free radicals formed. The free radical production and its removal is taking place in a balanced condition. When the free radical production is more and a corresponding removal is not done, the system would undergo a state called oxidative stress. The oxidative stress can be cellular, tissue level or in organ level and can be an important cause for early onset of various degenerative diseases as discussed in the previous section. The antioxidant defense system plays a vital role in removal of excess free radicals and maintains a balanced redox state. One important line of defense is a system of enzymes, including glutathione peroxidase, superoxide dismutase and catalase, which decrease concentrations of the most harmful oxidants in the tissues. Several essential minerals including selenium, copper, manganese and zinc are necessary for the formation or activity of these enzymes. Hence, if the nutritional supply of these minerals is inadequate, enzymatic defenses against free radicals may be impaired (Bagchi and Puri, 1998). The second line of defense against free radical damage is the presence of nonenzymatic antioxidants. Antioxidants are a group of substances which when present at lower concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative process, while often being oxidized themselves (Vaya and Aviram, 2001). Antioxidants are stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Some such antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body.

Other lighter antioxidants are found in the diet. Although about 4000 antioxidants have been identified, the best known are vitamin E, vitamin C and the carotenoids. Many other non-nutrient food substances, generally phenolic or polyphenolic compounds, display antioxidant properties and, thus, may be important for health (Bagchi and Puri, 1998). Both antioxidant defense systems jointly scavenge the excess free radicals produced in vivo. Therefore the antioxidant defense system helps to maintain health and protect from early onset of free radical mediated degenerative diseases.

1.7 Antioxidants

1.7.1 Endogenous antioxidants

ROS contribute to the formation of various pathological conditions. To counteract the effects of ROS, the body is endowed with a protective mechanism consisting of enzymatic and non-enzymatic endogenous antioxidants. Up regulation of enzymatic antioxidants have been reported to minimize free radical production and oxidative stress mediated tissue damage and hence the onset and progression of degenerative disease (Li et al., 2006).

Endogenous antioxidants are capable of different activities and work synergistically with exogenous antioxidants contributing the overall protective effect to the individuals by preventing or delaying the onset and progression of various free radical contributed degenerative disease (Serafini, 2006).Following section presents about various endogenous antioxidant and their importance.

Superoxide dismutase: Superoxide dismutase is the first antioxidant enzyme involved in the antioxidant defense system found in higher organism and microbes (Fridovich, 1983; Fridovich, 1986) and the major function is the removal of superoxide radicals formed by various reasons as presented in section.

Catalase: Catalase is a common peroxidase enzyme found virtually in all aerobic organisms that breakdown hydrogen peroxide which is produced by superoxide dismutase enzyme. Hydrogen peroxide is an important starting molecule for the production of hydroxyl radicals by Fenton's reaction. Catalase decomposes hydrogen peroxide into water and oxygen and thereby prevents the damaging effect caused by hydroxyl radicals (Deisseroth and Dounce, 1970).

Glutathione peroxidase: Glutathione peroxidase (GPX) is ubiquitous selenium containing antioxidant enzyme in all higher organisms that catalyze the decomposition of hydrogen peroxide to water by utilizing reduced glutathione as hydrogen atom source. There are several isozymes differentially located in various organs. GPX-1 is present in the cytoplasm of all mammalian cells and its preferred substrate is hydrogen peroxide. GPX-2 is an intestinal and extracellular enzyme. The GPX enzyme, which is more abundant in extracellular fluid, especially plasma, is isozyme-3. isozyme-4 also expressed in all cells in less abundance whose preferred substrate is lipid hydroperoxids (Muller et al., 2007).

Glutathione reductase: Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione. Oxidation of glutathione by GPX forms two glutathione molecules, which are linked to form glutathione disulfide (GSSG), a stable molecule. The reduction of glutathione disulfide catalyzed by GR produces two molecules of GSH, which is one important substrate for GPX for decomposing hydrogen peroxide to water. The substrate, GSH, used from the pool during the detoxification of hydrogen peroxide is maintained by the glutathione reductase.

Thiols: Thiols contain highly active SH group and therefore having antioxidant property. The most studied endogenous antioxidant thiols are lipoic acid and glutathione. Lipoic acid and dihydrolipoic acids are present in most kind of cells. Properties and therapeutic effects of LA and DHLA are well reviewed (Fuchs et al., 1997; Sies, 1997). DHLA is an

efficient scavenger of all oxygen radicals; however, LA is active only in the reaction with highly reactive hydroxyl radicals.

Ubiquinones: Ubiquinones are essential electron carriers in the mitochondrial electron transport chain. They shuttle electron from NADH and succinate dehydrogenase to the cytochrome b-c1 complex. There are two types of redox interaction, in which ubiquinone can manifest their antioxidant activity: the reaction with quinone and hydroquinone formation. The antioxidant activities of ubiquinone has been demonstrated in vitro and in vivo studies (Filipe et al.,2001; Robak et al., 1986; Silva et al., 2000)

Uric acid: Uric acid is another physiologically important antioxidant. Uric acid contains two active hydroxyl groups in the purine heterocycle. The physiological level of uric acid protects erythrocyte against free radical damage (Ames et al., 1981). It is also a major antioxidant in human airway mucosal surface (Peden et al., 1990).

*'**ADPH**:* NADPH is an indirect antioxidant due to its capacity to reduce various oxidized substrates. Recent study showed that NADPH possesses scavenging capacity against free radicals such as CO3•-, NO2•, ROO• and RO• (Kirsch and Groot, 2001).

Melatonin: Melatonin is a pineal hormone, which is synthesized from tryptophan. Melatonin is an effective scavenger of hydroxyl radicals, nitric oxide and peroxy nitrite (Reiter et al., 2000). It is an effective inhibitor of ironinitiated peroxidation of brain phospholipids liposome (Marshall et al., 1996).

1.7.2 Exogenous antioxidants.

Similar to endogenous antioxidants, some exogenous dietary compounds can neutralize the free radicals as well as enhance the activities of endogenous antioxidants. When the system is under oxidative stress and the endogenous antioxidants are not sufficient enough to scavenge the free radicals and ROS, the dietary antioxidants may be required

to maintain optimal cellular functions (Rahman, 2007). Some important dietary antioxidants are presented below. ***α-Tocopherol:*** α-Tocopherol is a lipid soluble phenolic antioxidant with an active hydroxyl group. Several authors reported the high antioxidant and antiradical activities of α-tocopherol (Burton et al., 1983; Doba et al., 1985; Lambelet and Loliger, 1984; Scarpa et al., 1984). However similar to many other antioxidants, α-tocopherol also shows pro-oxidant action under certain conditions (Terao and Matsushota, 1986; Upston et al., 1999; Weinberg et al., 2001).

Vitamin C: Ascorbic acid is a highly active free radical scavenger and strong reducing agent. Oxidation and reduction reactions of ascorbic acid with numerous oxidants and reductants are well studied (Afanas'ev, 1989). Other than its antioxidant properties pro-oxidant activities also well studied. It is known that the competition between antioxidant and pro-oxidant activities of ascorbic acid depends on the rate of reaction (Afanas'ev et al., 1987; McCay et al., 1978). Ascorbic acid at lower concentration enhanced lipid peroxidation but inhibited at higher concentration (Afanas'ev et al., 1989). Presence of other factor also promotes the pro-oxidant activity of ascorbic acid. In the presence of Fenton's reactants, ascorbic acid promotes the hydroxyl radical production by redox cycling of iron ion (Benherlal and Arumughan, 2008).

Carotenoids: Hundreds of carotenoids are found in nature but relatively a few are found in human tissues, the five main carotenoids are; β-carotene, lutein, lycopene, β-cryptoxanthin, and α-carotene (Bendich and Olson, 1989; Rock et al., 1996; Thurnham, 1994). The antioxidant properties of the carotenoids closely relate to the extended system of conjugated double bonds, which occupies the central part of carotenoid molecules, and to the various functional groups on the terminal ring structures (Mathews-Roth, 1974; Stryker, 1988; Thurnham, 1997). The reactive oxygen species scavenged by carotenoids are singlet oxygen and peroxyl radicals (Foote and Denny, 1968; Palozza and Krinsky, 1992). In this process the carotenoid absorbs the excess energy from singlet oxygen and then releases it as heat. Singlet oxygen is generated during photosynthesis; therefore, carotenoids are important for protecting plant tissues, but there is some evidence for an

antioxidant role in humans. β-Carotene has been used in the treatment of erythropoietic Protoporphyria (Mathews-Roth, 1986). Using in vitro studies, they showed that β carotene was effective in reducing the rate of lipid peroxidation at the low oxygen concentrations found in tissues (Johnson and GR, 1993; Terao, 1989).

Flavonoids: Flavonoids are naturally occurring low molecular weight phenolic compounds widely distributed in plant Kingdom. Huge amount of literature is available on the antioxidant activates of flavonoids. Flavonoids are reported to have multiple biological activities such as anti-inflammatory, antidiabetic, antiallergic, antiviral, anticancer etc. (Critchfield et al., 1996; Havsteen, 1983).

Since they are polyphenols, their antioxidant activities depend on the hydroxyl groups. Flavonoids are generally good scavengers of peroxyl radicals, hydroxyl radical and superoxide radicals (Denisov and Afanas′ev, 2005). Other nonflavonoid phenolic compounds also possess in vitro and in vivo antioxidant activity. One of the well-studied compound is resveratrol, which has been identified as potential antioxidant, anticancer and antimutagenic agent (Jang et al., 1997).

Steroids: Some steroid molecules such as estrone, estradiol, and estriol has phenolic hydroxyl group and therefore are able to react with free radicals. All the above said compounds are reported to inhibit liposomal lipid peroxidation (Nakano et al., 1987; Sugioka et al., 1987). The role of phenolic hydroxyl group in the steroid molecules have been studied using various steroids. Only phenolic hydroxyl group containing steroids inhibited lipid peroxidation (Huber et al., 1990).

1.7.3 Antioxidants and disease prevention

In vitro as well as in vivo cell culture studies showed that intra cellular and extracellular antioxidants may prolong the onset or progression of degenerative diseases such as

diabetes, cancer, and cardiovascular diseases etc. the literature evidences showing role of antioxidants in a few degenerative disease are presented below.

Cancer: In vitro and in vivo research indicated that some dietary antioxidants show anticancer activity. Strong antioxidants such as pyrrolidine dithiocarbamate (PDTC) and N-acetyl cysteine (NAC) inhibited growth of human colorectal cell in culture and when fed to mice with implanted tumors (Chinery et al., 1997). Another similar study showed that consumption of antioxidant rich tea reduced the risk of breast cancer several fold (Hirvonen et al., 2006). Though the mechanism of action of antioxidants and low cancer risks are not clear, there are some indications about the activation of tumor suppressor genes which are inactivated in almost one half of human tumors (Chinery et al., 1997). It was shown that the possible first step in the activation of tumor suppressor genes is by antioxidant-induced activation of protein kinase A. The reduced form of protein kinase A activated by associating with plasma membrane, and triggers the downstream events. The modulation of activity of protein kinases by antioxidants is one attractive way to improve current cancer therapies and prevention strategies. Antioxidants are also reported to induce apoptosis by the activation of nuclear factor *k*B (NF*k*B). Pyrrolidinedithiocarbamate (PDTC) inhibited translocation of NF*k*B from cytoplasm to nucleus and prevented expression of several antiapoptotic proteins (Gunawardena et al., 2005; Gunawardena et al., 2002). Antioxidants also inhibit or delay the onset and progression of cancer possibly by inhibition other factors such as angiogenesis and endothelial nitric oxide synthase. (Bai et al., 1998; Hesketh, 1997; Kuzumaki et al., 1998). Moreover, when used with chemotherapy agents such as 5-fluorouracil and doxorubicin, antioxidants enhance the cytotoxicity of chemotherapy agents and cause complete remissions, where only partial remission is possible with chemotherapy agents alone (Bach et al., 2001; Chinery et al., 1997).

Diabetes: Pancreatic β-cell dysfunction together with insulin resistance is associated with the development of type 2 diabetes. Various authors have shown the significance of hyperglycemia as a direct cause of β-cell glucose toxicity in vivo (Zangen et al., 1997)

and in vitro (Olson et al., 1993; Poitout et al., 1996; Robertson et al., 1992; Sharma et al., 1995). At the onset of insulin resistance and hyperglycemia, β-cell function progressively deteriorates and subsequently glucose induced insulin secretion becomes further impaired and β-cells number decreases as a result of degranulation (DeFronzo et al., 1992; Porte, 1991; Vinik et al., 1996; Yki-Jarvinen, 1992). Impairment of β-cell function happens at the level of insulin synthesis as well as insulin secretion (Olson et al., 1993; Poitout et al., 1996; Robertson et al., 1992; Sharma et al., 1995). One important reason attributed for the hyperglycemia induced dysfunction is through hyperglycemia mediated production of free radicals and ROS by the glycation of biomolecules (Hunt et al., 1991; Sakurai and Tsuchiya, 1988). Although the induction of the glycation reaction in diabetes was originally found in neural cells and the lens crystalline, which are known targets of diabetic complications, another target that accelerate the progression of DM was shown to be the β-cell (Ihara et al., 1999; Kaneto et al., 1996). Moreover the ROS thus produced also play significant role in the development of other complications related to diabetes (Baynes, 1991). In vitro studies that simulated β-cells in hyperglycemic condition showed several glycosylation end products as well as oxidative stress markers (Ihara et al., 1999). It has also been noted that under hyperglycemic condition, expression of cellular endogenous antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are down regulated in islet cells compared with other tissues and cells (Tiedg et al., 1997). Therefore, once β-cells face oxidative stress, they may be rather sensitive to it, suggesting that glycation and subsequent oxidative stress may in part mediate the toxic effect of hyperglycemia. As direct support for this, another study showed that glycation mediated ROS production reduces insulin gene transcription (Matsuoka et al., 1997). Conclusively the antioxidant treatment is beneficial for treating diabetes and can provide protection to β- cells against glucose toxicity. This is supported by a study (Kaneto et al., 1999) in which the cells under hyperglycemic condition treated with N-acetyl-L (NAC), vitamins C and E showed protective activity. Though vitamin C and E did not show significant effects when used alone, they exerted some beneficial effects when used in combination with NAC. A recent study of 2285 men and 2019 women, 40-69 years aged,

showed that intake of vitamin C, carotenoids and tocopherols reduced the development of type 2 diabetes (Montonen et al., 2004). Thus a sufficient supply of antioxidants may prevent or delay β-cell dysfunction in diabetes by providing protection against glucose toxicity.

