# *In vitro* pharmacological investigations of dichloromethane fraction of *Opuntia elatior*

***A DISSERTATION SUBMITTED TO THE DEPARTMENT OF PHARMACY, EAST WEST UNIVERSITY IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF PHARMACY***

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# Declaration By The Research Candidate

I, Silvia Islam, hereby declare that the dissertation entitled "***In vitro* pharmacological investigations of dichloromethane fraction of *Opuntia elatior***" submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy is a complete record of original research work carried out by me, under the supervision and guidance of Abdullah-Al-Faysal, Lecturer, Department of Pharmacy, East West University and the thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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# Certificate By The Supervisor

This is to certify that the thesis entitled "***In vitro* pharmacological investigations of dichloromethane fraction of *Opuntia elatior***" submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of pharmacy was carried out by Silvia Islam, ID# 2010-1-70-049 in 2015, under the supervision and guidance of me. The thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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# Endorsement By The Chairperson

This is to certify that the thesis entitled "***In vitro* pharmacological investigations of dichloromethane fraction of *Opuntia elatior***" submitted to the Department of Pharmacy, East West University, Dhaka, in the partial fulfillment of the requirement for the degree of Bachelor of pharmacy was carried out by Silvia Islam, ID# 2010-1-70-049 in 2015.

Dr. Shamsun Nahar Khan

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Thank you

*Dedication*

*This Research Paper is dedicated*

*to*

*My beloved parents and*

*My beloved teachers*

# Abstract

The purpose of the study was to evaluate the cytotoxic and antioxidant activity of dichloromethane fraction of *Opuntia elatior* (Family: Cactaceae) extract.

The powdered of *Opuntia elatior* were extracted with methanol and then partitioned with pet-ether, DMSO, ethyl acetate and crude fraction was taken for experiment. The aqueous fraction was used to evaluate cytotoxic and antioxidant activities. The cytotoxic activity was measured by brine shrimp lethality bioassay. $LC_{50}$ value of aqueous fraction of *Opuntia elatior* was 12.5µg/ml in brine shrimp lethality test. The fraction contained 16.47 mg AAE/g of total phenolic content, 68.1 mg AAE/g of total reducing power content and 35.88 mg AAE/g total flavonoid content. The results of study clearly indicate the presence of cytotoxic and poor antioxidant properties of dichloromethane extract. The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.

**Keyword:** *Opuntia elatior*, Brine shrimp lethality bio-assay, phenolic content, flavonoid content, reducing power assay.

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# Chapter One

# INTRODUCTION

# 1.1 General Introduction

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs. The development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. Plant derived medicines are used in self–medication in all cultures. Only a fraction of the world's available plants have been studied. Discovery and use of synthetic drugs have caused side effects or adverse reactions that were not for seen in preclinical and clinical examinations. As a result, a resurgence of interest in the study and use of medicinal plants has been taken place during the last two decades. As a result of modern isolation techniques and pharmacological testing procedures, new plant drugs found their way into modern medicine as purified substances rather than in the form of galenical preparations (Reddy *et al.,* 2010). Compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al.,* 1996).

Ethnobotany, the scientific study of the relationships that exist between humans and plants, is a recognized way to discover new effective medicines for future and further use. In ancient Greece, plants were classified and descriptions of them were given by scholars. It aids in the identification process. Researchers identified in 2001, 122 compounds that were isolated and identified from "ethno medical" plant sources, are used in modern medicine. The current use of the active elements of the plants is 80% similar to those of ethno medical use (Fabricant & Farnsworth, 2001).

## 1.1.1 Medicinal Plants

Medicinal plants are plants which have a recognized medical use. It's containing essential bioactive ingredients are used to cure disease or disorder since time immortal. One of the aims of medicinal plant research is the isolation andidentification of markers/ bioactivecompounds. Isolation of the markers compounds and bioactive plant constituents has always been a challenging task for the researchers. Separation of these components from the medicinal plants includes the use of combination of chromatographic techniques such as column chromatography,

preparative thin layer chromatography, preparative high performance liquid chromatography, droplet counter current chromatography, centrifugal thin layer chromatography, etc. Centrifugal thin layer chromatography which makes use of centrifugal force for separation of multi-component system offers extensive platform for the isolation of phytoconstituents from medicinal plants. This review focuses on basic principle, instrumentation and advantages of centrifugal thin layer chromatography (Agrawal& Desai, 2015).

### 1.1.2 Medicinal Plants as Drugs

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal and human body are generally designated as medicinal plants (Ghani, 1998).

**According to the World Health Organization (WHO),**

"A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis" (Sofowara,1982).

When a plant is designated as 'medicinal', it is implied that the plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation. "Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes".Many of the plants could be used as stimulants, poisons, hallucinogens or as medicine because of the presence of unique or rich biological-active plant chemicals (i.e. Chemical compounds that have a biological effect on another organism (Hamburger& Hostettmann, 1991).

## 1.2 Medicinal Plants – History and Context

Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. Written evidence of herbal remedies dates backover 5,000 years, to the Sumerians, who created lists of plants. A number of ancient cultures wrote on plants and their medical uses. In ancient Egypt, herbs are mentioned in Egyptian medical papyri, depicted in tomb illustrations, or on rare occasions found in medical jars containing trace amounts of herbs. The earliest known Greek herbals were those of Diocles of Carystus, written during the 3rd century B.C,

and one by Krateuas from the 1st century B.C. Only a few fragments of these works have survived intact, but from what remains scholars have noted that there is a large amount of overlap with the Egyptian herbals. Seeds likely used for herbalism have been found in the archaeological sites of Bronze Age China dating from the Shang Dynasty. Over a hundred of the 224 drugs mentioned in the Huangdi Neijing, an early Chinese medical text, are herbs. Herbs were also common in the medicine of ancient India, where the principal treatment for diseases was diet. De Materia Medica by Pedanius Dioscorides, a Roman physician, is a particularly important example of such writings. The documentation of herbs and their uses was a central part of both Western and Eastern medical scholarship through to the 1600s, and these works played an important role in the development of the science of botany (Nunn, 2002; Hong, 2004; Ackerknecht, 1982).

Human beings have used plants for the treatment of diverse ailments for thousands of years. According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements, since they cannot afford the products of Western pharmaceutical industries, together with their side effects and lack of healthcare facilities. Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products tomaintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses (Ernst, 2007).

Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety margins and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Ernst, 2007). Even with the advent of modern or allopathic medicine, Balick and Cox (1996) have noted that a number of important modern drugs have been derived from plants used by indigenous people.

## 1.2.1 Characteristics of Medicinal plants

Medicinal plants have many characteristics when used as a treatment, as follow:

• Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.

• Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.

• Preventive medicine- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment (Hasan, 2012).

## 1.2.2 Significances of Medicinal Plants to Mankind

Even if we only consider the impact of the discovery of the penicillin, obtained from micro-organisms, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (Yue-Zhong Shu, 1998). The vast majority of these cannot yet be synthesised economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburger & Hostettmann, 1991).

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❖ Plants are valuable for modern medicine in four basic ways:

a) They are used as sources of direct therapeutic agents.
b) They serve as raw materials base for elaboration of more complex semi synthetic chemical compounds.
c) The chemical structures derived from plant sources can be used as models for new synthetic compounds.
d) Finally plants can be used as taxonomic markers for the discovery of new compounds(Reddy, *et al.*2010).

### 1.2.3 Global scenario of Medicinal plants

According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Herbal medicine is a common element in Ayurvedic, homeopathic, naturopathic, traditional, and oriental, Native American & Indian medicine. Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. The present global herbal market is worth about US$ 62 billion per annum. The annual growth of herbal market is about 15 percent and the global herbal market by 2050 is expected to be about US$ 5 trillion.

Thus, the modern social context and economic view of health services, the needs of the pharmaceutical market and the recognition that research on medicinal plants used in folk medicine represents a suitable approach for the development of new drugs (Calixto, 2000) have led to an increase in the number of publications in this field, and private and governmental institutions are now financially supporting research programs worldwide (Rates, 2001).