Cardio vascular disease: Quite a lot of recent studies have demonstrated that altered oxygen utilization and/or increased formation of ROS contribute to atherogenesis and CVD progression. Several sources of oxygen/nitrogen species do occur in CVD. Intracellular ROS are formed during mitochondrial electron transport chain and are controlled by antioxidant defense system. However several studies suggested that oxidative stress as well as polymorphic variations in endogenous antioxidants are linked to increased risk for atherosclerosis and CVD (Hiroi et al., 1999). Immediate targets of ROS are long-chain free fatty acids in the cytosolic compartment and membrane-bound lipids however chemically vulnerable substrates to ROS are the polyunsaturated fatty acids in the lipoproteins. Free radicals attack plasma low density lipoprotein (LDL) that is oxidatively modified to oxidized low density lipoprotein (oxLDL) leading to the attraction of blood monocytes beneath the endothelium. Monocytes differentiate into macrophages that are converted to foam cells, full of cholesterol and oxidized lipids. Foam cells form the early atherosclerotic lesions, which are documented as the pathogenesis of CVD (Berliner et al., 1995). Based on the 'oxidation theory' for atherosclerosis, dietary antioxidants have attracted considerable attention as preventive and therapeutic agents. There is adequate evidence from observational, in vitro, ex vivo, controlled intervention and animal model studies that consumption of certain extracts contain vitamin C and E, β carotene, and polyphenols results to a reduction in oxidative stress and myocardial infarction biomarkers (Kalioraa et al., 2006). There is a large body of observational studies on the dietary antioxidant intake link to prevention of CVD progression. Amongst the most established are; inverse correlation between death for myocardial infarction and Vitamin E (Stephens et al., 1996), inverse correlation between vitamin C intake and carotid wall thickening (Kritchevsky et al., 1995), vitamin C

deficiency and associated increased risk of myocardial infarction (Nyyss¨onen et al., 1997), Correlation between flavonoid intake and reduction in CVD mortality (Hertog et al., 1993), inverse correlation between carotenoids level and myocardial infarction (Street et al., 1994), the relationship between adipose tissue lycopene and the risk for myocardial infarction (Kohlmeier et al., 1997) etc.

1.8 Plant based different drugs

This is to say that either the drugs currently contain plant-derived materials, or synthesized materials from agents originally derived from plants. Some medicines, such as the cancer drug Taxol (from *Taxus brevifolia*) and the anti-malarial quinine from *Cinchona pubescens* and are manufactured from plants. (Medicinehunter.com, 2015)

1.8.1 Plant-based anticancer agents

Cancer is one of the second largest health problems worldwide after CVD and the new incidence is close to 6 million per year (Heron, 2010). The development of new anticancer drugs and more effective treatment strategies are the fields of utmost importance in drug discovery and clinical therapy. Much of the research in this area is currently focused on cancer-specific mechanisms and the corresponding molecular targets (McLaughlin et al., 2003). Conventional cancer therapy includes surgery, irradiation, and chemotherapy. Chemotherapy is based on the systemic administration of anticancer drug targeting localized tumors and metastized cancer cells. Chemotherapy is a rapidly developing field of cancer treatment with new drugs continuously being tested and developed, which includes plant secondary metabolites. Some secondary metabolites are considered as metabolic waste products. However, a significant portion of the products derived from secondary pathways serve either as protective agents against various pathogens or growth regulatory molecules to the plants. The protective nature of these secondary metabolites can be attributed to their capacity to induce toxicity to

invading organisms. This cytotoxicity can be exploited to treat fast proliferating cancer cells under optimized dose that are in sub-lethal levels to normal cells. The phytochemical classes which are known to have anticancer activities are; aldehydes, alkaloids, annonaceous acetogenins, flavonoids, glycosides, lignans, lipids (unsaponified), phenols and derivatives, and terpenoids (Kintzios and Barberaki, 2004).

Several phytochemicals having anticancer property are being clinically used at present and many are under clinical evaluation. In the process of identification of novel anticancer agents from plant materials, knowledge about the possible mechanisms of action of the active principles could contribute in developing structure function relation as well as developing new drug candidates by advanced computer aided designs and chemoinformatics studies. Mechanism of action and signaling pathways of several phytochemicals are known and based on these compounds' structure, many modified compounds have been synthesized with enhanced bioavailability and efficacy. A few standard anticancer phytochemicals viz. camptothecin, Taxol, combretastatin A-4, and podophyllotoxin are reviewed below

***Camptothecin*:** Camptothecin is a naturally occurring alkaloid isolated from the wood of Chinese tree *C.acuminata* (Wall, 1998). Preliminary studies showed substantial antitumor activity in standard in vitro systems and subsequently extended the studies in animals and human volunteers. Due to the severe toxicity, this has been withdrawn from the phase III clinical trials.

However, work had been continued to synthesize its analogues with low nonspecific toxicity. Presently the first generation analogues of camptothecin such as hycamtin and camptosar are marketed for the treatment of ovarian cancer and colon cancer (Gore et al., 2001; Saltz et al., 2000). Initial studies on the mechanism of antitumor activity showed that camptothecin selectively inhibited topoisomerase enzyme. Topoisomerases are enzymes involved in the uncoiling of super coiled double stranded DNA during replication (Potmesil and Kohn, 1991)

Paclitaxel: paclitaxel is a complex polyoxygenated diterpenoid isolated from the pacific yew, *Taxus brevifolia* (Wani et al., 1971). The drug Taxol was developed from paclitaxel by the National Cancer Institute, USA and commercially produced by Bristol-Myers Squibb. Taxol exhibits a unique mode of action by stabilizing microtubulin while the other anticancer agents destabilize this process (Schiff et al., 1979). The major drawback of taxol is its poor bioavailability.

Combretastatins: combretastatins are mitotic agents isolated from the bark of the South African tree *Combretum caffrum*. It has been found to be a potent cytotoxic agent that strongly inhibits the polymerization of tubulin by binding to the colchicine site. In vitro studies have shown that combretastatins competes with colchicine for binding sites on tubulin. Hence, it is a member of the colchicine-like inhibitors of microtubulin assembly (Hamel, 1996)

Podophyllotoxin: podophyllotoxin is a well-known naturally occurring aryltetralin lignans. Podophyllotoxin was first isolated in 1880 from the North American plant *Podophyllum peltatum* (Mukherjee et al., 2006). Podophyllotoxin shows strong cytotoxic activity against various cancer cell lines. It is effective in the treatment of Wilm's tumors, various genital tumors and in non-Hodgkin's and other lymphomas and lung cancer (Subrahmanyam et al., 1998; Utsugi et al., 1996). Podophyllotoxin acts as an inhibitor of assembly of microtubules and arrests the cell cycle in metaphase (Buss and Waigh, 1995; Gordaliza et al., 2000).

Other than the above discussed phytochemicals, several cytotoxic compounds have been isolated from plant sources. Many of them are currently in clinical trials or preclinical trials or undergoing further investigation. Betulinic acid, a pentacyclic triterpene, a secondary metabolite isolated from *Betula* species (Cichewitz and Kouzi, 2004), and *Zizyphus* species (Nahar et al., 1997; Pisha et al., 1995) is a potent selective cytotoxic agent against human melanoma cell lines (Balunas and Kinghorn, 2005). The development of systemic and topical formulations of the agent for potential clinical trials

by the NCI is ongoing. Pervilleine A was isolated from the roots of *Erythroxylum pervillei* Baill (Silva et al., 2001). Pervilleine A is also a selective cytotoxic against multidrug resistant (MDR) oral cancer cell in the presence of other anticancer agents. Pervilleine A is currently in preclinical development. Silvestrol was first isolated from the fruits of *Aglaila sylvestre* (Hwang et al., 2004). Silvestrol exhibited cytotoxicity against lung and breast cancer cell lines (Cragg et al., 1997). Biological studies are ongoing to determine the mechanism(s) of action for silvestrol. Two novel alkaloids, schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana* (Shoeb, 2005; Shoeb et al., 2005). Both of the alkaloids exhibited significant cytotoxicity against human cancer cells. Some important phytochemicals with potent cytotoxic activity in various cancer cells are presented below (Table 1-2).

Several plant-based synthetic compounds are also known to have potent anticancer activity. Flavopiridol is a rohitukine derived synthetic flavone, which was isolated from *Dysoxylum binectariferum* (Kelland, 2000), is a potent cytotoxic agent that enhances radioresponsiveness in various tumors (Christian et al., 1997; Kathy et al., 2004). Synthetic roscovitine, a derivative of olomucine originally isolated from *Raphanus sativus*, is a strong cytotoxic agent which is now under Phase II clinical trials in Europe (Cragg and Newman, 2005; Cragg et al., 1997). Importance of phytochemicals as anticancer drug is evidenced by their huge demand. it is estimated that camptothecin, a plant derived cytotoxic agent, accounts for nearly one-third of global anticancer market (Oberlines and Kroll, 2004). Though there are more than 270000 higher plants in this planet. Only a small portion has been explored phytochemically for anticancer potential. Therefore it is assumed that plants can provide potential chemical entities for development of new anticancer drugs.

Table 1-2 Anticancer activities of a few phytochemicals in various cancer cells

Cancer type	Cell type	Compounds	References
Human oral cancer	HSC-2, HSG, SCC-25	Flavanones, isoflavans, EGC, chalcones, EGCG, curcumin, genistein, ECG, quercetin, cisplatin	(Elattar and Virji, 2000; Elattar and Virji, 2000; Fukai et al., 2000; Sakagami et al., 2000; Shi et al., 2001)
Human breast cancer	MCF-7	Flavanones, daidzein, genistein, quercetin, luteolin	(Han et al., 2001; Pouget et al., 2001)
Human thyroid cancer	ARO, NPA,WRO	Genistein, apigenin, kaempferol, chrysin, luteolin, biochanin A	(Yin et al., 1999; Yin et al., 1999)
Human lung cancer	SK-LU1, SW900, H441, H661, haGo-K-1, A549	Flavone, quercetin	(Bai et al., 1998; Caltagirone et al., 1997)
Human prostate cancer	LNCaP, PC3, DU145	Catechin, epicatechin, quercetin, kaempferol, luteolin, genistein, apigenin, myricetin, silymarin	(Agarwal, ; Bhatia and Agarwal, 2001; Kampa et al., 2000; Knowles et al., 2000)
Human colon cancer	Caco-2, HT-29, IEC-6, HCT-15	Flavone, quercetin, genistein, anthocyanin	(Kamei et al., 1998; Kuntz et al., 1999; Kuo, 1996; Kuo et al., 1997; Wenzel et al., 2000)
Human leukaemia	HL-60, K562, Jurkat	Apigenin, quercetin, myricetin, chalcones	(Chung et al., 1999; Csokay et al., 1997; Vincenzo et al., 2000; Wang et al., 1999)
B16 mouse melanoma	4A5	Chalcones	(Iwashita et al., 2000)

Colon cancer	SW620	2-hydrocycinnaldehyde	(Lee et al., 2007)
Prostate cancer	LNCaP, PC-3/AR	camptothecin	(Liu et al., 2010)
nasopharyngeal	KB-VIN	quassinoids	(Murakami et al., 2004)
Leukemia, fibrosarcoma, lung cancer, colon cancer, melanoma, breast cancer	P-388,KB, HT-1080, LU-1, COL-2, MEL-2, BC-1	22-Hydroxytingenone	(Bavovada et al., 1990)
leukemia	P388	4-Hydroxy-2-cyclopentenone	(Perry et al., 1991)
Cervix	HeLa	6,7-dehydrototarol	(Moujir et al., 2008)
leukemia	P-388	wikstromol	(Torrance et al., 1979)
Larynx and Lung cancer	HEp-2, PC-13	3-(3,3-dimethylallyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone	(Arisawa et al., 1990)

1.8.2 Plant-based antidiabetic agents

Diabetes mellitus (DM) is a growing public health concern worldwide. DM is a chronic metabolic disease primarily due to the disorder of carbohydrate metabolism. Cause of which is the deficiency or diminished effectiveness of insulin resulting in hyperglycemia, as a result of uncontrolled hepatic glucose output and reduced uptake of glucose by skeletal muscle (fasting plasma glucose > 7.0mM/l or post prandial plasma glucose > 11.1 mM/l), and glycosuria. Secondary abnormalities of hyperglycemia may occur in the metabolism of proteins, fats, water, and electrolytes in tissues/organs (Leon and Stanley, 2009), sometimes with serious consequence including ophthalmic, cardiovascular, and renal diseases (Dean et al., 2006). Several statistical reports on the risks of diabetic death, warrants the need for new and alternative therapeutical/management methods to control this metabolic disorder.

According to the National Vital Statistics Report (NVSR), more than 180 million people worldwide have diabetes and the number is likely to more than double by 2030. It was estimated that in 2005, 1.1 million people died from diabetes and almost 80% of diabetes occurred in low and middle income countries. NVSR projects that diabetes death will increase over 80% in upper middle income countries by 2015 (Heron, 2010).

Several antidiabetic drugs have been isolated from plants and are currently being practiced; e.g. the α-glucosidase inhibitor acarbose and galegine that contributed to the discovery and development of the biguanides (Alan, 2010). On the other hand though we have evidences for the antidiabetic properties of traditionally used herbs, often, neither their mechanism nor their active components have been defined. Despite the lack of robust scientific data to support the efficacy of many such plants, they are still the main source of medication for patients with diabetes in many parts of the world. But currently, there is considerable interest in exploring traditionally known plant for identifying active principle. Some recent studies validated the antidiabetic potential of plants that are used in traditional medicine viz. *Melothriamaderaspatana* (Balaraman et al., 2010),

Dichrostachys glomerata (Kuate et al., 2010), *Oreocnide integrifolia* (Ansarullah et al., 2010), *Moringa. Oleifera* (Jaiswal et al., 2009), *Ricinus communis* (Shokeen et al., 2008) etc. Some active principles viz. phenylethyl cinnamaldehyde (Phuwapraisirisan et al., 2008) and aegline-2 (Narender et al., 2007) have also been recently isolated from *Aegle marmelos*, a traditionally used plant to treat diabetes. The active principles thus isolated can improve glucose metabolism as well as the overall condition of individuals with diabetes not only by hypoglycemic effects but also by improving lipid metabolism, antioxidant status etc. (Bailey and Day, 1989). Therefore investigations on antidiabetic agents from traditionally known plant might be useful in the clinic or that might have novel effects, such as stimulation of β-cell proliferation and therefore it is possible that novel mechanisms of action and novel compounds will be discovered.

Table 1-3 Anti diabetic activity of a few plants and their parts

Plant	Parts	Reference
A. marmelos .	Seed and fruit	(Kamalakkannan and Prince, 2003; Kesari et al., 2006)
Melothria maderaspatana.	aerial parts	(Balaraman et al., 2010)
Ricinus communis	root	(Shokeen et al., 2008)
Cynodon dactylon	leaves	(Singh et al., 2007)
Coccinia indica.	aerial parts	(Balaraman et al., 2010)
Murraya koenigii.	leaves	(Kesari et al., 2005)
S. cumini	**seeds**	**(Anandharajan et al., 2006)**
Ficus benghalensis.	stem bark	(Kar et al., 2003)
Dichrostachys glomerata.	Stem bark	(Kuate et al., 2010)
G. sylvestre.	leaves	(Kar et al., 2003)

Momordica charantia.	fruits	(Reyes et al., 2006)
Pterocarpus marsupium.	bark	(Vats et al., 2002)
Swertia punicea.	whole plant	(Tian et al., 2010)
Terminalia. bellirica.	fruit pulp	(Kar et al., 2003)
Tinospora. cordifolia	root	(Stanely et al., 2000)
Trigonella. foenum.	seed	(Zia et al., 2001)
Moringa. oleifera.	stem bark	(Jaiswal et al., 2009)
Ocimum sanctum.	leaves	(Vats et al., 2004)
Carissa edulis	leaves	(El-Fiky et al., 1996)
Oreocnide integrifolia	leaves	(Ansarullah et al., 2010)
Luffa aegyptiaca	seeds	(El-Fiky et al., 1996)
Tamarindus indica	seed	(Maiti et al., 2004)
Premna integrifolia	Whole plant	(Alamgir et al., 2001)
Tragia involucrata	leaf	(Kar et al., 2003)

Chapter 2: Pharmacognosy of *Enhydra fluctuans*

Pharmacognosy of *Enhydra fluctuans*

2.1 Names of *Enhydra fluctuans*

2.1.1 Common names

Commonly known as Enhydra (Engl.), Kankong-kalabau (Tag.), Buffalo spinach (Engl.), Marsh herb (Engl.), Water cress (Engl.), Zhao ju (Chin.). Some compilations refer to Enhydra fluctuans as Enydra fluctuans.(Stuartxchange.com)

2.1.2 Synonyms

Enydra anagallis Gardner, *Meyera fluctuans* (Lour.)Spreng.(Stuartxchange.com)

2.1.3 Other vernacular names

Bengali: Hingcha, helencha, hinche, hingcha, hincha,

India: harkuch, haruch, matsayaakshi,

Indonesia: Godobos.

Thai: Phak bung ruem.

Vietnamese: Câyraungo, Rau ngo, Ngotrâu, Ngodât,

Ngoh__ng

Sanskrit: achari, bramhi, chakrangi, helanchi, hilamochika, himamocika, jalabramhi, mambi, matsyakshi, matsyangi, mochi, rochi, sasasrutih, shankhadhara, trinittaparni, vishaghni.(Stuartxchange.com)

2.2 Plant classification

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: Enhydra

Specific Epithet: fluctuans Lour

Species: *Enhydra fluctuans* Loureiro. (Review)

2.2.1 The family Asteraceae

The Asteraceae are herbs, shrubs, or less commonly trees and are arguably the largest family of flowering plants, comprising about 1,100, 12 sub families and 23,000 currently accepted species that are characterized by having the flowers reduced and organized into an involucrate pseudanthium in the form of a head or capitulum. The leaves are alternate, opposite, or less commonly whorled, and range from simple to pinnately or palmately compound; stipules are absent. Subtending and often partly enclosing the florets of the head is one or more series of usually green, free or variously connate bracts called involucral bracts or phyllaries. Another kind of bract called a receptacular bract or chaff

may be associated with each disk floret throughout the head. The flowers are of two basic types: those with tubular actinomorphic corollas and those with strap-shaped or radiate zygomorphic corollas, often within the same head. This family has a remarkable ecological and economic importance and is present from the Polar Regions to the tropics, colonizing all available habitats. The Asteraceae may represent as much as 10% of autochthonous flora in many regions of the world.

Most members of Asteraceae are herbaceous, but a significant number are also shrubs, vines, or trees. The family has a worldwide distribution and is most common in the arid and semiarid regions of subtropical and lowers temperate latitudes.

The Asteraceae are an economically important family. Some members provide products, including cooking oils, lettuce, sunflower seeds, artichokes, sweetening agents, coffee substitutes, and herbal teas. Several genera are popular with the horticultural community, including marigold, pot marigold (also known as calendula), cone flowers, various daisies, fleabane, chrysanthemums, dahlias, zinnias, and heleniums. Asteraceae are important in herbal medicine, including Grindelia, Echinacea, yarrow, and many others. A number of species have come to be considered invasive, including, most notably in North America, dandelion, which was originally introduced by European settlers who used the young leaves as a salad green.

Plants in Asteraceae are medically important in areas that don't have access to Western medicine. They are also commonly featured in medical and phytochemical journals because the sesquiterpene lactone compounds contained within them are an important cause of allergic contact dermatitis. Allergy to these compounds is the leading cause of allergic contact dermatitis in florists in the US. Pollen from ragweed Ambrosia is among the main causes of so-called hay fever in the United States. (Herbarium.usu.edu)

2.2.2 The species *Enhydra fluctuans*

Enhydra fluctuans Lour. (Family: Asteraceae) is Perennial herb of swampy ground in coastal areas, till recently considered as a single species under the first name, but now recognized to be two: E. fluctuans only in the Niger Delta, but widespread in the tropics, and E. radicans from Senegal to Dahomey and Fernando Po.No usage of either species is recorded for the Region. The leaves of E. fluctuans are somewhat bitter and are eaten as a salad or vegetable in several tropical countries. In Zaïre *E. fluctuans* has been reported a favourite food of the hippopotamus. This plant is a prostate, spreading, annual herb. The stems are somewhat fleshy, 30 centimeters or more in length, branched, rooting at the lower nodes, and somewhat hairy. The leaves are stalkless, linear-oblong, 3 to 5 centimeters in length, pointed or blunt at the tip, usually truncate at the base, and somewhat toothed at the margins.

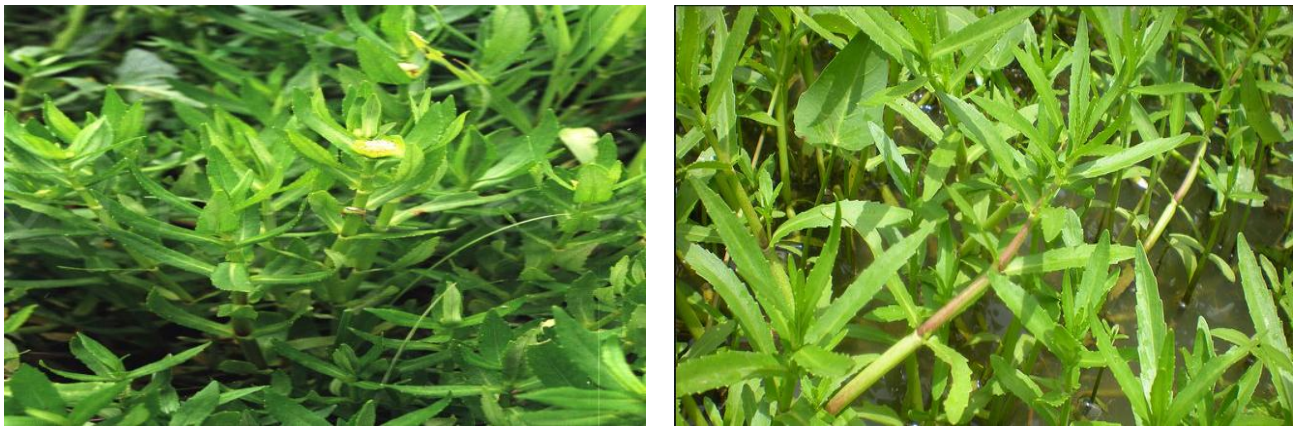


Figure: *Enhydra fluctuans*

The flowering heads are without stalks, are borne singly in the axils of the leaves, and excluding the bracts, are less than 1 centimeter in diameter. The outer pair of the involucral bracts is ovate and 1 to 1.2 centimeters long; the inner pair is somewhat smaller. The flowers are white or greenish-white. The acheness is enclosed by rigid receptacle-scales. The pappus is absent.Flower colour: beige, white. (Find Me A Cure)

2.2.2.1 Natural habitat

Enhydra fluctuans is a tropical herb, more sensitive to cold especially when very young. The species grows in and along ditches, water courses, margins of fish ponds and rice fields in the open, from sea-level up to 1,800 m. It is able to reproduce by fragmentation and may be so abundant that it clogs water courses. (Find Me A Cure)

2.2.2.2 Geographic distribution

This species is an old world species, possibly of Indochinese origin, which occurs in tropical Asia and Africa. It is common to all countries of Southeast Asia.(Find Me A Cure)

a. Native

Bangladesh; Benin; Burkina Faso; Cambodia; Cameroon; Congo, The Democratic Republic of the; Côte d'Ivoire; Ghana; India; Indonesia; Kenya; Lao People's Democratic Republic; Malaysia; Mozambique; Myanmar; Nigeria; Philippines; Rwanda; Senegal; South Africa; Sri Lanka; Sudan; Tanzania, United Republic of; Thailand; Togo; Uganda; Viet Nam; Zambia; Zimbabwe.(Find Me A Cure)

b. Present - origin uncertain

Australia

2.2.2.3 Edible uses

According to Burkill the young parts are used as a salad in several countries, including Malaya. Sometimes they are steamed before they are eaten. Guerrero reports that in the Philippines the leaves are pressed and applied to the skin as a cure for certain herpetic eruptions. In bengal it is

washed, chopped and cooked as Sag fry or boiled with rice and eaten with boiled rice with boiled potato, salt and mastered oil. Burkill reports that the young parts and the leaves of the plant are somewhat bitter and are used by the Malays as a laxative. Caius says that the leaves are useful in diseases of the skin and of the nervous system. The fresh juice of the leaves is prescribed in Calcutta as an adjunct to tonic metallic medicines, and is given in neuralgia and other nervous diseases. The leaves are antibilious. The expressed juice of the leaves is used as a demulcent in cases of gonorrhea; it is taken mixed with the milk of either a cow or a goat. As a cooling agent, the leaves are pounded and made into a paste which is applied cold to the head.

Watt quotes Forsyth, who states that the plant is useful in torpidity of the liver. An infusion should be made the previous evening. It is boiled with rice and taken with mustard oil and salt. (Find Me A Cure)

2.2.2.4 Medical uses

Laxatives, etc.; paralysis, epilepsy, convulsions, spasm; skin, mucosa. They are said to be a laxative, antibilious and demulcent. They are used in India in skin and nervous affections, and in the Philippines are applied to certain herpetic eruptions. (Find Me A Cure)

2.2.2.5 Chemical constituents

Plant is rich in protein and is a good source of _-carotene. It also contains saponins, myricyl alcohol, kaurol, cholesterol, sitosterol, glucoside, sesquiterpene lactones including germacranolide, enhydrin, fluctuanin and fluctuandin, a number of diterpenoid acids and their isovalerate and angelate derivatives, stigmasterol, cholesterol, sitosterol, glucoside, other steroids and gibberellins A9 and A13 have been isolated from this plant.(Mbpd.info)

2.2.2.6 Characteristics

Climate: Tropical

Habitat: Hydrophytic

Habit: Herb

Flower colour: Beige, white. (Find Me A Cure)

Chapter 3: Literature review

Literature review

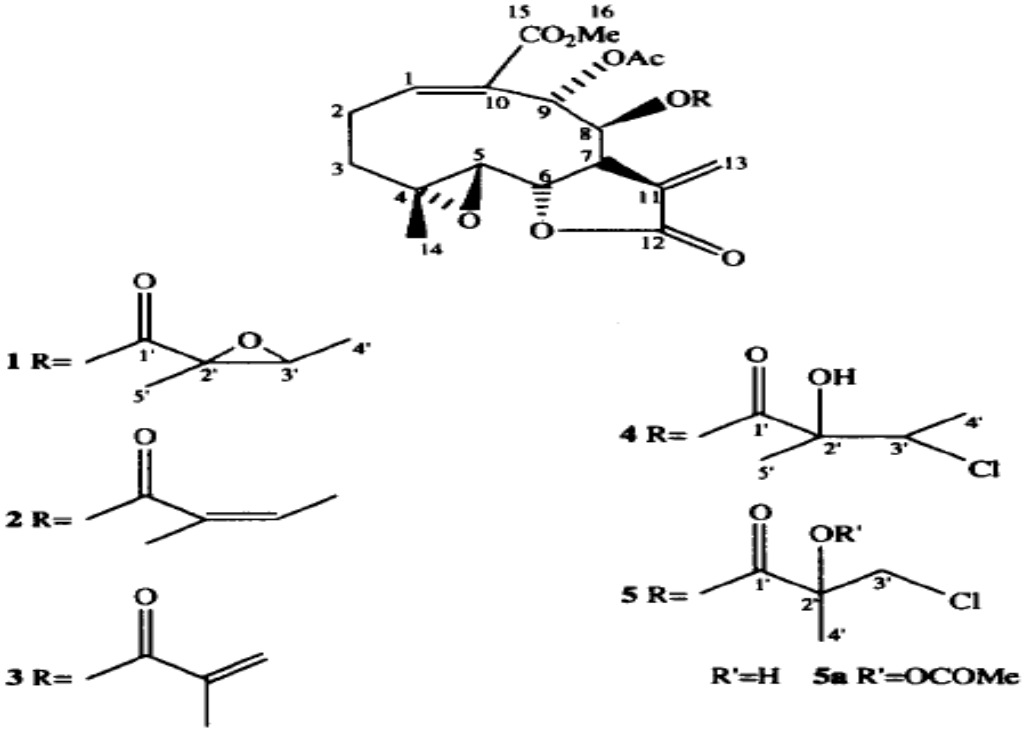
3.1 Chemical investigation

Previously the plant *Enhydra fluctuans* was studied for phytochemical investigation and it was investigated that it contains sesquiterpene lactone and flavonoids. Some of them are illustrated below:

- **Sesquiterpene lactones**

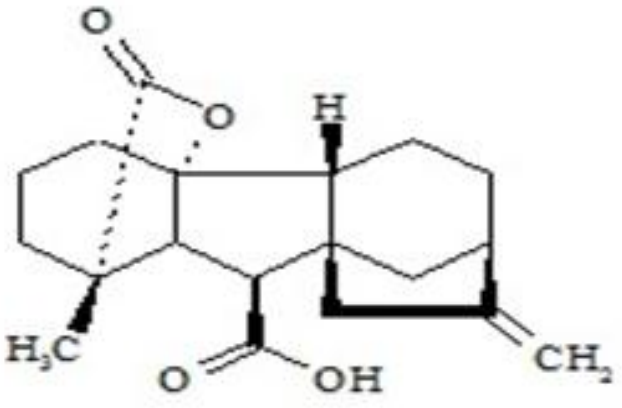
1. The structures given on next page are obtained from the petrol extract of *Enhydra fluctuans*-enhydrin (1), fluctuanin (2), fluctuadin (3) and two new chlorine containing melampolides (4 and 5) from the leaves of *E.fluctuans*. (N. R. Krishnaswamy 1995)

   The highly oxygenated gramacronalide enhydrin was isolated from *E. fluctuans* and its structure was also confirmed by x-ray analysis. (Wagner and Wolff)