### 1.2.4 Classification of medicinal plants

Of the 250,000 higher plant species on earth, more than 80,000 species are reported to have at least some medicinal value and around 5000 species have specific therapeutic value. They are classified according to the part used, habit, habitat, therapeutic value etc, besides the usual botanical classification (Joy*et.al,*. 1998).

**Table-1.1: Based on part used**(Joy *et.al,*. 1998).

| Part | Name of plant |
|---|---|
| **Whole plant** | *Phyllanthusneruri, Saraca indica* |
| **Root** | *Panax ginseng, Polemonium reptans* |
| **Stem** | *Tinosporacordifolia, Acoruscalamus* |
| **Bark** | *Saracaasoca, Cinchona rubra* |
| **Leaf** | *Medicago sativa, Indigoferatinctoria, Lawsoniainermis, Aloe vera* |
| **Flower** | *Biophytum sensitivum*, Mimusopselenji, *Bellis perennis, Calendula officinalis* |
| **Fruit** | *Solanum* species, *Arctium lappa* |
| **Seed** | *Daturastramonium, Apium graveolens* |

**Table-1.2: Based on habit**(Joy *et.al,*. 1998).

| Part | Name of plant |
|---|---|
| **Grasses** | *Cynodondactylon* |
| **Sedges** | *Cyperusrotundus* |
| **Herbs** | *Rosemarinus officinalis* |
| **Shrubs** | *Solanum* species |
| **Climbers** | *Asparagus racemosus* |
| **Trees** | *Azadirachtaindica* |

**Table-1.3: Based on habitat**(Joy *et.al,.* 1998).

| Habitat | Name of plant |
|---|---|
| **Tropical** | *Andrographispaniculata* |
| **Sub-tropical** | *Menthaarvensis* |
| **Temperate** | *Atropabelladonna* |

**Table-1.4: Based on therapeutic value**(Joy *et.al,.* 1998).

| Therapeutic value | Name of plant |
|---|---|
| **Antimalarial** | *Cinchona officinalis, Artemisia annua* |
| **Anticancer** | *Catharanthusroseus, Taxusbaccata* |
| **Antiulcer** | *Azadirachtaindica, Glycyrrhizaglabra* |
| **Antidiabetic** | *Catharanthusroseus, Momordicacharantia* |
| **Anti-cholesterol** | *Allium sativum* |
| **Anti-inflammatory** | *Curcumadomestica, Desmodiumgangeticum* |
| **Antiviral** | *Acacia catechu* |
| **Antibacterial** | *Plumbagoindica* |
| **Antifungal** | *Allium sativum* |
| **Antiprotozoal** | *Cephaelisipecacuanha* |
| **Antidiarrhoeal** | *Psidiumguajava, Curcuma domestica* |
| **Hypotensive** | *Coleus forskohlii, Aliumsativum* |
| **Tranquilizing** | *Rauvolfiaserpentina* |
| **Anaesthetic** | *Erythroxylum coca* |
| **Spasmolytic** | *Atropabelladona, Hyoscyamusniger* |
| **Diuretic** | *Phyllanthusniruri, Centellaasiatica* |
| **Astringent** | *Piper betle, Abrusprecatorius* |
| **Anthelmentic** | *Quisqualisindica, Punicagranatum* |
| **Cardiotonic** | *Digitalis* sp., *Thevetia*sp |
| **Antiallergic** | *Nandinadomestica, Scutellariabaicalensis* |
| **Hepatoprotective** | *Silybummarianum, Andrographispaniculata* |

### 1.2.5 Advantages of Drug Discovery from Natural Resources

Usage of botanical sources as starting point in the drug development program is associated with few specific advantages:

- Mostly, the selection of a candidate species for investigations can be done on the basis of long-term use by humans (ethnomedicine). This approach is based on an assumption that the active com-pounds isolated from such plants are likely to be safer than those derived from plant species with no history of human use. At certain time point afterward, one may attempt upon synthesis of active molecule and reduce pressure on the resource. Drug development from *Rauwolfia serpentine*, *Digitalis purpurea*, etc. in the past fall under this category of approach.

- Sometimes, such approaches lead to development of novel molecules derived from the source due to inherent limitations of the original molecule. For instance, podophyllin derived from *Podophyllum hexandrum* was faced with dose-limiting toxicities. Such limitations could be overcome to a great extent by semi-synthesis of etoposide, which continues to be used in cancer therapy today. Similar was the case with camptothecin (originally isolated from *Camptotheca sp*. and subsequently from*Mappia sp*.), which led to development of novel anticancer molecules like topotecan and irinotecan.

- Natural resources as starting point has a bilateral promise of delivering the original isolate as a candidate or a semi-synthetic molecule development to overcome any inherent limitations of original molecule.

### 1.2.6 Tribal Medicines

In different localities of Rangamati and Bandarban Districts of Bangladesh a survey was carried out between 2001 and 2002 to document medicinal plants. Kaukhali proper is about 10 km west to Rangamati town. Betbunia is a Union under Kaukhali P.S. situated about 9 km south of Kaukhali proper and about 18 km south-west to Rangamati town. Being a hilly area they are rich in floral diversity. Inhabitants of those areas are mostly tribal, dominated by Chakma and Marma. Many of them still depend on local medicinal plants for the treatment of different diseases. A good

number of Bangali families are also living there. They also use quite a good number of medicinal plants for the treatment of different diseases. In recent years due to development of good communication, modern doctors and medicines have reached there, resulting decline in the use of traditional medicine. Therefore the knowledge of traditional use of medicinal plants by the local people is likely to be lost in near future, and for this it is necessary to document as much as possible the existing available information. Keeping this in mind this survey was done to document those valuable ethno-medico-botanical knowledge. The survey was carried out for about a year. During this work 34 species representing 23 genera and 17 families were documented which are used for the treatment of 31 diseases. Local names of those plants, locality and method of use are mentioned (Yusuf *et.al,*. 2006).

**Table-1.5: Some tribal medicinal plants & their uses** (Yusuf *et.al,*. 2006).

| Scientificname | Tribal name | Locality | Disease |
|---|---|---|---|
| ***Alstoniascholaris* (Apocynaceae)** | Marma-Chailoi | Betbunia | Arthritic pain |
| ***Leeaindica (Leeacea)*** | Chakma-Haskura | Toolaban-Marissa | Sore, leprosy, eczema, itching |
| ***Eupatorium odoratum (Asteraceae)*** | Tonchongya-Demrapata Gach | Naramuk-Rajsthali | Bleeding |

# 1.3 Approaches for isolation of active compounds from natural origin

## 1.3.1 Random approach

Two approaches have been followed for screening of the plants selected randomly for the purpose of new drug discovery (Katiyar et al., 2012):

**a) Screening for selected class of compounds like alkaloids, flavonoids, etc.:** While this route is simple to perform, however, it is flawed in the sense that it

provides no idea of the biological efficacy. However, chances of getting novel structures cannot be denied following this approach.

**b) Screening of randomly selected plants for selected bioassays:** Central Drug Research Institute, a premier R and D organization of Council of Scientific and Industrial Research of India, followed this approach about three decades ago. They screened almost 2000 plants for biological efficacy. However, the screening did not yield any new drug. National Cancer Institute (NCI) of National Institute of Health, USA, studied about 35,000 plant species for anticancer activity, spending over two decades from 1960 to 1980. It resulted in proving two success stories, which were those of paclitaxel and camptothecin. This route, therefore, has been applied for both focused screening as well as general screening, showing some success in focused screening. If target-based bioassays are used, e.g. screening against PTP1B, chances of success would probably be more. This approach, however, needs a huge library of extracts, which very few organizations in the world are having.

## 1.3.2 Ethnopharmacology approach

The approach of ethnopharmacology essentially depends on empirical experiences related to the use of botanical drugs for the discovery of biologically active New Chemical Entities (NCEs). This process involves the observation, description, and experimental investigation of indigenous drugs, and is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines like anthropology, archaeology, history, and linguistics. This approach based on ethnomedicinal usage history has seen some success, e.g. *Andrographis paniculata*was used for dysentery in ethnomedicine and the compounds responsible for the activity were isolated as andrographolide. Morphine from *Papaver somniferum*, Berberine from *Berberis aristata*, and *Picroside* from *Picrorhiza kurroa* are some examples of this approach. Some of the plants which are not selected on the basis of ethnomedical use also had some success stories, like L-Dopa from *Mucuna prurita* and paclitaxel from *Taxus brevifolia* (Katiyar et al., 2012).