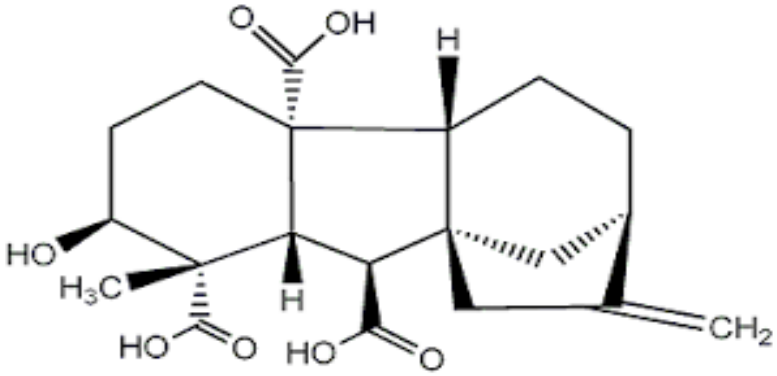


- **Flavonoids**

1. Gibberellins A9 and A13 were isolated from Enhydra fluctuans and identified on the basis of m.p., m.m.p., IR MS and chromatography. (Sciencedirect.com, 2015)

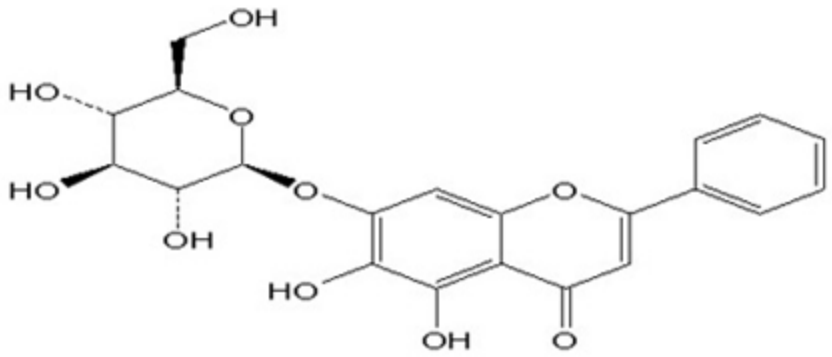


Gibberellin A9

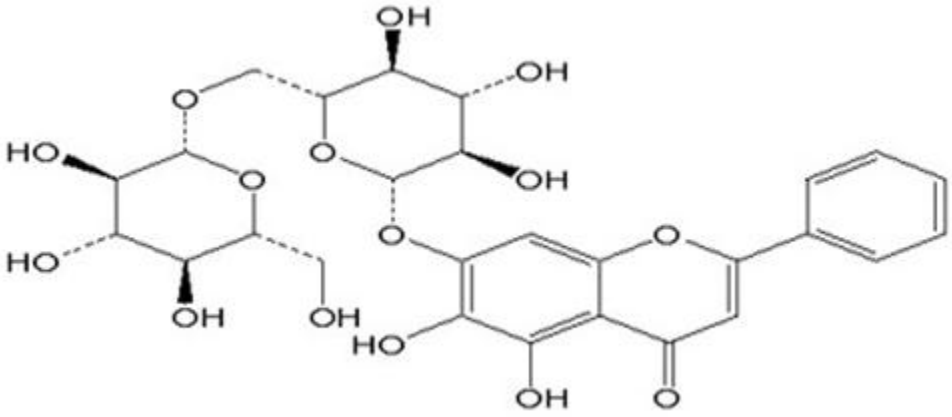


Gibberellin A13

2. Two flavonoids, baicalein 7-O-glucoside and baicalein 7-O-diglucoside, were isolated from the ethyl acetate fraction which showed analgesic and anti-inflammatory activity in different animal models. (Sannigrahi et al., 2011)

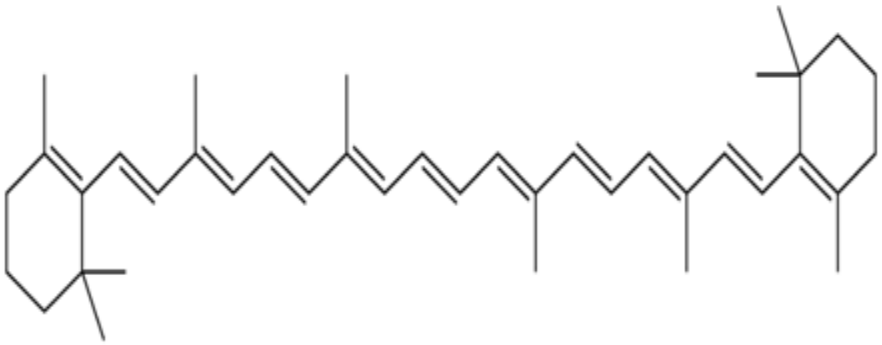


Baicalein-7-O-glucoside



Baicalein-7-O-diglucoside

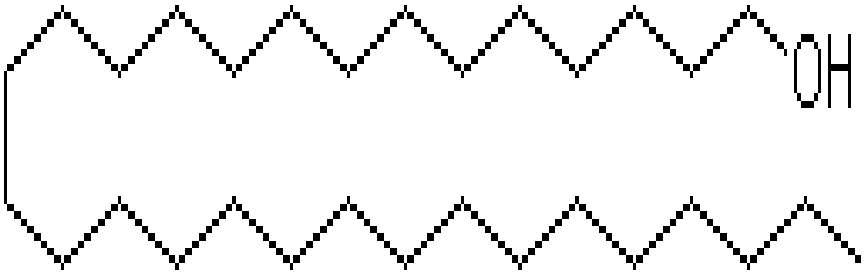
- An analysis on *E. fluctuans* showed that it is a good source of beta carotene (3.7 to 4.2 mg/100 g on a fresh weight basis). (Dewanji et al., 1993)



β- carotene

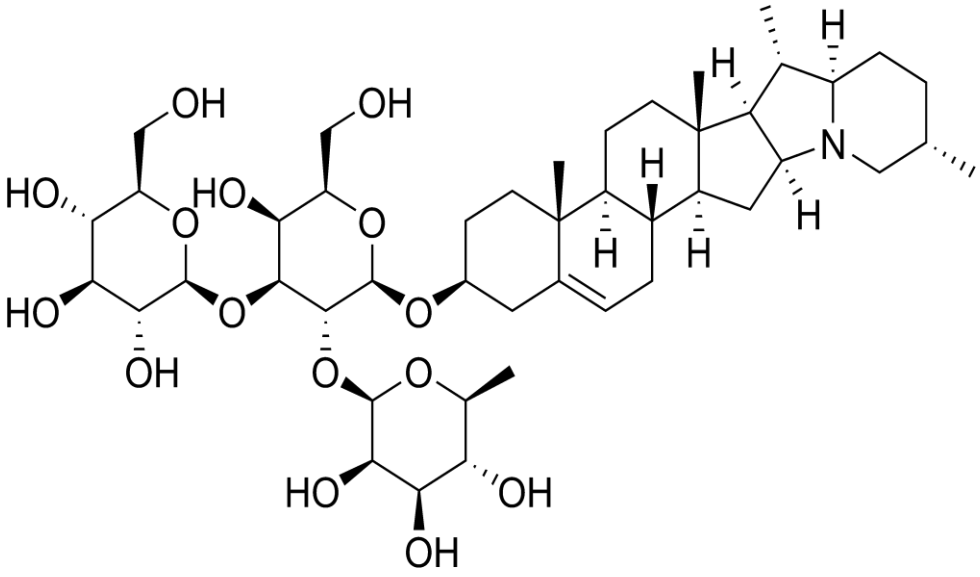
- Phtochemical analysis of *E. fluctuans* yielded alcohol, glycosides, and steroids. (Ghani, 2003).

- **Alcohol**



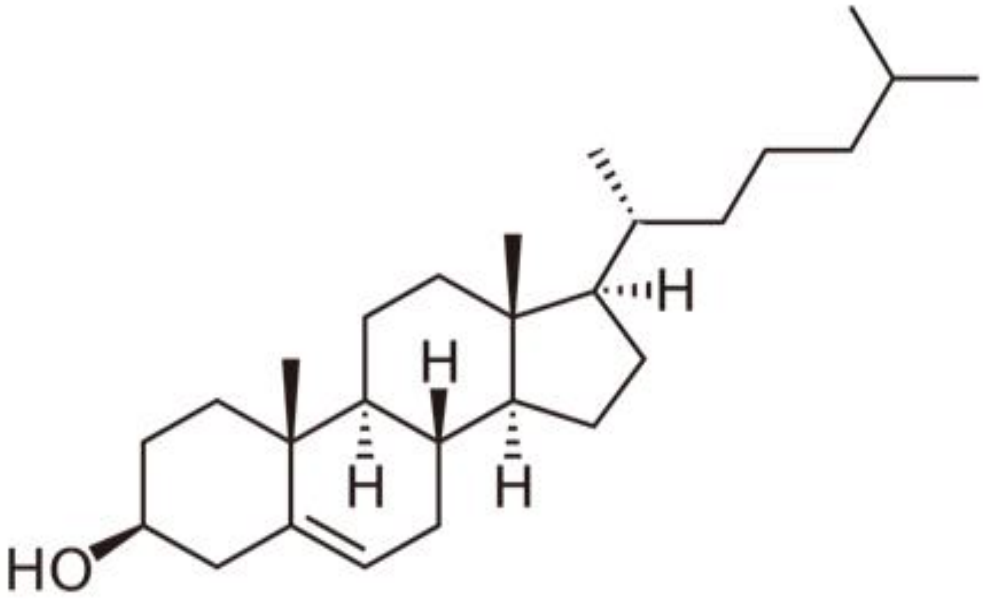
Myricyl alcohol

- **Glycoside**

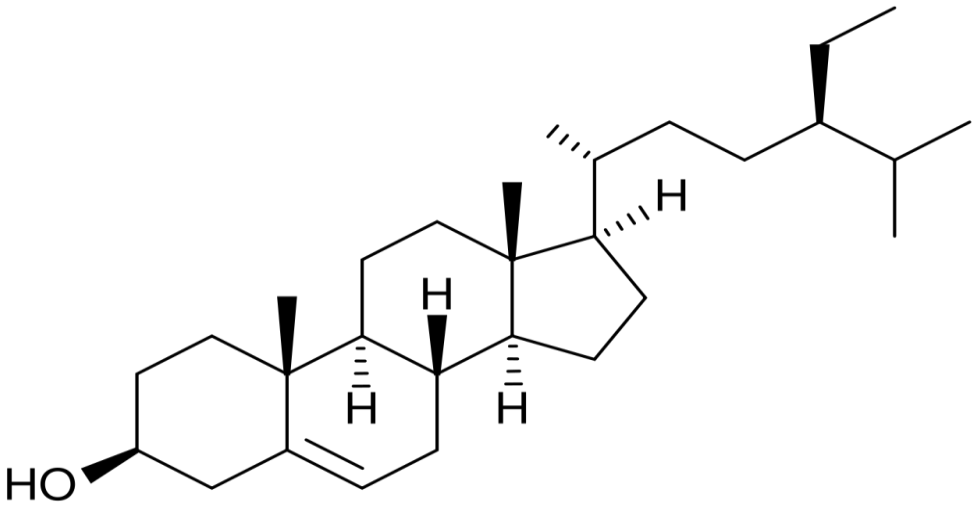


Saponin

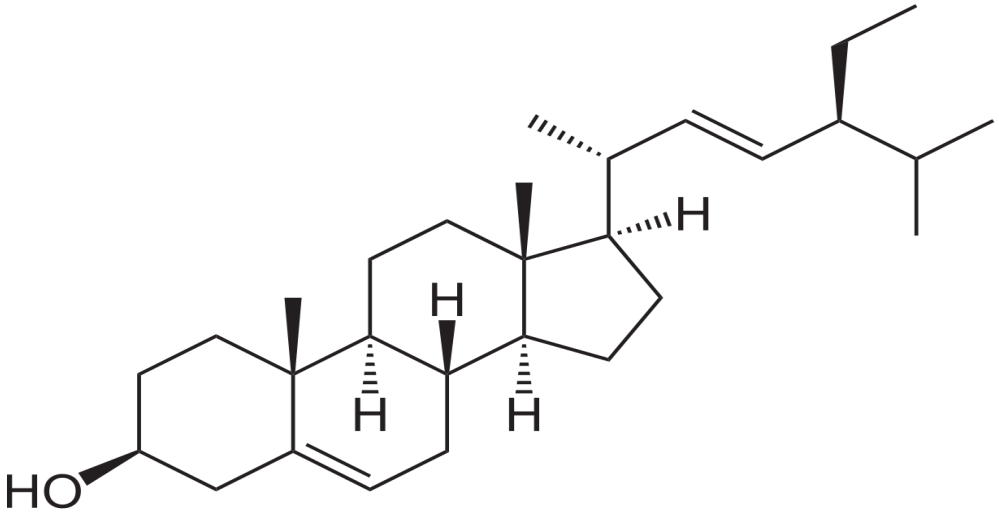
- **Steroids**



Cholesterol



Sitasterol



Stigmasterol

3.2 Pharmacological study

1. Antidiabetic activity

A study carried out by the Meitei-pangal community of the Thoubal district of Manipur states that Enhydra fluctuans extract can be effectively used as an antidiabetic plant by boiling and cutting it at the nodes. It was also found that the tribal practitioners of the Marakh sect of the Garo tribe living in Mymensingh of Bangladesh uses twelve medicinal plants for treatment of diabetes out of which *Enhydra fluctuans*is one of them.(Sarma et al.)

2. Antioxidant activity

The Ethanol (EEF), Chloroform (CEF) and Pet-Ether (PEEF) extracts of *Enhydra fluctuans* were evaluated for reducing power, total phenolic content, the DPPH scavenging activity, NO-scavenging activity and super oxide scavenging activity. In all the tests the ethanol extract was found to have higher antioxidant activity. (Sarma et al.)

3. Antimicrobial and activity

Enhydra fluctuanshas been found to have potent antibacterial activity against many gram positive as well as gram negative organisms. Some of them are *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus typhi, Staphylococcus aureus, Shigella boydii, Bacillus subtilis*.The plant has also been found to be possessing significant antifungal activities against selected fungi like *Aspergillus niger, Fusariumspp.and Aspergillus fumigatus.*(Sarma et al.)

4. Anti-inflammatory activity

The flavonoid isolated from leaves of *Enhydra fluctuans* shows anti-inflammatory activity by inhibiting COX-2 and 5-LOX. Moreover, flavonoid isolated from leaves of *Enhydra fluctuans* exhibits in vitro on key enzymes of arachidonic acid cascade involved in the mediation of inflammation. Based on it, in vivoanti-inflammatory activity of flavonoid fraction was evaluated by using carrageenan induced paw oedemaand cotton pellet induced granuloma. It significantly reduced the inflammation in such cases. (Sarma et al.)

5. Anti-cancer activity

Flavonoids obtained from *Enhydra fluctuans*(FEF) were screened for anticancer activity against Ehrlich's ascites carcinoma (EAC) bearing Swiss albino mice. Two flavonoids, baicalein 7-O-glucoside and baicalein 7-O-diglucoside, were isolated from the ethyl acetate fraction. Treatment with FEF caused a significant decrease in the tumour cell volume and increase of life span.(Sarma et al.)