### 1.3.3 Traditional system of medicine approach

Countries like India and China have a rich heritage of well-documented traditional system of medicine in vogue. Though these codified systems of medicine use largely botanical sources asmedicines, however, these stand apart from ethnomedicine specifically on three accounts (Katiyar et al., 2012):

- The ethnomedicinal practice is based on empirical experiences. On the other hand, these codified systems built up the empirical practices on strong conceptual foundations of human physiology as well as of pharmacology (though the tools of their investigations in those times were far different from the existing ones).

- The pharmaceutical processes have been more advanced as against the use of crudely extracted juices and decoctions in ethnomedicinal practices. Due to this phenomenon, the concept of standardization was known to the system.

- They are well documented and widely institutionalized. On the other hand, the ethnomedicinal practices are localized and may be largely controlled by few families in each of the community.

However, in terms of historicity, ethnomedicinal practices might be older than codified systems of medicine (Katiyar et al., 2012).

Discovery of artemisinin from *Artemesia alba* for malaria, guggulsterones from *Commiphora mukul* (for hyperlipidemia), boswellic acids from *Boswellia serrata* (anti-inflammatory), and bacosides from *Bacopa monnieri* (nootropic and memory enhancement) was based on the leads from these codified systems of medicine prevailing in China and India. However, it can be stated that such approach for selecting candidates in drug discovery programs has not been adopted much so far. Nonetheless, the approach has a distinct promise in terms of hit rates. But the distinct example for this approach has been the discovery of reserpine from *Rauwolfia serpentine*, which was based on the practices of Unani medicine (Katiyar et al., 2012).

## 1.4 Phytochemistry

Phytochemistry can be defined as the biochemical study of plants which is concerned with the identification, biosynthesis, and metabolism of chemical constituents of plants, especially used in regard to natural products. Phytochemistry is considered as one of the early subdivisions of organic chemistry. It has been of great importance in the identification of plant substances of medicinal importance (Website 1).

Phytochemistry is the study of phytochemicals produced in plants, describing the isolation, purification, identification, and structure of the large number of secondary metabolic compounds found in plants. Effect of extracted plant phytochemicals depends on:

- The nature of the plant materials
- Its origin
- Degree of processing
- Moisture content (Tiwari& Kumar, 2011)

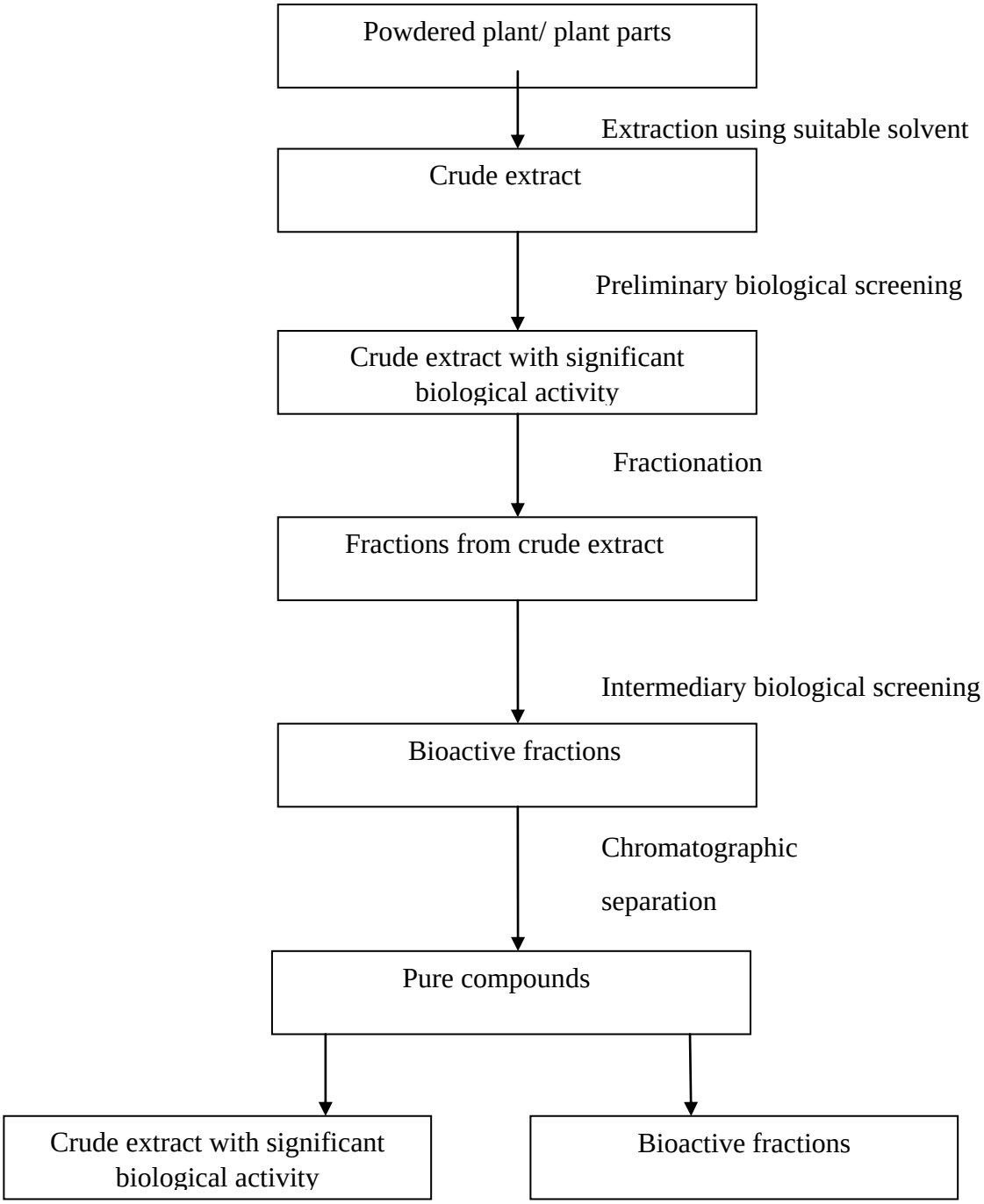
## 1.5 Procedure for Development

Since drug development is an expensive practice, careful phytochemical analysis and pharmacological screening and if promising clinical tests are required. The way of developing drugs from plants involves several stages (Ghani, 1998), which include:

a) Selection and correct identification of the proper medicinal plant.
b) Extraction with suitable solvent(s).
c) Detection of biological activity of crude extract and establishment of a bioassay system to permit the identification of the active fractions and rejection of the inactive ones.
d) Fractionations of crude extract using the most appropriate chromatographic procedures, biological evaluation of all fractions and separation of the active fractions.
e) Repeated fractionation of active fractions to isolate pure compound(s).
f) Elucidation of chemical structure of pure compound(s) using spectroscopic methods.
g) Evaluation of biological activity of pure compound(s)
h) Toxicological tests with pure compound(s).

i) Production of drug in appropriate dosage forms.

### 1.5.1 Schematic Diagram of Bioactivity Guided Research of Medicinal plants



## 1.5.2 Bioactive Compounds in Medicinal Plants

All plants produce chemical compounds as part of their normal metabolic activities. These phytochemicals are divided into (Bernhoft, 2010):

a) Primary metabolites such as sugars and fats, which are found in all plants; and
b) Secondary metabolites—compounds which are found in a smaller range of plants, serving a more specific function. For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination.

It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs—examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove. Toxic plants even have use in pharmaceutical development (Bernhoft, 2010).

Plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs (Bernhoft, 2010):

- Alkaloids are a class of chemical compounds containing a nitrogen ring. Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the psychedelic psilocin; the stimulant caffeine; nicotine; the analgesic morphine; the antibacterial berberine; the anticancer compound vincristine; the antihypertension agent reserpine; the cholinomimeric galatamine; the spasmolysis agent atropine; the vasodilator vincamine; the anti-arhythmia compound quinidine; the anti-asthma therapeutic ephedrine; and the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste.

- Polyphenols (also known as phenolics) are compounds contain phenol rings. The anthocyanins that give grapes their purple color, the isoflavones, the phytoestrogens from soy and the tannins that give tea its astringency are phenolics.
- Glycosides are molecules in which a sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis, which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body. An example is the cyanoglycosides in cherry pits that release toxins only when bitten by an herbivore.
- Terpenes are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers, which are often strong smelling and thus may have had a protective function. They are the major components of resin, and of turpentine produced from resin. (The name "terpene" is derived from the word "turpentine"). Terpenes are major biosynthetic building blocks within nearly every living creature. Steroids, for example, are derivatives of the triterpene squalene. When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives. Vitamin A is an example of a terpene. The fragrance of rose and lavender is due to monoterpenes. The carotenoids produce the reds, yellows and oranges of pumpkin, corn and tomatoes.

The goals of using plants as sources of therapeutic agents are (Bernhoft, 2010):

- to isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine , reserpine, taxol, vinblastine, vincristine etc

- to produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesics), taxotere which are based respectively on galegine, Δ9-tetrahydrocannabinol, morphine, taxol
- to use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, and
- to use the whole plant or part of it as a herbal remedy, e.g., cranberry, garlic etc.

## 1.6 Antioxidant Activity

Antioxidants are substances that may protect human cells against the effects of free radicals. Dietary plants contain variable chemical families and amounts of antioxidants. It has been hypothesized that plant antioxidants may contribute to the beneficial health effects of dietary plants. Studies suggest that a diet high in antioxidants from fruits and vegetables is associated with a lower risk of cancer, cardiovascular disease, Parkinson's disease and Alzheimer's disease. Such diseases have been found to be the result of damage of cells due to free radical generation (Singh et.al. 2013).

### 1.6.1 Free Radicals and Oxidative Stress

Free radicals are natural by-products of human metabolism. These are charged molecules which attack cells, breaking cellular membranes and reacting with the nucleic acids, proteins, and enzymes present in the cells. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to lose their structure, function and eventually result in cell dysfunction. They are continuously produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc. (Li & Trush, 1994). Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems which scavenge these free radicals preventing them from causing deleterious effects

in the body (Nose, 2000). The antioxidant defense systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental conditions or infections, it leads to oxidative stress (Finkel & Holbrook, 2000).

When the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system, oxidative stress occurs in cellular system, including the superoxide anion radical, the hydroxyl radical, hydrogen peroxide and the peroxyl are greatly reactive molecules, which consequently generate metabolic products that attack lipids in cel1 membrane or DNA (Halliwell & Gutteridge, 1995). Oxidative stress, involves a series of free radical chain reaction processes, is associated with several types of biological damage, DNA damage, diabetes, respiratory tract disorders, carcinogenesis and cellular degeneration related to aging (Anderson et al., 2000). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng et al., 1997). Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna et al., 2009). The interest in natural antioxidants, especially of plant origin, has greatly increased in recent years as the possibility of toxicity of synthetic antioxidants has been criticized (Jayaprakash and Rao, 2000).

Several herbs and herbal formulations are available for the scavenging activity. In addition to this there is a global trend to revive the traditional systems of medicines and renewed interest in the natural remedies for treating human ailments. Antioxidants have important preventive roles, not only on undesirable changes in the flavor and nutritional quality of food, but also on tissue damage in various human diseases. Almost all organisms are well protected against free radical damage by either enzymes or compounds, such as ascorbic acid, α- tocopherol and gluthione (Singh et.al., 2013).

Phenolic compounds from medicinal plants, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins, possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals.

They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers. (Kähkönen et al., 1999; Proestos et al., 2006) There is a growing interest all over the world for discovering the untapped reservoir of medicinal plants. Hence, the present study was aimed at determining the antioxidant capacities of the plant chosen.

## 1.6.2 Classification of Antioxidants

It is of two types(Gupta et al., 2006):

**1. Based on solubility:**

(a) Hydrophilic antioxidants- They are soluble inwater. Water soluble antioxidants react with oxidantsreact with oxidants in the cell cytoplasm and bloodplasma.

(b) Hydrophobic antioxidants- They are soluble in lipids. Lipid soluble antioxidants protect cell membranes from lipid peroxidation.

**2. Based on line of defense:**

(a) First line defense (preventive antioxidant):

These are enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GTX), glutathione reductase and some minerals like Se, Mn, Cu etc. SOD mainly acts by quenching of superoxide ($O_2$), catalase by catalyzing the decomposition of hydrogen peroxide ($H_2O_2$) to water and oxygen. GTX catalyzes the reduction of $H_2O_2$ and lipid peroxide generated during lipid peroxidation to water using reduced glutathione as substrate.

(b) Second line defense (Radical scavenging antioxidant):

These are glutathione, Vit C, uric acid,albumin, biliribin, vit E, carotenoids, flavonoid etc. β-carotene is an excellent scavenger of singlet oxygen. Vit C interacts directly with radicals like $O_2$, OH. GSH is a good scavenger of many free radicals like $O_2$, OH and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone.

(c) Third line defense (Repair and de-novo enzymes):

These are a complex group of enzymes forrepair of damaged DNA, protein, oxidized lipids andperoxides and also to stop chain propagation ofperoxyl lipid radical. These enzymes repair thedamage to biomolecules and reconstitute the damagedcell membrane.

## 1.7 Cytotoxic Screening

Cytotoxicity is the quality of being toxic to cells. Treating cells with the cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis). To measure the cytotoxicity of a compound derived from plant, a bioassay can be employed in order to provide an estimation of concentration or potency of a substance (drugs, hormones, vitamins, toxins, and antitoxin) by measurement of the biological response that it produces.

Some of the traditional medicine involves the use of crude plant extracts which may contain an extensive diversity of molecules, often with indefinite biological effects. However, most of the available information regarding the medicinal potential of these plants is not provided with credible scientific data. For this reason, several researches have been conducted to determine the toxicity of medicinal plants. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp (*Artemia* sp.) Lethality Assay (BSLA). BSLA is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds. The low cost and ease of performing the assay and the commercial availability of inexpensive brine shrimp eggs makes BSLA a very useful bench top method. This assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts (Olowa and Nuneza, 2013).

In this present study, methanolic extracts of the selected medicinal plant were tested *in vivo* for their cytotoxic effect against the brine shrimp nauplii and relate toxicity results with their known ethno-pharmacological activities.

## 1.8 Review on Plant(*Opuntia elatior*)

**Figure 1.1: *Opuntia elatior***

### 1.8.1 Vernacular Names

**Table 1.6: Showing the vernacular names of *Opuntia elatior* in different regions**(Kirtikar and Basu, 1999)

| Region/ Tribal name | Vernacular names |
|---|---|
| **Arabic** | Jhakawoon |
| **Bengal** | Negphana, Phenimansa |
| **Burma** | Kalzaw, Shasounglitwa |
| **Canaresa** | Chappatigalli, Dabbugalli, Nagadali |
| **Deccan** | Chappal, Chappalsend, Nagphansi |
| **English** | Prickly pear, Slipper Thorn |
| **French** | Raquette |
| **Gujrati** | Chorhathalo, Zhorhatheylo |
| **Hindi** | Haththathoira, Nagphana, Nagphani |
| **Malayam** | Nagamullu, Nagatali, Palakakkalli |
| **Marathi** | Chapal, Nagaphana Samar |
| **Murang** | Philorotpaw |
| **Portuguese** | Palmatoria d'inferno |
| **Sanskrit** | Bahudugdhika, Bahushala, Gula |
| **Sinhales** | Kodugaha |

| | |
|---|---|
| **Tamil** | Kalli, Manjarnagadali, Mullukkalli |
| **Telegu** | Nagadali, Nagajemudu, Nagamullu |
| **Tulu** | Kalli |
| **Urdu** | Nagaphani, Thuar |
| **Uriya** | Nagophenia, Nagopheni, Poturiyasiju |