6. Anti-diarrheal activity

The methanolic and aqueous extract was evaluated in experimental diarrhoea, induced by castor oil in mice. Both methanolic and aqueous extracts, given orally at a dose of 250mg/kg body weight showed significant anti-diarrhoeal activity. Results indicated strong anti-diarrheal activity of *Enhydra fluctuans.* (Sarma et al.)

7. Hepatoprotective activity

The flavonoid rich ethyl acetate fraction of *E. fluctuans* has significant hepatoprotective effects. The possible mechanisms of protection include the inhibition of lipid peroxidation and increase in the content of enzymatic defense

system, which cause the recuperation of biological parameters and the integrity of the tissue.

8. Analgesic activity

The methanol extract of *Enhydra fluctuans*, given orally at the dose of 250 and 500 mg/kg, was evaluated for its analgesic activity using the acetic acid induced writhing and the tail-flick methods. The extract showed promising activity in both tests. (Sarma et al.)

9. Neuroprotective potential

Identification and characterization of new medicinal plants to cure neurodegenerative diseases and brain injuries resulting from stroke is the major and increasing scientific interest in recent years. The Indian system of medicine out of the numerous medicinal plants showing promising activity in neuro-psychopharmacology *Enhydra fluctuansis* one of them. Some neuro-pharmacological effects of three fractions (Benzene, Chloroform and Ethyl Acetate) of methanolic extract of *Enhydra fluctuans* were studied in mice using various models. The study concludes that different fractions of *Enhydra fluctuans* aerial parts possess central nervous system depressant activity. (Sarma et al.)

10. Phagocytic and cytotoxic

The activity of aqueous extract of this plant have showed effective results on neutrophil phagocytic function. Different concentrations of the leaf extract were subjected to study its effect on different in-vitro methods of phagocytosis such as neutrophil locomotion, chemotaxis, and immune stimulant activity of phagocytosis of killed Candida albicans and qualitative nitro blue tetrazolium test using human neutrophils. (Sarma et al.)

Chapter 4: Materials

Materials

The following materials were used during the course of phytochemical study:

4.1 Solvents

- Petroleum ether
- Methanol
- Chloroform
- Acetic acid
- Ethyl acetate
- Toluene
- Acetone
- Benzene
- Dichloromethane

4.2 Glassware

- Thin layer chromatography (TLC) tank
- TLC plates, size in cm (20 x 20), (20 x 5)
- Precoated TLC plates
- Quick fit flasks
- Capillary tube
- Micropipette 1000 microliter

4.3 Equipment

- Rotary evaporator
- Grinding Machine
- Electronic balance
- Distill water maker
- UV Light

4.4 Silica gel

- TLC grade (PF-254)

4.5 Spray Reagent

- Vanillin-H_2SO_4

4.6 Filter aids

- Filter paper (Whatman no 1)
- Cloth
- Cotton pad
- 1000 ml Beakers

4.7 Figures of Equipment

Rotary Evaporator

Grinding Machine

Electronic balance

Distill water maker

Chapter 5: Method (Phytochemical investigation)

Method (Phytochemical investigation)

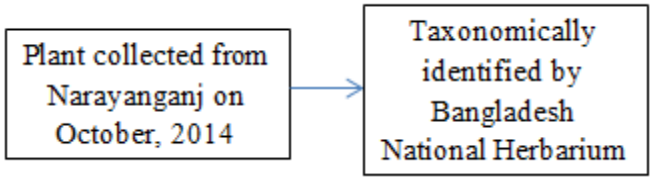
5.1 Selection of plants

Fresh plants of *Enhydra fluctuans* (family asteracea)were selected for biological investigation.

5.2 Collection of the plant part

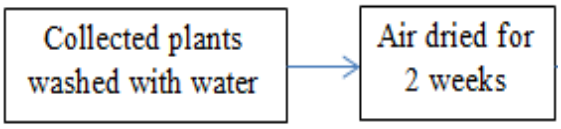
For this present investigation the plants of *Enhydra fluctuans* was collected from Naryangonj on October 2014.

The specimen of plant was taxonomically identified at the Bangladesh National Herbarium, Mirpur, Dhaka. These arefamiliar plant and widely distributed all over Bangladesh.



5.3 Drying of the plant part

The collected plants were washed with water and unwanted materials were discarded. Collected plants were air and sun dried for 14 days.

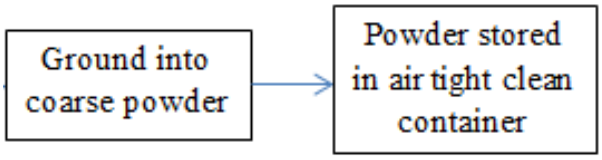


5.4 Storage and Preservation of plant part

Most plant parts from desired plants have undergone a period of storage before they were finally used for research purpose in the laboratory. During this period many undesirable changes may occur in the plant parts if they were not properly stored and preserved against the reabsorption of moisture, oxidation, excessive heat, humidity, direct sunlight, growth of molds and bacteria and infestation by insects and rodents. Proper storage and preservation of plant parts are thus are very important factors in maintaining a high degree of quality in them. All efforts towards proper storage should be geared to protect the drugs from all the above deteriorating factors and agents.

5.5 Grinding of the plant parts

The dried small pieces of plant parts were grinded into small fine particles by a grinder machine from. The powder was stored in an air tight container and kept in a cool dark and place until analysis commenced.



5.6 Cold extraction

On 27th November, 2014, 500gm of coarse plant powder was soaked in 1500ml distilled methanol at room temperature. The solvent was added in powder in such a way so that the solvent front remains at least three inches above the powder. The mixture was then kept in an air tight container for 72 hours. The container was occasionally stirred to soak the powder homogenously.

On 2nd December 2014, 177.05gm of coarse plant powder was again soaked in 750ml distilled methanol at room temperature. The solvent was added in powder in such a way so that the solvent front remains at least three inches above the powder. The mixture was

then kept in an air tight container for 72 hours. The container was occasionally stirred to soak the powder homogenously.

On 17th January, 2015, 500gm of coarse plant powder was again soaked in 1500ml distilled methanol at room temperature. The solvent was added in powder in such a way so that the solvent front remains at least three inches above the powder. The mixture was then kept in an air tight container for 72 hours. The container was occasionally stirred to soak the powder homogenously.

5.7 Filtration of extract

After three days of cold extraction, the solvent was decanted and filtered through two-folded fine cotton cloth and 1480ml of filtrate was obtained.

5.8 Drying of extract

Using rotary evaporator, the methanolic extract of plant was evaporated at 55-60 degree Celsius temperature and a rotation speed of 160-180 rpm for 1 month. After this drying process, a slurry concentration were obtained, which were kept in small 50 ml beakers for further drying. During transfer to the beaker the extracts were rinsed by acetone.

5.9 Separation of oil part

The crude extract was kept untouched for several days. And after this, an oil layer was formed on the upper surface of the extract. The oil was then separated from the upper surface of the extract through decantation. The oil portion separated from extract was 27ml. the extract portion remained after decantation is termed as crude extract by which further analysis was commenced.

5.10 Thin Layer Chromatography (TLC)

5.10.1. Principle

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. It may be performed on the analytical scale as a means of monitoring the progress of a reaction, or on the preparative scale to purify small amounts of a compound. TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and speed of separation. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots.

They all have a stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates.

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

As the solvent moves past the spot that was applied, equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is

used to visualize the plates. (The plate itself contains a fluorescent dye which glows everywhere except where an organic compound is on the plate.).

5.10.2 Retention Factor

After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent. The Rf formula is

Rf = distance traveled by sample / distance traveled by solvent

The Rf value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions, the compound with the larger Rf value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower Rf value.

Rf values and reproducibility can be affected by a number of different factors such as layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and solvent parameters. These effects normally cause an increase in Rf values. However, in the case of layer thickness, the Rf value would decrease because the mobile phase moves slower up the plate.

If it is desired to express positions relative to the position of another substance, x, the Rx (relative retention value) can be calculated:

Rx=distance of compound from origin / distance of compound x from origin

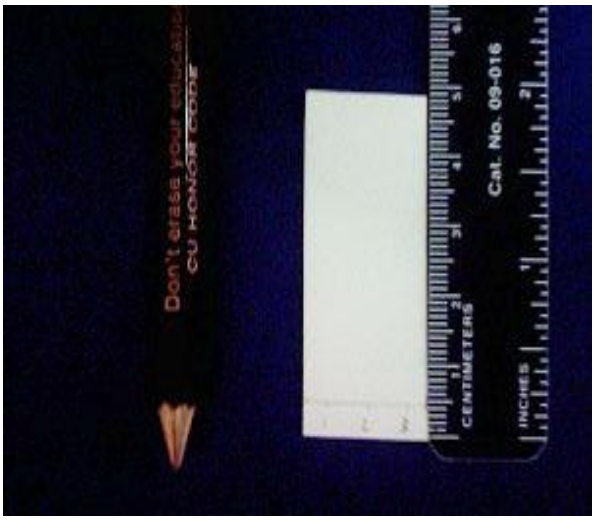
Rx can be greater than 1.

5.10.3 Materials & reagents

1. TLC plate
2. TLC tank
3. Cutter
4. Scale
5. Pencil
6. Solvents & reagents
   - ✓ Methanol
   - ✓ Ethanol
   - ✓ Ethyl acetate
   - ✓ Benzene
   - ✓ Diluted sulfuric acid
7. Hot plate

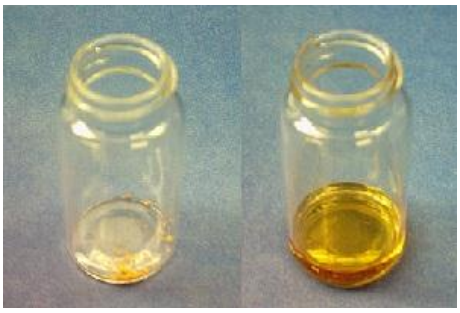
5.10.4 Test TLC procedure

1. Each large TLC sheet (made up of metal & coated with silica) was cut horizontally into plates which are 5 cm tall by 1.5cm in widths. Handle the plate was carefully handled so that the coating of adsorbent was not damaged.

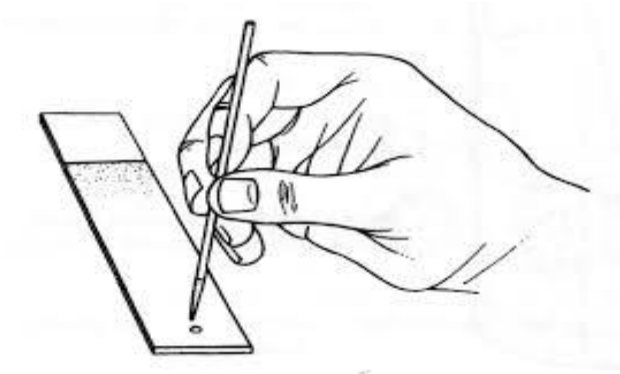


2. 0.5 cm from the bottom of the plate was measured. Using a pencil, draw a line was drawn across the plate at the 0.5 cm mark. This is the origin: on which the extract was spotted. The name of the samples spotted on the plate was lightly marked under the line.

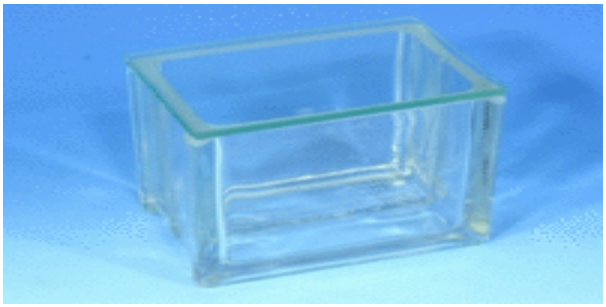
3. ~1 mg of the hard extract was dissolved in 1 ml of methanol in a watch glass to make the solution of the extract.

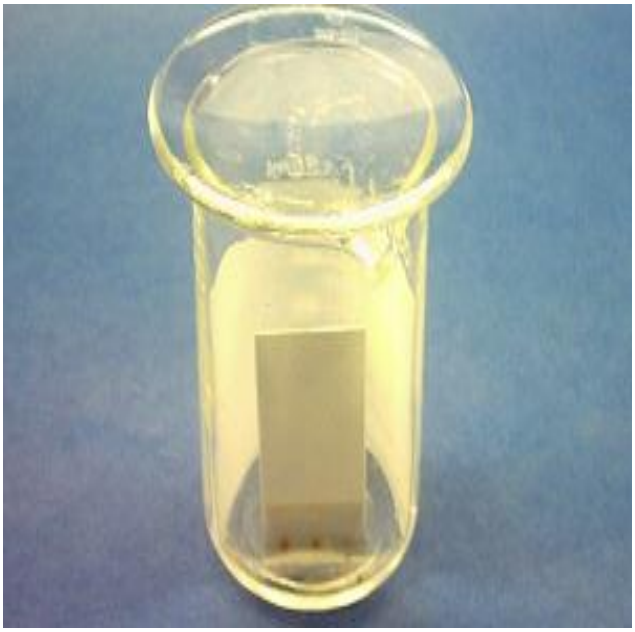


4. The prepared extract solution was then spotted on TLC plate



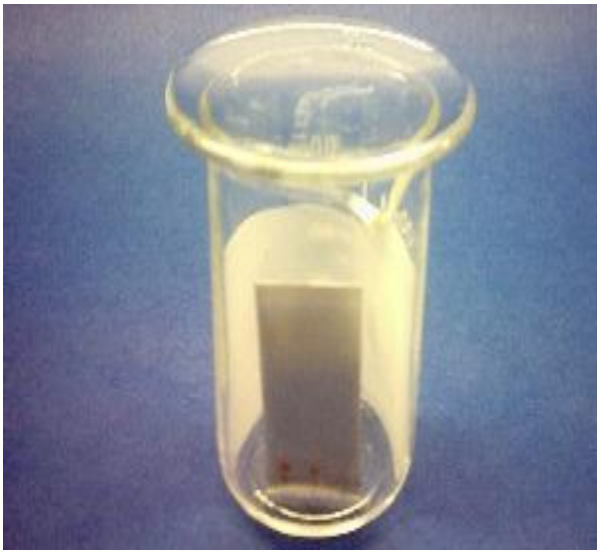
5. Measured solvents were taken in TLC tank

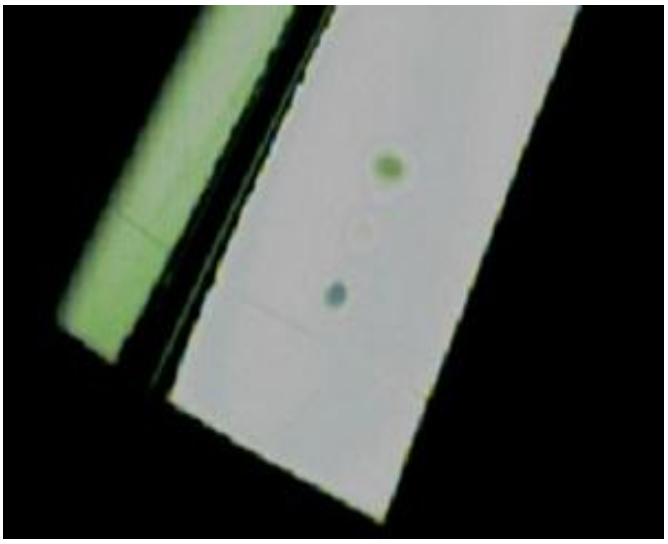




6. The prepared TLC plate was placed in the tank in such a way so that the solvent front remains under the drawn line, the tank was covered with the lid, and left undisturbed on the bench top. The solvent rose up the TLC plate by capillary action. Make sure the solvent does not cover the spot

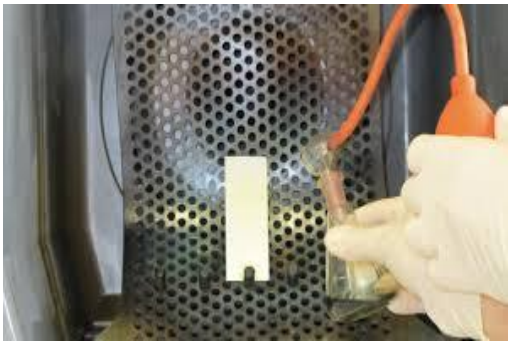
7. The plate was developed until the solvent is about half a centimeter below the top of the plate. The plate was removed from the beaker and the solvent front was immediately marked with a pencil. The plate was allowed to dry.