### 1.8.2 Taxonomy of *Opuntia elatior*(Robinson, 1974).

Kingdom: Plantae

Subkingdom: Viridiplantae

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Archichlamydeae

Order: Caryophyllales

Family: Cactaceae

Subfamily: *Cereoideae, Opuntioideae, Pereskioideae*

Tribe: *Opuntieae*

Genus: *Opuntia*

Species: *Opuntia elatior*

### 1.8.3 Synonyms:

*O. nigricans*Haw.;*O. burgeriana; Cactus tuna* var.*elatior; C. elatior*

### 1.8.4 Scientific Name

*Opuntia elatior*

### 1.8.5 Plant Family

Cactaceae

## 1.8.6 Cactaceae Family

The Cactaceae is a family belonging to the order Caryophyllales. Cacti typically are found in dry and arid desert or semi-desert regions with high average daytime temperatures and cold nights, and high evaporation rates. Cacti range from Canada to Argentina, predominantly occurring in the warm and arid reaches of the continents of both North and South America across a wide range of different habitats like deserts, sandy coastal stretches, scrublands, dry deciduous forests, high alpine steppes and tropical rain forests (Barthlott and Hunt, 1993; Gibson and Nobel, 1986; Nyffeler, 2001). The main diversity centers are Mexico and south-west USA, central Andes, Brazil, Paraguay, Uruguay and Argentina with Mexico being the richest and most endemic region (Boyle and Anderson, 2002; Ortega-Baes). The family is classified into three subfamilies: Pereskioideae, Opuntioideae, and Cactoideae (Schumann, 1899; Barthlott and Hunt, 1993).

## 1.8.7 Physical description of Cactaceae Family

Cacti show remarkable variation in growth form including large tree-like or columnar forms, solitary or clumped globular or globose habits, and even epiphytes and climbers. Cacti are characterized by highly organized fleshy stems and branches either bearing reduced or highly modified leaves or leaves are often replaced by specialized spines, hairs, bristles or scales borne to a central swollen fleshy structure called the areoles, which in turn is understood according to botanists to be a reduced form of a branch. Most cacti members are characterized by the presence of areole an important diagnostic character of the family. Among other important reproductive diagnostic characters of the family include presence of composite tubular floral structure, pericarpels and tepals. Cacti are champions of adaptations to difficult environment and are a great model of understanding plant evolutionary biology.

True leaves are quite rare among the family members to cope with their harsh desert environment. Most terrestrial cacti have small or large spines as an effective protective measure against herbivores as well as for preventing the loss ofmoisture; while epiphytic members and climbers of the family usually have hairs or bristles providing similar function. This areolar arrangement of spines, bristles or scales show wide diversity among different members of the family showcasing spectacular

morphological adaptations. The root system of several species have been found to be long, branched with intricate interwoven networks reaching great depths below the arid soil surface in search of available water of the water table. Cacti flowers are highly colorful and attractive bearing both accessory and reproductive whorls including intergrading sepals and petals forming composite tubular structure (with wide morphological modifications among different members), multiple stamens, single style and multi-lobed stigma. The flowers vary in color, shape and form across different genera and species demonstrating a kaleidoscope of color and spectacular forms adorning their dry, desert gardens.

## 1.8.8 Botanical Description of *Opuntia* Species

### (i) *Opuntia* genus

A general characterization of each of the varieties is given followed by a particular descriptor
(Ochoa, 2003).

- Plant descriptors
- Plant Size

(a)Small (height < 1.5 m) (b) Medium (1.6 – 2.0 m) (c) Large (> 2.1 m)

- Plant Shape (Figure 1.2)

(a) Flat (b) Round (c) Elongate (width < height)

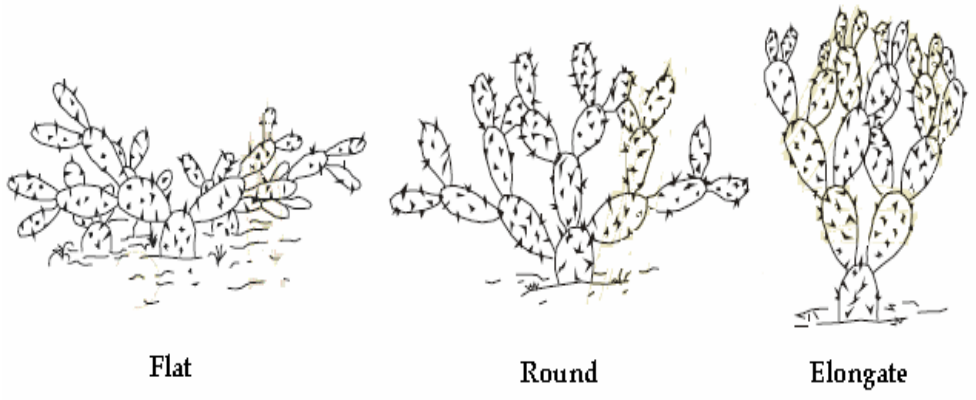



**Figure 1.2: The plant shape of *Opuntia* spp.**

- Habitus (Figure 1.3)

(a) Upright (b)Medium(c)Spreading (d)Prostrate (e)Shrubby (f) Arborescent

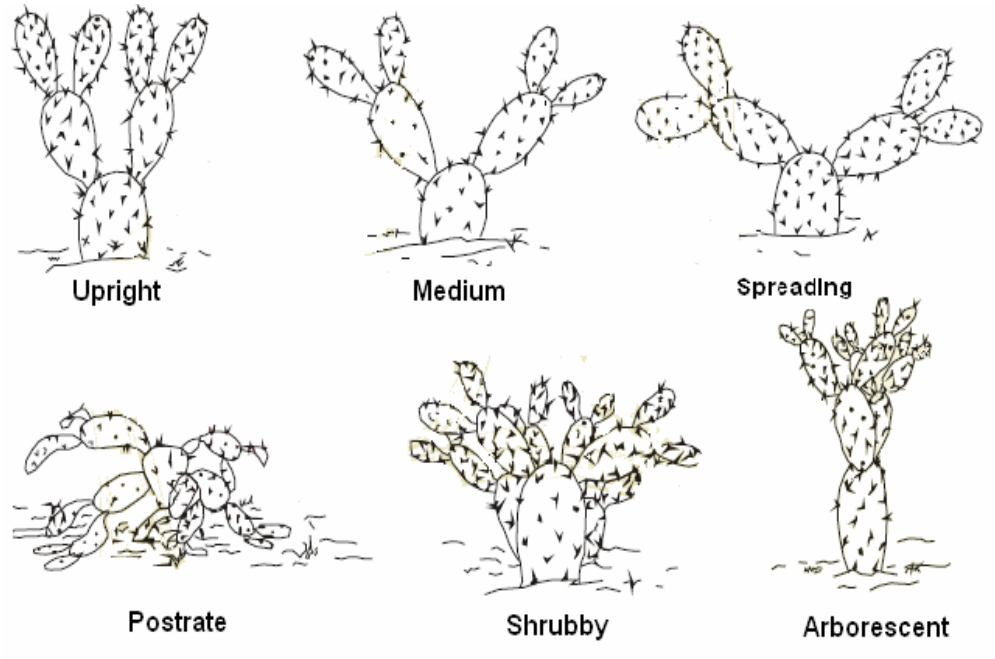



**Figure 1.3: Habitus of *Opuntia* spp.**

- Phylloclades descriptors
- Cladodes Shape (Figure 1.4)

(a) Ovate (b) Round (c) Elliptic

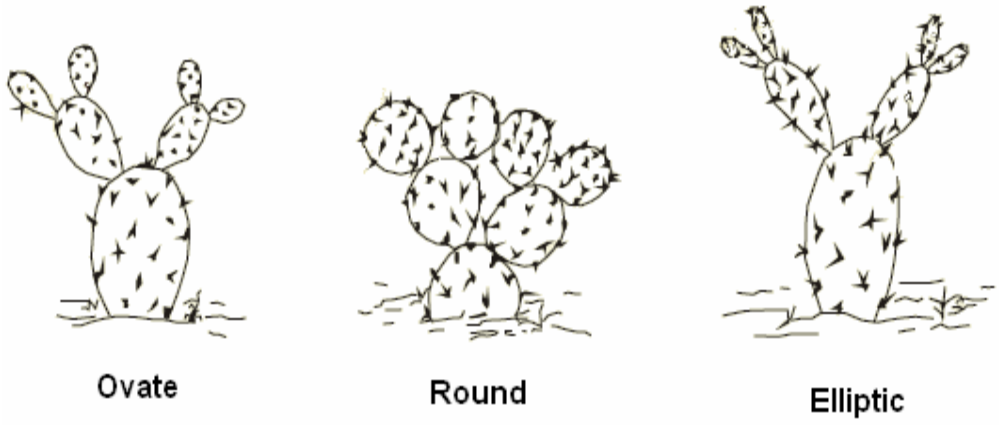



**Figure 1.4: Phylloclades Shape**

- Spines

(a) Absent (b) Few (c) Intermediate (d)Few

- Glochides:

They are very little thorns that shoot up from the areoles of a dense fascicule having their front free end some what rised so that they act as a hook penetrating the skin easily thought it is hard to take them out.

(a) Absent (b) Few (c) Intermediate (d) Many

- Fruit descriptor
- Shape (Figure 1.5)

(a) Ovoid (b) Round (c) Elliptic (d) Oblong

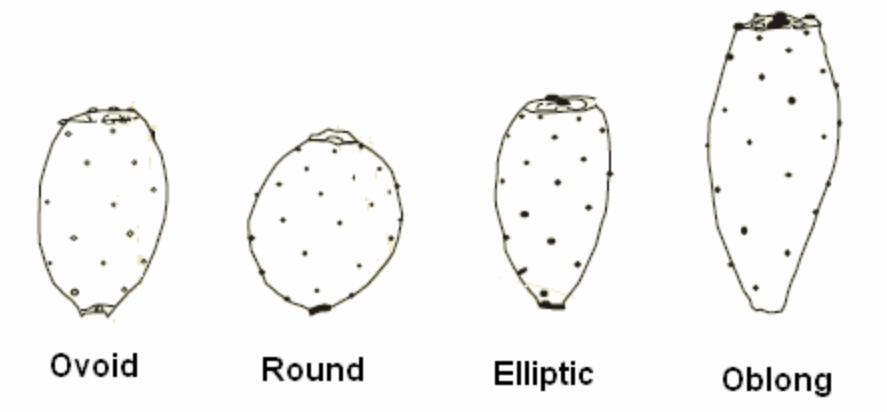



**Figure 1.5: Shape of Fruit of *Opuntia* spp.**

- Recepticular Scar Position (Figure 1.6): This characteristic is included because its importance in the spines removal process.

(a) Elevated (b) Flattened (c) Sunken


**Figure 1.6: Recepticular Scar Position in Fruit of *Opuntia* spp.**

- Fruit Color

(a) Green (b) White (c) Light Yellow (d) Yellow (e) Orange (f) Pink (g) Red (h) Purple

**(ii) *Opuntia elatior* Mill.**

Subarborescent or shrubby, 3 meter high or more. Leaves 7.5 mm long, subulate, recurved, reddish at the tips. Joints variable in size, about 18-30 cm in height by 10-18 cm in width, obovate or elliptic, rather thin, not undulate, dull bluish green. Areoles bearing about 4-5 cm increasing up to 10 cm, rather slender straight prickles which are grey and opaque except when quite young, the largest 3-5 cm. long; glochidia inconspicuous, almost hidden amongst woolly hairs, rusty-brown. Flowers 5 cm. across, yellow or orange. Perianth rotate, the outer segments short, ovate, acute, red in the centre, yellow at the edges, the inner spathulate, acute. Stamens a little shorter than the perianth.Style exceeding the stamens; stigmas 6 in number.Berry pyriform, angular or more or less warty, bearing tufts of glochidia and occasionally a few prickles, reddish purple when ripe (Kirtikar and Basu, 1999).

## 1.8.9 Traditional Uses of *Opuntia* species

The plant is bitter, hot; laxative, stomachic, carminative, diuretic, antipyretic, alexiteric; cures biliousness, burning, leucoderma, "vata", urinary complaints, tumors, ascites, loss of consciousness, piles, inflammations, vesicular calculi, anaemia, ulcers, cures bronchitis of children, ophthalmia, liver complaints lumbago and enlargement of the spleen. The cladodes are very tasty, stomachic; cure inflammations, ascites, tumors, pains. They mashed up and applied as a poultice are said to allay heat and inflammation. The hot cladode applied to boils hastens suppuration; it made into a pulp is applied to the eyes in cases of ophthalmia. In South Africa and in Australia a decoction of the stem has been used as a diabetes remedy. The flowers cure bronchitis and asthma. The fruit is considered a refrigerant, and is said to be useful in gonorrhea. The baked fruit is said to be given in whooping cough andsyrup of the fruit is said to increase the secretion of bile and control spasmodic cough and expectoration (Kirtikar and Basu, 1999). In addition to food, Indian fig is used to treat whooping cough, diabetes, prostate problems, rheumatism, nosebleed, and in dentistry in central Mexico (Duke and Vasquez, 1994). Sicilians use the fruits as Mexicans do, boiling the juice into syrup and also producing a jam. A tea is made from the flowers and

drunk for kidney problems. Dried flowers are also ground into a paste and applied to the skin for measles. The Sicilians do not eat the stem joints, however, which Mexicans call nopales and nopalitos. Instead, stem jointsare fed to livestock on occasion because of their high water content (Barbera et al., 1992).

In addition to the excellent quality and favor of the fresh fruit, the young phylloclades serve both as a vegetable and salad dish and the immature fruit is used to make mock gherkins (Gurbachan singh, 2003). Although traditionally appreciated for its pharmacological properties by the Native Americans, cactus pear is still hardly recognized because of insufficient scientific information (Feugang *et al.*, 2006).

## 1.8.10 Chemical constituents of *Opuntia elatior*

Stems contain malate of manganese, a fatty acid, citric acid, wax, resin and sugar. Fruits contain carbohydrates (mucilage, sugars), albuminoids, fat, vitamin C and other fruit acids. Ripe fruits contain a red pigment, betanin. Flowers contain flavonoids, glycosides of *iso*-rhamnetin, quercetin, *iso*-quercitrin and narcissin. The plant also contains β-sitosterol, opuntiol and opuntiol acetate. A polysaccharide containing galactose and arabinose in 3:1 molar ratio has been isolated from pods (Ghani, 2003).

# Chapter Two

# LITERATURE REVIEW

## 2.1 Analgesic & Anti-inflammatory

Park et al. (2001) studied the various fractionation of the methanol extract of stems of *Opuntia ficus-indica* Mill. for anti-inflammatory action using adjuvant-induced pouch granuloma model in mice and identified β-sitosterol as an active anti-inflammatory compound. Lyophilized aqueous extract (100–400 mg/kg, i.p.) of the fruits of *Opuntia dillenii* (Ker-Gawl) Haw was evaluated for analgesic activity using writhing and hot plate test in mice and rat, respectively and also anti-inflammatory activity using carrageenan-induced paw edema in rats, the results exhibited dose dependent action (Loro et al., 1999).