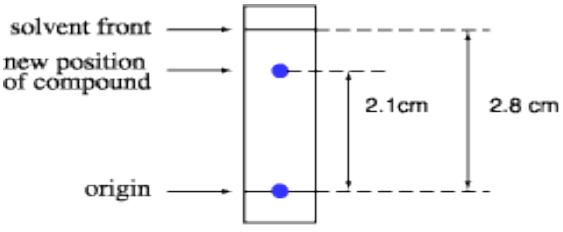
8. The plate was then kept under UV lamp to observe any band if appeared.

9. The TLC plate was then sprayed by sulfuric acid in fume hood.



10. The plate was then heated on hot plate so that the band color can be appeared clearly.

11. Then Rf valued by measuring the distance traveled by the sample and the distance travled by the solvent in the TLC plate.



$$R_f = \frac{2.1}{2.8} = 0.75$$

5.10.5 Different solvents used as mobile phase and results based on this in test TLC

Test TLC was performed for the crude extract of *Enhydra fluctuans* on the basis of trial & error for several times by changing the ratio of solvents used as mobile phase.

- **Using polar solvents**

1. **Set-A:**

Reagent	Amount
Ethyl acetate	8 ml
Ethanol	2 ml

❖ **Result:**

- An orange band was observed under UV lamp.
- Distance traveled by the sample = 5.5
- Distance travled by the solvent = 5.6
- Therefore Rf value = 5.5/5.6 = 0.98

Set-B:

Reagent	Amount
Ethanol	10 ml

❖ **Result:**

- Two orange bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 1.3
- Distance traveled by the sample for band (2) = 5.4
- Distance travled by the solvent = 5.6
- Therefore Rf value for band (1) = 1.3/5.6 = 0.232

  Rf value for band (2) = 5.4/5.6= 0.964

2. Set-C:

Reagent	Amount
Ethyl acetate	10 ml

❖ Result:

- Two orange and one green bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 0.7
- Distance traveled by the sample for band (2) = 1.5
- Distance traveled by the sample for band (3) = 4.7
- Distance travled by the solvent = 5.
- Therefore Rf value for band (1) = 0.7/5 = 0.14

  Rf value for band (2) = 1.5/5 = 0.3

  Rf value for band (3) = 4.7/5 = 0.94

- **Using non polar solvents**

1. **Set A:**

Reagent	Amount
Benzene	9 ml
Ethanol	1 ml

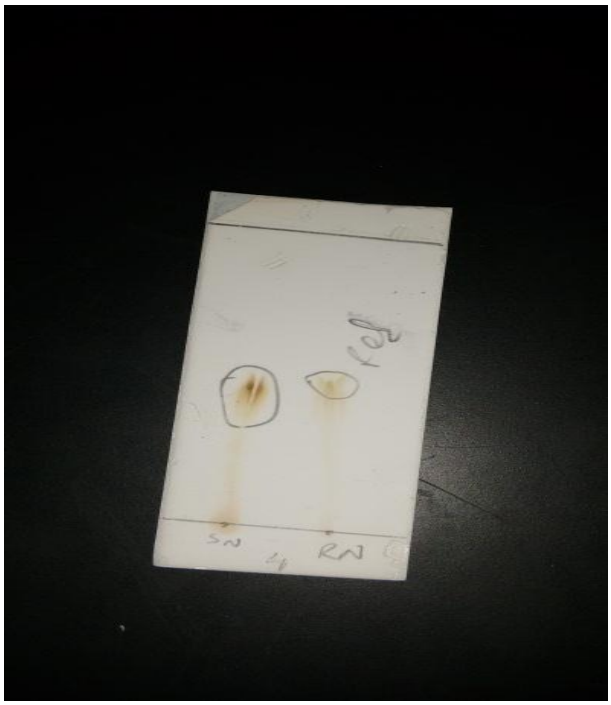
❖ **Result:**

- An orange and a green bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 1.2
- Distance traveled by the sample for band (2) = 2.4
- Distance traveled by the solvent = 7.3
- Therefore Rf value for band (1) = 1.2/7.3 = 0.164

  Rf value for band (2) = 2.4/7.3= 0.329

2. **Set B:**

Reagent	Amount
Benzene	6 ml
Ethanol	4 ml



❖ **Result:**

- An orange band was observed under UV lamp.
- Distance traveled by the sample for band = 2.6
- Distance traveled by the solvent = 5.2
- Therefore Rf value for band = 2.6/5.2 = 0.5

3. **Set B:**

Reagent	Amount
Benzene	5 ml
Ethanol	5 ml

❖ **Result:**

- An orange band was observed under UV lamp.
- Distance traveled by the sample for band = 2.6
- Distance traveled by the solvent = 5.2
- Therefore Rf value for band = 2.6/5.2 = 0.5

5.11 Preparative Thin Layer Chromatography

5.11.1 Apparatus and reagents

- Ethanol
- Ethyl acetate
- Chloroform
- Spatula
- Silica gel
- Distilled Water
- Glass rod
- Preparative TLC tank
- Test TLC plates
- Test TLC tank
- Sulfuric acid
- Heater
- Forcep
- UV spectrometer

5.11.2 TLC plate preparation

TLC plates had prepared by Silica gel and distilled water. 500 gm of silica gel was mixed with distilled water of a suitable amount to make a suitable paste that could be made as a layer on a square shaped glass plate. The paste was made and its viscosity was checked by making a sample plate. Then the paste was smoothly applied on the glass plates to make a suitable TLC plate. Then the plates were kept for 24 hour to let them dry.

Fig: Preparative TLC plate

5.11.3 Sample Introduction

When the TLC plates were completely dried, sample was introduced on side the plate by using micropipette. A few microgram of the sample extract was diluted by ethanol in a small beaker then the diluted sample was introduced by the micropipette. After this, the plate was dried for few minutes.

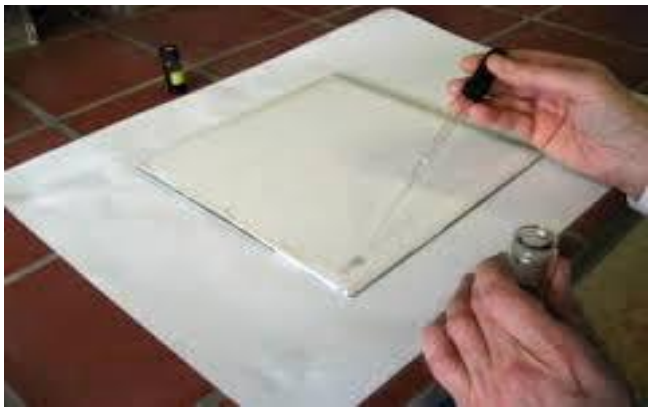


Fig: Sample application on preparative TLC plate

5.11.4 Tank preparation

Fig: preparative TLC tank

The Tank for Preparative TLC is a larger one then the test TLC tank. The tank was cleaned well first then dried by electrical drier. The TLC reagents Ethanol 350 ml was introduced into the tank and the closed it for few moments to saturate the internal environment by the reagent.

5.11.5 Operation

The prepared TLC plate was introduced to the tank very carefully that must not touch the wall of the tank and the reagent must not cross the sample line. Then the tank closed by the closure and waited to run the reagent through the plate. When the reagent reaches the top, it came out from the tank and let it for drying.

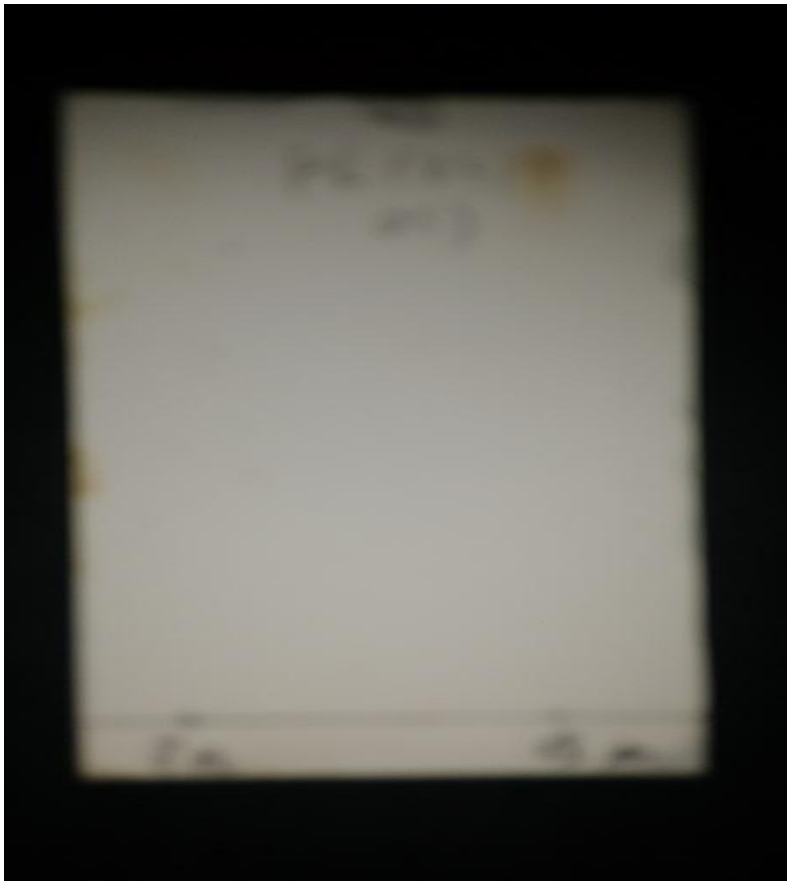
5.11.6 Observation

After drying the plate it was observed under UV light at Wavelength 257nm and three bands were observed. After that the plates were placed into the Fume hood for charring with H_2SO_4. Then a single blue band was clearly observed.

5.11.7 Band collection

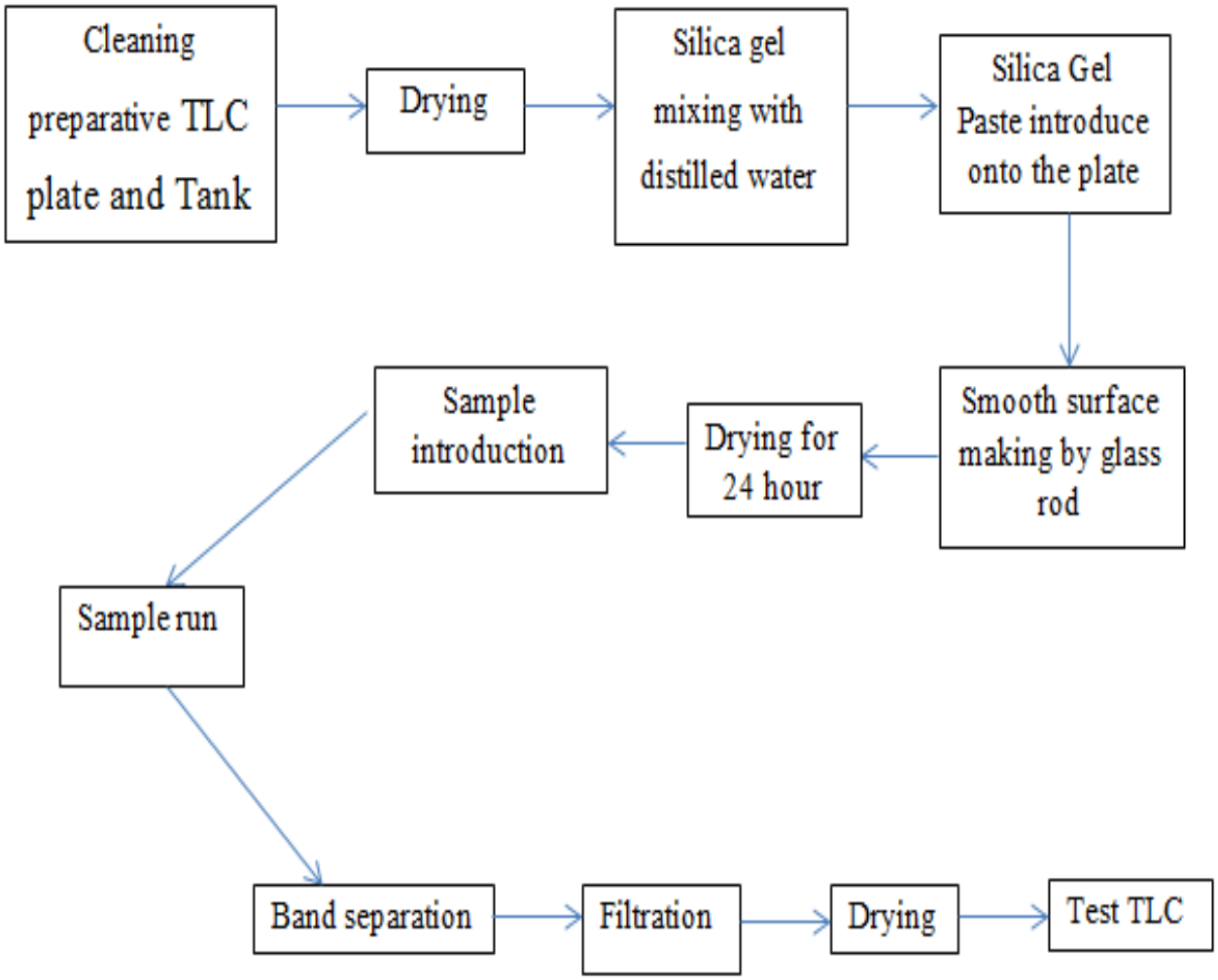
The band was collected by a clean Spatula into a small beaker. Then the contents of the beaker were dissolved in chloroform and ethyl acetate and kept for one day. Then the contents were filtered by filter paper and kept the filtrate for 3 days to make that contents dried and to remove solvents. Then the dried content was dissolved by acetic acid and chloroform for test TLC purpose. Then the sample was introduced onto test TLC plate and a test TLC was done with Ethanol.