## 2.2 Anticancer

Most recent studies suggests that the cactus pear fruit extract (i) inhibits the proliferation of cervical, ovarian and bladder cancer cell lines *in vitro*, and (ii) suppresses tumor growth in the nude mice ovarian cancer model *in vivo*. These experiments showed that inhibition was dose- (1, 5, 10 and 25% cactus pear extract) and time- (1, 3 or 5 day treatment) dependent on *in vitro*-cultured cancer cells. The intra-peritoneal administration of cactus extract solution into mice did not affect the animal body weight, which indicated that cactus did not have a significant toxic effect in animals. Growth inhibition of cultured-cancer cells was associated with an increase in apoptotic cells and the cell cycle arrest at the G1-phase. Moreover, the induced growth inhibition seems dependent on the P53 pathway, which is the major tumor suppressor. Annexin IV was increased and the VEGF decreased in the tumor tissue obtained from animals having received the cactus solution. The antiproliferative effect of betanin, isolated from the fruits of *Opuntia ficus indica*, was evaluated on human chronic myeloid leukemia cell line (K562). The results show dose and time dependent decrease in the proliferation of K562 cells treated with betanin with an $IC_{50}$ of 40 μM. Further studies involving scanning and transmissionelectron microscopy revealed the apoptotic characteristics such as chromatin condensation, cell shrinkage and membrane blebbing. Agarose electrophoresis of genomic DNA of cells treated with betanin showed fragmentation pattern typical for apoptotic cells. Flow cytometric analysis of cells treated with 40 mM betanin showed 28.4% of cells in sub G0/G1 phase. Betanin treatment to the cells also induced the release of cytochrome *c* into the cytosol, PARP cleavage, down regulation Bcl-2, and reduction in the membrane potentials. These studies demonstrate that betanin induces apoptosis in K562 cells

through the intrinsic pathway and is mediated by the release of cytochrome *c* from mitochondria into the cytosol, and PARP cleavage. The mechanisms responsible for executing the antiproliferative effects include: (i) induction of alterations in the cell differentiation pattern, which plays a vital role in the invasiveness and metastatic progression of the tumors, (ii) blockade of pre neoplastic cell expansion or induction of apoptosis, and (iii) intervention of metabolic activation of carcinogens by scavenging ROS (Sreekanth et al., 2007).

## 2.3 Antidiabetic

The prickly pear cactus stems have been used traditionally to treat diabetes in Mexico (Domínguez López, 1995). Nowadays, *Opuntia* species is amongst the majority of products recommended by Italian herbalists that may be efficacious in reducing glycemia (Cicero et al., 2004). The hypoglycemic activity of broiled stem of *Opuntia streptacantha* Lemaire was demonstrated using different extract preparation and dosed in diabetic and non-diabetic human volunteers by Meckes-Lozyoa and Roman-Ramos (1986), Frati et al. (1989, 1989a, 1990, 1991), and Roman-Romas et al (1991). Some studies have demonstrated the hypoglycemic activity of the prickly pear cactus extract on non-diabetics and diabetic-induced rats or diabetic humans (Ibanez-Camacho et al., 1979, 1983). The anti-hyperglycemic effect of 12 edible plants was studied on rabbits, submitted weekly to subcutaneous glucose tolerance tests after gastric administration of a juice of stems of *Opuntia streptacantha* (dose, 4 ml/kg) which decrease significantly the area under the glucose tolerance curve and the hyperglycemic peak (Roman-Ramos et al., 1995). The hypoglycemic activity of a purified extract from stems of *Opuntia fuliginosa* Griffiths was evaluated on Streptozotocin-induced diabetic rats. Blood glucose and glycated hemoglobin levels were reduced to normal values by a combined treatment of insulin and *Opuntia* extract. When insulin was withdrawn from the combined treatment, the prickly pear extracts alone maintained normoglycemic state in the diabetic rats. The magnitude of the glucose control by the small amount of *Opuntia* extract required (1 mg/kg body weight per day) to control diabetes contrast with the high quantities of insulin required for an equivalent effect. Plasma glucose concentrations in Streptozotocin-induced diabetic and non-diabetic rats were reduced by the orally administration of *O. megacantha* leaf extracts (20 mg/100 g body weight). The results suggest that leaf extracts not only reduce blood glucose levels, but may be toxic to the kidney as shown

by the elevation in plasma urea and creatinine concentrations and the reduction of plasma $Na^{+}$ concentration. The seed oil from fruits of *Opuntia ficus-indica* is rich in polyunsaturated fatty acids with an exceptional level of linoleic acid (700g/kg). In this study, evaluated the effect of seed oil supplemented diet on rats, the results indicated a significant decrease in serum glucose concentration (22%) over the control group and an increase in the concentration of glycogen in liver and muscle. Blood cholesterol and low density lipoprotein-cholesterol decreased in the treated group and high density lipoprotein-cholesterol concentration increased during the treatment. These findings support the nutritional value of cactus pear as a natural source of edible oil containing essential fatty acids (Ennouri et al., 2006, 2006a).

## 2.4 Antihyperlipidemic & Hypercholesterolemic

Experimental evidence suggested that cactus pear reduces cholesterol levels in human blood and modify low density lipoprotein composition (Fernandez et al., 1992) have found that the cholesterol, low density lipoprotein and triglyceride plasma levels of rats were strongly reduced after 30 days of a daily administration (1 g/kg) of lyophilized cladodes of *Opuntia ficus-indica* L. Mill. Sterols which comprise the bulk of the unsaponifiables in many oils are of interest due to their ability to lower blood low density lipoprotein-cholesterol by approximately 10–15% as part of a healthy diet (Jones et al., 2000) investigated the effects of diets enriched with cactus pear oil and seeds on serum and liver parameters, the results indicated a significantly decreased blood cholesterol and low density lipoprotein-cholesterol and increased high density lipoprotein-cholesterol.

## 2.5 Antioxidant

The antioxidative action is one of many mechanisms by which fruit and vegetable substances might exert their beneficial health effects. The presence of several antioxidants (ascorbic acid, carotenoids, reduced glutathione, cysteine, taurine and flavonoids such as quercetin, kaempferol and isorhamnetin) has been detected in the fruits and vegetables of different varieties of cactus prickly pear. More recently, the antioxidant properties of the most frequent cactus pear betalains (betanin and indicaxanthin) have been revealed (Tesoriere et al., 2005). Numerous *in vitro* studies have demonstrated the beneficial effect of phenolics and betalains. These are generally attributed to the ability of antioxidants to neutralize reactive oxygen species such as singlet oxygen, hydrogen peroxide or $H_2O_2$or suppression of the

xanthine/xanthineoxidase system, all of which may induceoxidative injury, i.e. lipid peroxidation. Regular ingestion of prickly pear (*Opuntia robusta*) is able to significantly reduce in-vivo oxidation injury in young patients suffering from familial isolated hypercholesterolemia and oxidation injury determined via 8-epi-$PGF_{2\alpha}$in plasma, serum and urine. The findings on a decrease of 8-epi-$PGF_{2\alpha}$were more pronounced in females than in males, the highest significance being found in urine, while, in contrast, the effects on total- and low density lipoprotein-cholesterol were more pronounced in males. Thus, this may have a significant cardiovascular benefit. Kuti (2004) investigated antioxidant compounds in extracts from four *Opuntia* species (*O. ficus-indica, O. lindheimeri, O. streptacantha, O. stricta var. stricta*) fruit. ZEN is one of the most widely distributed fusarial mycotoxins which are encountered at high incidence in many foodstuffs. In this study, the effect of a single dose of ZEN (40 mg/kg b.w.) alone and with extract of cactus cladodes (25, 50 and 100 mg/kg b.w.) on the induction of oxidative stress was monitored in kidney and liver by measuring the MDA level, the protein carbonyls generation, the catalase activity and the expression of the heat shock proteins (Hsp). The results clearly showed that ZEN induced significant alterations in all tested oxidative stress markers, while the combined treatment of ZENwith the lowest tested dose of cactus extracts (25 mg/kg b.w.) showed a total reduction of ZEN induced oxidative damage for all tested markers.

Su-Feng Chang et al. (2008) investigated the antioxidant activity and inhibitory effect of extracts from *Opuntia dillenii* Haw fruit on low-density lipoprotein peroxidation. The results indicated that the antioxidant activity of methanolic extracts in Trolox equivalent antioxidant capacity and oxygen-radical absorbance capacity assays were in the order of seed > peel > pulp. Among the extracts, seed extracts 10 μg/ml) possessed the highest inhibitory effect on the formation of thiobarbituric acid reactive substances and relative electrophoretic mobility and contained the highest amounts of polyphenols and flavonoids (212.8 and 144.1 mg/100 g fresh seed), respectively.