5.11.8 Result



- A pink band was observed under UV lamp.
- Distance traveled by the sample for band = 3.75
- Distance traveled by the solvent = 4
- Therefore Rf value for band = 3.75/4 = 0.9375

5.11.9 Flow chart of entire process



5.11.10 Discussion

Result of preparative TLC said that the sample contain some different compounds which gave the bands. The bands were for a single compound for each band as the test TLC for each band showed only a single band.

5.12 Crystal Separation

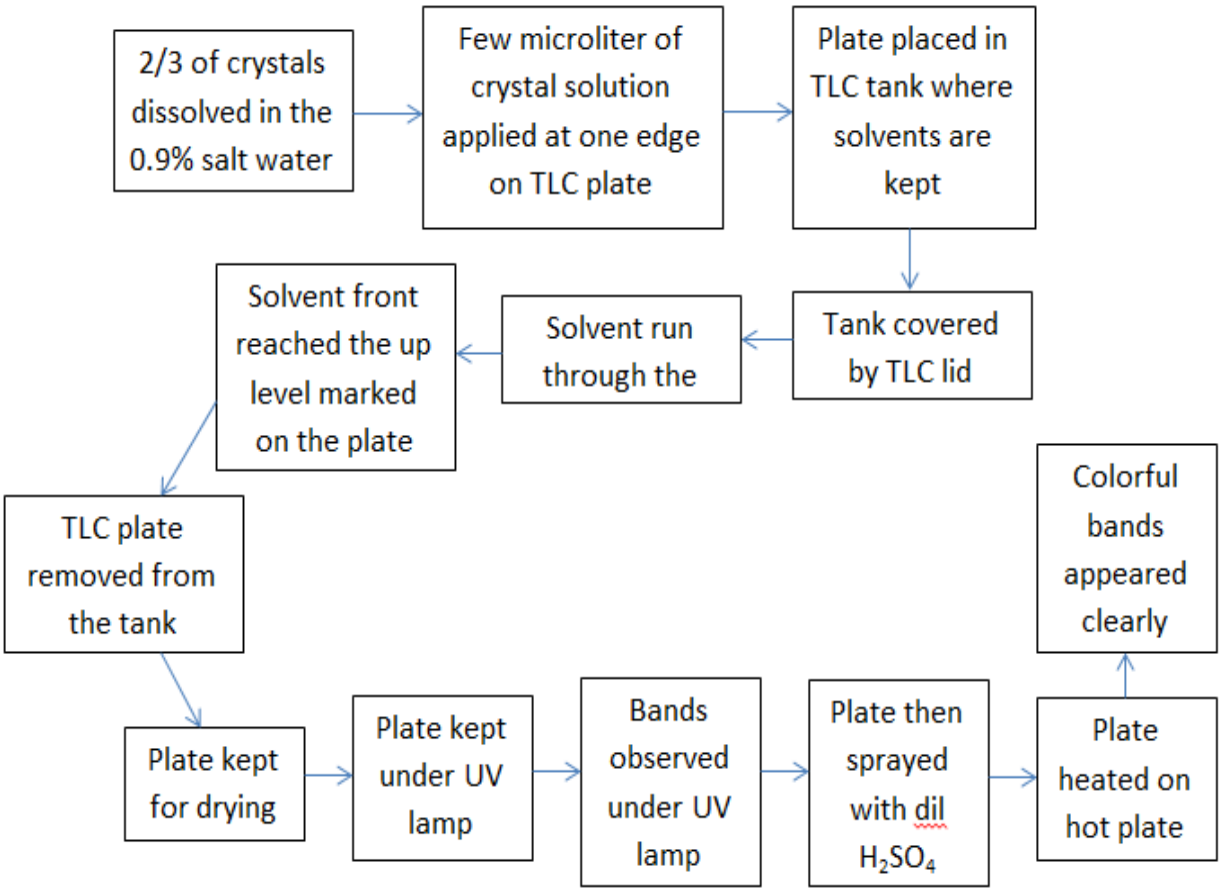
Crystals were found in the main methanolic extract of the plant. The extract was vigorously rinsed by methanol. The extract was dissolved in methanol except the crystals. The extract was come out form the crystal in the beaker by continuous rinsing with pure methanol.

Fig: Crystal form methanolic extract of plant

5.12.1 Thin Layer Chromatography for crystals

Two methods of TLC were used for the crystals.

5.12.1.1 Method 1 by using 0.9% salt water



- **Different solvents used as mobile phase**

3. **Set-A (Polar solvent)**

Reagent	Amount
Ethyl acetate	8 ml
Ethanol	1.2 ml
Water	0.8 ml

❖ **Result**

No band was found.

4. **Set-B (Semi Polar solvent):**

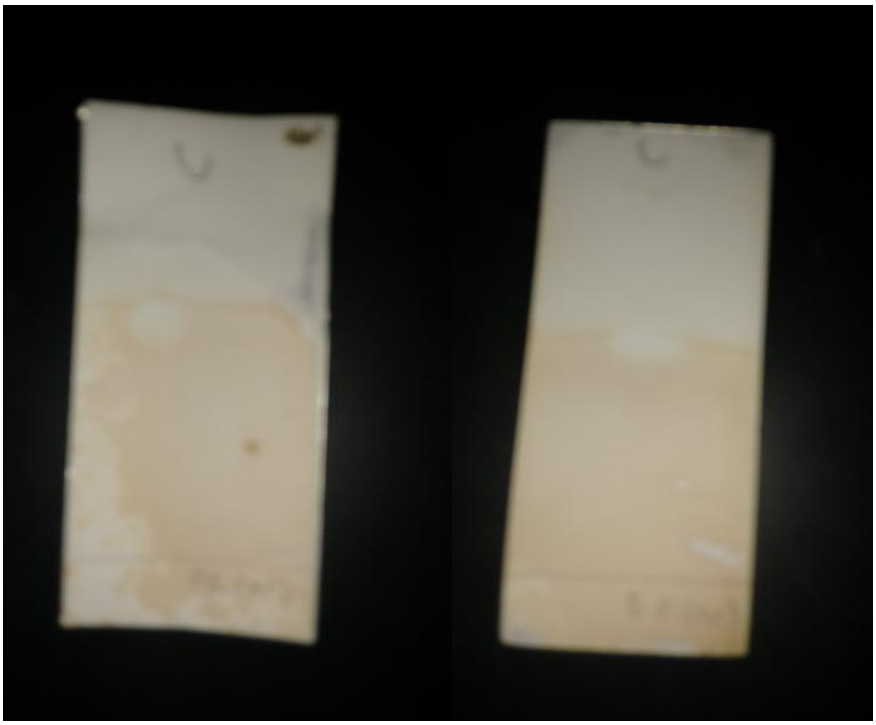
Reagent	Amount
Chloroform	5 ml
Ethyl acetate	4 ml
Acetic acid	1 ml

❖ **Result**

No band was found.

5. Set-C (Non Polar solvent):

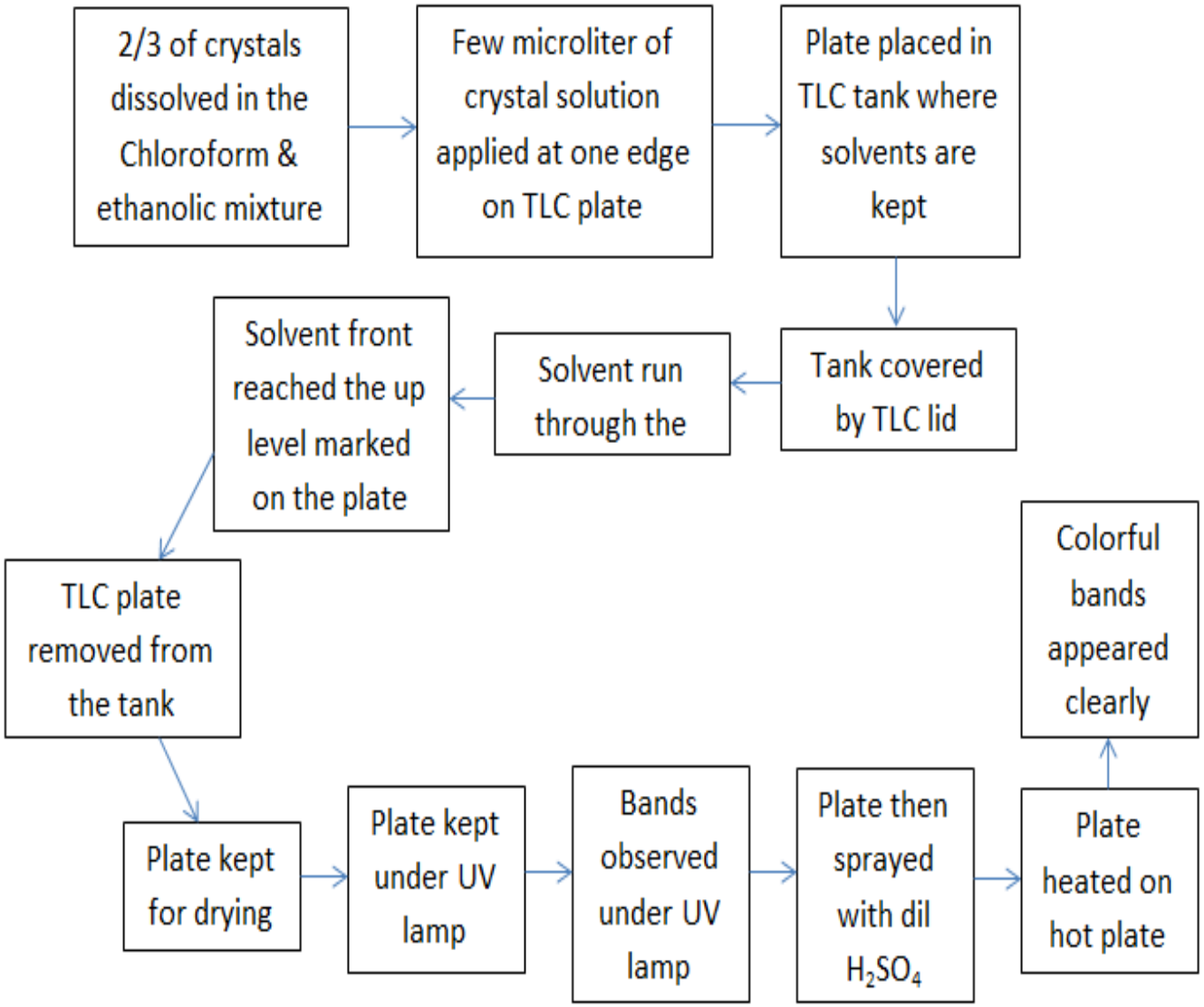
Reagent	Amount
Benzene	9 ml
Ethanol	1 ml



❖ Result:

- A clear white band was observed under UV lamp.
- Distance traveled by the sample = 4.75
- Distance travled by the solvent = 5
- Therefore Rf value = 4.75/5= 0.95

5.12.1.2 Method 2 by using Chloroform & ethanolic mixture



- **Different solvents used as mobile phase**

6. Set-A (Polar solvent)

Reagent	Amount
Ethyl acetate	8 ml
Ethanol	1.2 ml
Water	0.8 ml

❖ **Result:**

- A vertical white band was observed under UV lamp.
- Distance traveled by the sample = 5.1
- Distance travled by the solvent = 5.6
- Therefore Rf value = 5.1/5.6 = 0.91

7. Set-B (Semi Polar solvent):

Reagent	Amount
Chloroform	5 ml
Ethyl acetate	4 ml
Acetic acid	1 ml

❖ **Result**

No band was found.

8. Set-C (Non Polar solvent):

Reagent	Amount
Benzene	9 ml
Ethanol	1 ml

❖ **Result:**

No band was found

5.13 Infrared Spectroscopy of the crystals

5.13.1 Infrared (IR) spectroscopy

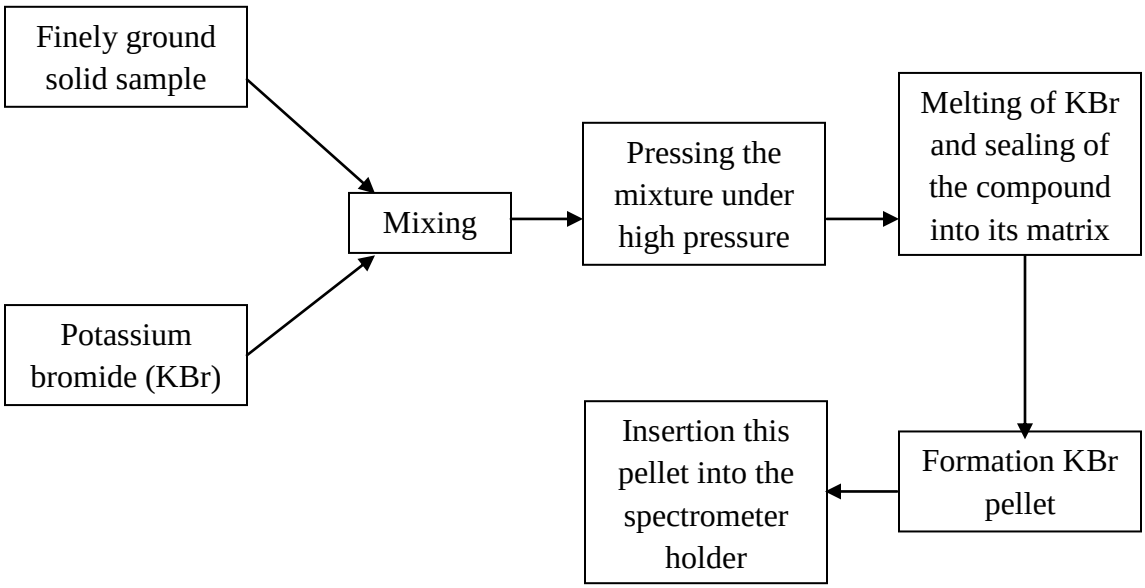
IR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantify some components of an unknown mixture and for the analysis of solids, liquids, and gases. The term Fourier Transform Infrared Spectroscopy (FTIR) refers to a development in the manner in which the data is collected and converted from an interference pattern to a spectrum. It is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular "fingerprint". The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum.

Fig: IR spectroscopy

5.13.2 Principle

Molecular bonds vibrate at various frequencies depending on the elements and the type of bonds. For any given bond, there are several specific frequencies at which it can vibrate. According to quantum mechanics, these frequencies correspond to the ground state (lowest frequency) and several excited states (higher frequencies). One way to cause the frequency of a molecular vibration to increase is to excite the bond by having it absorb light energy. For any given transition between two states the light energy (determined by the wavelength) must exactly equal the difference in the energy between the ground state and the first excited state.

5.13.3 Preparation of sample for FTIR



5.13.4 IR instrumentation & process

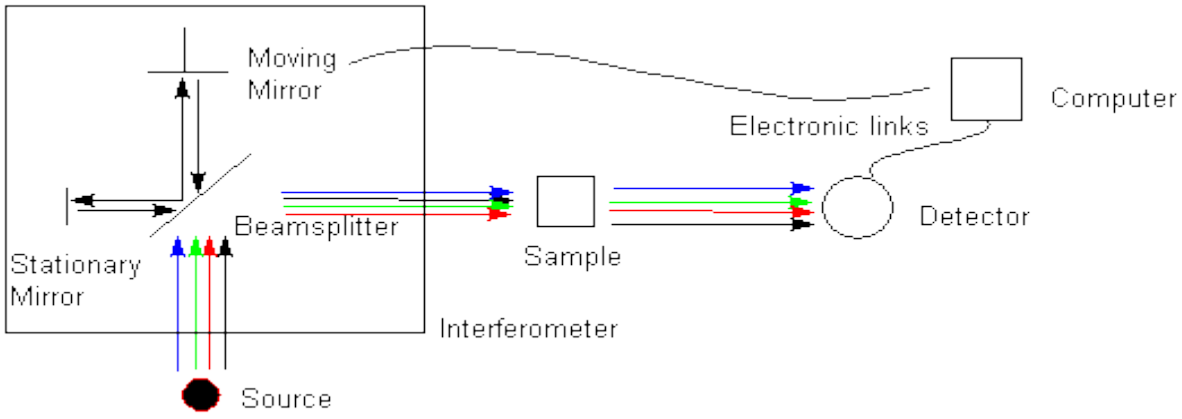


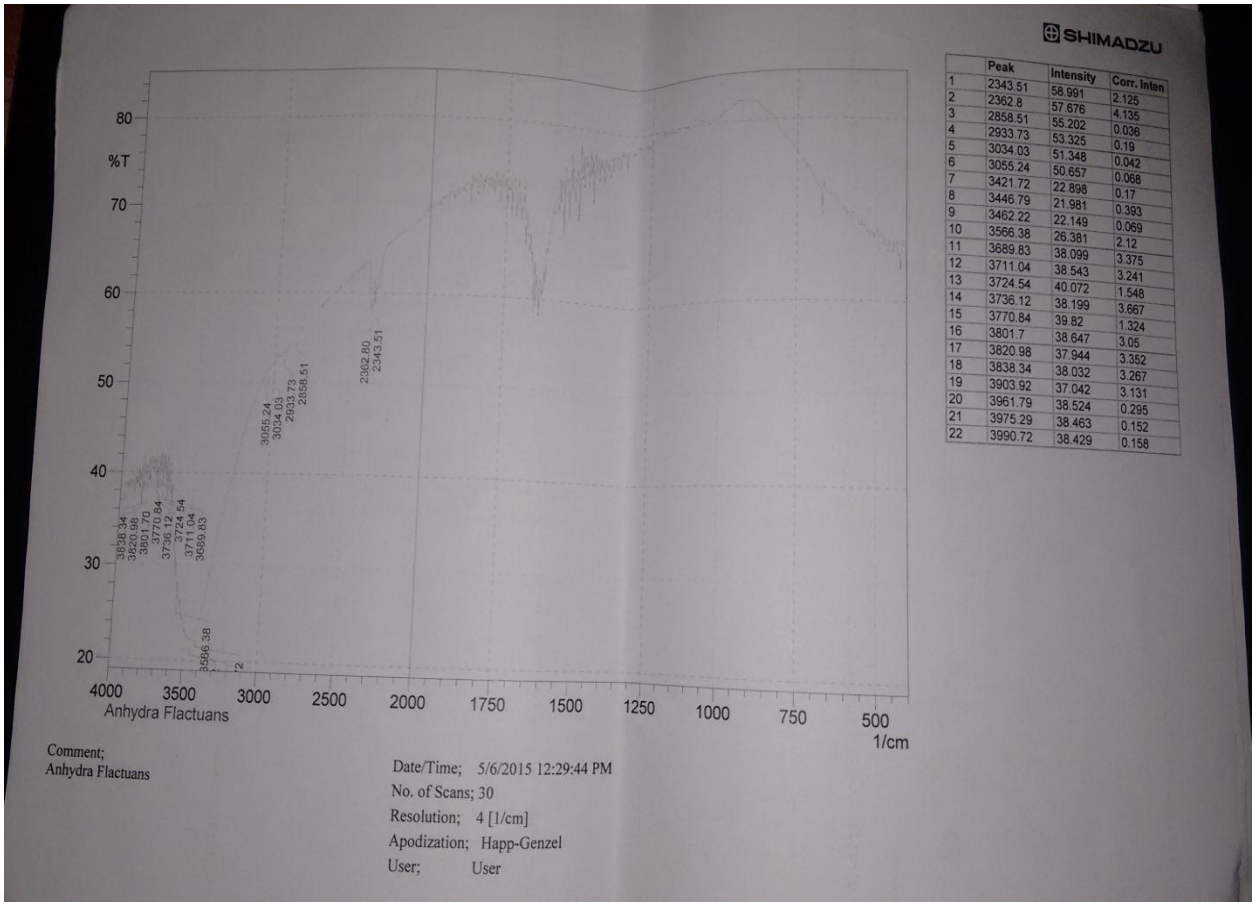
Figure: FTIR instrument and process

- The FTIR uses an inferometer to process the energy sent to the sample
- In the inferometer, the source energy passes through a beam splitter, a mirror placed at 45° angle to the incoming radiation. This allows the incoming radiation to pass through but separates it into perpendicular beams, one undeflected, the other oriented at 90° angle.
- One beam, the one oriented at 90° goes to a stationary or fixed mirror, and returned to the beam splitter.
- The undeflected beam goes to a moving mirror and also returned to the beam splitter.
- When the beams meet at the beam splitter, they recombine, but the path length differences of the two beams causes both constructive and destructive interferences.
- The combined beam containing these interference patterns is called the interferogram having a wide range of wavelengths.
- The interferogram is oriented towards the sample by the beam splitter.
- As it passes through the sample, the sample simultaneously absorbs all of the wavelengths that are normally found in its infrared spectrum.
- The modified interferogram signal that reaches the detector contains information about the amount of energy that was absorbed at every wavelength.

- The computer modifies the modified interferogram to a reference laser beam to have a standard of comparison.

5.13.5 Result

IR spectrum of the crystal obtained from the methanolic extract of *Enhydra fluctuans*:



	Peak	Intensity	Corr. Inten
1	2343.51	58.991	2.125
2	2362.8	57.676	4.135
3	2858.51	55.202	0.036
4	2933.73	53.325	0.19
5	3034.03	51.348	0.042
6	3055.24	50.657	0.068
7	3421.72	22.898	0.17
8	3446.79	21.981	0.393
9	3462.22	22.149	0.069
10	3566.38	26.381	2.12
11	3689.83	38.099	3.375
12	3711.04	38.543	3.241
13	3724.54	40.072	1.548
14	3736.12	38.199	3.667
15	3770.84	39.82	1.324
16	3801.7	38.647	3.05
17	3820.98	37.944	3.352
18	3838.34	38.032	3.267
19	3903.92	37.042	3.131
20	3961.79	38.524	0.295
21	3975.29	38.463	0.152
22	3990.72	38.429	0.158

The spectrum showed lots of overlapping peak which is very difficult to interpret.

Chapter 6: Results & Discussions

6.1 Result and Observation

6.1.1 Test TLC result & observation

Several numbers of Thin Layer Chromatography (TLC) tests were performed on the methanolic extract of *Enhydra fluctuans,* which are given below:

6.1.1.1 Using Polar solvent

1.

Reagent	Amount
Ethyl acetate	8 ml
Ethanol	2 ml

❖ **Result:**

- An orange band was observed under UV lamp.
- Distance traveled by the sample = 5.3
- Distance travled by the solvent = 5.6
- Therefore Rf value = 5.3/5.6 = 0.87

2.

Reagent	Amount
Ethanol	11 ml

❖ **Result:**

- An orange and a green bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 1.3
- Distance traveled by the sample for band (2) = 5.4

- Distance travled by the solvent = 5.6
- Therefore Rf value for band (1) = 1.3/5.6 = 0.232

  Rf value for band (2) = 5.4/5.6= 0.964

3.

Reagent	Amount
Ethyl acetate	11 ml

❖ **Result:**

- Two orange and one green bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 0.7
- Distance traveled by the sample for band (2) = 1.5
- Distance travled by the solvent = 5.
- Therefore Rf value for band (1) = 0.7/5 = 0.14

  Rf value for band (2) = 1.5/5 = 0.3

6.1.1.2 Using non polar solvents

4.

Reagent	Amount
Benzene	9 ml
Ethanol	1 ml

❖ **Result:**

- Two orange bands were observed under UV lamp.
- Distance traveled by the sample for band (1) = 1.2
- Distance traveled by the sample for band (2) = 2.4
- Distance traveled by the solvent = 7.3
- Therefore Rf value for band (1) = 1.2/7.3 = 0.164

  Rf value for band (2) = 2.4/7.3= 0.329

5.

Reagent	Amount
Benzene	6 ml
Ethanol	4 ml

Result:

- An orange band was observed under UV lamp.
- Distance traveled by the sample for band = 2.6
- Distance traveled by the solvent = 5.2
- Therefore Rf value for band = 2.6/5.2 = 0.5

6.

Reagent	Amount
Benzene	5 ml
Ethanol	5 ml

❖ **Result:**

- An orange band was observed under UV lamp.
- Distance traveled by the sample for band = 4.8
- Distance traveled by the solvent = 5.2
- Therefore Rf value for band = 4.8/5.2 = 0.96

6.1.2 Preparative TLC result & observation

Through preparative TLC, A blue band was observed under UV lamp which was further investigated by test TLC experiment.

- A pink band was observed under UV lamp.
- Distance traveled by the sample for band = 3.75
- Distance traveled by the solvent = 4
- Therefore Rf value for band = 3.75/4 = 0.9375

6.1.3 IR result & observation

Peaks were appeared within this (2343-3990 cm^{-1}) range which indicates the presence of the following functional groups in crystal compound.

1. C-H of alkane
2. C-H of alkene
3. C-H of aromatics.
4. O-H of free and H bonded & free alcohols and carboxylic acids
5. N-H of primary and secondary amines and amides.
6. S-H of mercaptans

6.2 Discussion

6.2.1Test TLC

Some of the tests showed bands and some didn't. The reason behind, may be either there are no such compound to show band by those solvents or there were some technical error during the test period or there may be something error in the procedure. Rf values were measured from the experiment of Thin Layer Chromatography where bands were observed. The Rf values were calculated by dividing the distance travelled by the sample from the distance travelled by the solvent.

Many compounds of this plant were studied from the literature reviews of many journals and websites. Then their Rf values were collected to match with the calculated Rf values which was got from Thin Layer Chromatography experiment and it was observed that Rf values of most of Thin Layer Chromatography results nearly matches with the exact Rf values of those compounds isolated previously from this plant.

But the Rf values were not exactly matched with the Rf values of pure compound previously isolated from this plant. These errors may be occurred owing to carelessness or technical error occurred during the experiment process or may be the extract was contaminated or partially impured by any impurities or due to the contamination of the stationary phase which was silica plate. Due to all of these reasons, we couldn't come at exact decision for a particular compound identification.

6.2.2 Preparative TLC

When preparative TLC was performed, one clear blue band was observed. When the band was separated by using spatula and was dissolved in chloroform and ethyl acetate and test TLC was performed with the same solvent system. A single spot was observed in test TLC. This means that, the band in the preparative TLC plate was for a single compound. The Rf value from the test TLC was calculated. . The Rf value was calculated by dividing the distance travelled by the sample from the distance travelled by the solvent.

Many compounds of this plant were studied from the literature reviews of many journals and websites. Then their Rf values were collected to match with the calculated Rf values which was got from Thin Layer Chromatography experiment and it was observed that Rf values of most of Thin Layer Chromatography results nearly matches with the exact Rf values of those compounds isolated previously from this plant.

But the Rf values were not exactly matched with the Rf values of pure compound previously isolated from this plant. These errors may be occurred owing to carelessness or technical error occurred during the experiment process or may be the extract was contaminated or partially impured by any impurities or due to the contamination of the stationary phase which was silica plate. Due to all of these reasons, we couldn’t come at exact decision for a particular compound identification.

6.2.3. Infrared Spectroscopy

After IR spectroscopy of the crystal of *E. fluctuans* extract crystals, The IR spectrum showed lots of peaks which are clustered together and these can't be properly read out. The reason of this problem could be that, the crystals were not pure crystal or there may be an error in operating the machine or may be some error in procedure.

After interpreting these peaks appeared in (2343-3990 cm^{-1}) range, it can be guessed that the following functional groups are present in crystal compound.

- ✓ C-H of alkane
- ✓ C-H of alkene
- ✓ C-H of aromatics.
- ✓ O-H of free and H bonded alcohols and carboxylic acids
- ✓ N-H of primary and secondary amines and amides.
- ✓ S-H of mercaptans

Chapter 7: Conclusion

Conclusion

Although, an extensive amount of research work has been done on *Enhydra fluctuans* to date, the isolated compounds from this plant are belonged to flavonoids, sesquiterpene lactone, saponins, tannins, glycosides, alcohols, protein and nutrients. Consequently, a broad field of future research remains possible in which the isolation of new active principles from this species would be of great scientific merit.

Hence, a detailed study is required to understand the structure– activity relationship of these chemical compounds isolated from this plant. As literature showed, many plant extracts having cytotoxic activity, antitumor, antimicrobial, antibacterial, analgesic, anti-inflammatory, hypothermia, diuretic, and anti-oxidative activities, hence, the particular constituent responsible for the activity may be isolated for further process. In addition, some plant extracts were only screened for their preliminary *in vitro* activities; so, the advance clinical trial of them deserves to be further investigated. Herein, we described the possible applications in clinical research but further investigations on phytochemical discovery and subsequent screenings are required for opening new opportunities to develop pharmaceuticals based on *Enhydra fluctuans*.

Enhydra fluctuans on which this research was done is an important medicinal plant. The extract of this plant was collected and some important tests including test TLC and Preparative TLC and IR spectroscopy were carefully performed on the extract and crystals obtained from the extract of this plant to determine the chemical constituents of the plant extract.

However, further studies are necessary to elucidate new compounds from this plant. This report may serve as a footstep to use this plant as a new source of medication.

Chapter 7: References

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