## 2.6 Antiulcer

In Sicily folk medicine, *Opuntia ficus-indica* (L.) Mill.cladodes are used for the treatment of gastric ulcer and cicatrisant action. Galati et al. (2001, 2002a) studied the effect of lyophilized cladodes (1 g/kg) using ethanol-induced ulcer model in rat. In this study, the ultra structural changes were observed by transmission electronic

microscopy confirming the protective effect exercised by administration of lyophilized cladodes. Probably, the mucilage of *Opuntia ficus-indica* is involved.

## 2.7 Antiviral

An interesting study by Ahmad et al. (1996) demonstrated that administration of a cactus stem extract (*Opuntia streptacantha*) to mice, horses, and humans inhibits intracellular replication of a number of DNA- and RNA-viruses such as Herpes simplex virus Type 2, Equine herpes virus, pseudorabies virus, influenza virus, respiratory syncitial disease virus and HIV-1. An inactivation of extra-cellular viruses was also reported by the same authors. However, the active inhibitory component(s) of the cactus extract used in this study was not investigated, and as of yet, no further study dealt with this specific topic. Mtambo et al. (1999) evaluated the efficacy of the crude extract of *Opuntia vulgaris* against Newcastle virus disease in domestic fowl in Tanzania.

## 2.8 Diuretics

Galati et al. (2002) studied the diuretic activity of *Opuntia ficus-indica* (L.) Mill. waste matter in rat. Acute and chronic diuretic activity of 15% infusion of cladodes, flowers and fruits were assayed. Natriuresis, kaliuresis and the activity on fructose-induced hyperuricemia was also studied. The results show that *O. ficus-indica* cladode, fruit and flower infusions significantly increase diuresis. This effect is more marked with the fruit infusion and it is particularly significant during the chronic treatment. The fruit infusion shows also antiuric effect. In this study, cladode, flower and fruit infusions showed a modest but not significant increase in natriuresis and kaliuresis.

## 2.9 Immunomodulatory

Research provide a molecular basis to explain a portion of the beneficial therapeutic properties of extracts from *O. polyacantha* on human and murine macrophages demonstrated that all four fractions had potent immunomodulatory activity, inducing production of reactive oxygen species, nitric oxide, TNFα, and interleukin 6. Modulation of macrophage function by *Opuntia* polysaccharides was mediated through activation of nuclear factor κB.

## 2.10 Improve Platelet Function

Prickly pear is traditionally used by Pima Indians as a dietary nutrient against diabetes mellitus. Wolfram et al. (2003) examined the effect of daily consumption of 250g in 8 healthy volunteers and 8 patients with mild familial heterozygous hypercholesterolemia on various parameters of platelet function. Beside its action on lipids and lipoproteins, prickly pear consumption significantly reduced the platelet proteins (platelet factor 4 and β-thromboglobulin), ADP-induced platelet aggregation and improved platelet sensitivity (against $PGI_2$ and $PGE_1$) in volunteers as well as in patients. Also plasma 11-DH-$TXB_2$ and the WU-test showed a significant improvement in both patients and volunteers. In contrast, collagen-induced platelet aggregation and the number of circulating endothelial cells showed a significant response in patients only. Prickly pear may induce at least part of its beneficial actions on the cardiovascular system via decreasing platelet activity and thereby improving haemostatic balance.

### 2.11 Neuroprotective

Jungsook Cho et al. (2003) isolated the flavonoids quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether from the ethyl acetate fractions of the fruits and stems of *Opuntia ficus-indica var. saboten* and evaluated their protective effects against oxidative neuronal injuries induced in primary cultured rat cortical cells and their antioxidant activities by using lipid peroxidation, 1,1-diphenyl-2-picrylhydrazyl, and xanthine oxidase bioassays. Quercetin was found to inhibit $H_2O_2$- or xanthine / xanthine oxidase-induced oxidative neuronal cell injury, with an estimated $IC_{50}$ of 4–5 μg/ ml and no more protection at concentrations of 30μg/ml and above while (+)-dihydroquercetin concentration-dependently inhibited oxidative neuronal injuries, but it was less potent than quercetin. On the other hand, quercetin 3-methyl ether potently and dramatically inhibited $H_2O_2$ - and xanthine / xanthine oxidase-induced neuronal injuries, with $IC_{50}$values of 0.6 and 0.7 μg/ ml, respectively. In addition, quercetin and quercetin 3-methyl ether were shown to inhibit xanthine oxidase activity *in vitro*, with respective $IC_{50}$values of 10.67 and 42.01 μg/ ml and quercetin-3-methyl ether appears to be the most potent neuroprotectant of the three flavonoids isolated from this plant.

Jung-Hoon Kima et al. (2006) examined the methanol extract of *Opuntia ficus-indica* (MEOF) as a neuroprotective action against *N*-methyl-d-aspartate (NMDA)-, kainate

(KA)-, and oxygen–glucose deprivation (OGD)-induced neuronal injury in cultured mouse cortical cells and also evaluated the protective effect in the hippocampal CA1 region against neuronal damage evoked by global ischemia in gerbils. Treatment of neuronal cultures with MEOF (30, 300, and 1000 μg/ml) inhibited NMDA (25 μM)-, KA (30 μM)-, and OGD (50 min)-induced neurotoxicity dose-dependently. The butanol fraction of *Opuntia ficus indica* (300 μg/ml) significantly reduced NMDA (20 μM)-induced delayed neurotoxicity by 27%. Gerbils were treated with MEOF every 24 h for 3 days (0.1, 1.0, and 4.0 g/kg, p.o.) or for 4 weeks (0.1 and 1.0 g/kg, p.o.), and ischemic injury was induced after the last dose. Neuronal cell damage in the hippocampal CA1 region was evaluated quantitatively at 5 days after the ischemic injury. When gerbils were given doses of 4.0 g/kg (3 days) and 1.0 g/kg (4 weeks), the neuronal damage in the hippocampal region was reduced by 32 and 36%, respectively. These results suggestedthat the preventive administration of *Opuntia ficus-indica* extracts may be helpful in alleviating the excitotoxic neuronal damage induced by global ischemia.

## 2.12 Antispermatogenic

A methanolic extract from *O. dillenii* Haw.defatted with chloroform and petroleum ether exerted antispermatogenic effects in animal tests on rats. According to (Gupta et al., 2002), the flavone derivatives vitexin and myricetin were found to be the active principles. When 250 mg extract per kg body weight was applied, the weight of testis, epididymis, seminal vesicle, and ventral prostate were reasonably, that of Sertoli cells, Leydig cells, and gametes considerably reduced. The motility of the sperms was also diminished.

## 2.13 Wound healing

In traditional medicine extracts of polysaccharide-containing plants are widely employed for the treatment of skin and epithelium wounds and of mucous membrane irritation. The extracts of *Opuntia ficus-indica* cladodes are used in folk medicine for their antiulcer and wound-healing activities. The methanolic extract of *Opuntia ficus-indica* stems and its hexane, ethyl acetate, *n*-butanol and aqueous fractions (100 mg/site) exhibited wound healing activity in rats by measuring the tensile strength of skin strips from the wound segments. The extract and less polar fractions showed significant effects (Park & Chun, 2001).

Trombetta et al. (2006) described the wound-healing potential of two lyophilized polysaccharide extracts obtained from *O. ficus-indica* (L.) cladodes applied on large full-thickness wounds in the rat. The wound-healing effect is more marked for polysaccharides with a molecular weight ranging $10^4$ – $10^6$Da than for those with molecular weight>$10^6$Da, author supposed that the fine structure of these polysaccharides and their particular hygroscopic, rheologic and viscoelastic properties may be essential for the wound-healing promoter action.

## 2.14 Monoamino-oxidase inhibition

Besides catecholmethyltransferases, the monoamino-oxidases (MAOs) are usually involved in the catabolism of catecholamines, thus regulating the overall amine pool. In cladodes and fruits from the Korean *O. ficus-indica var. saboten* Makino, methyl esters derived from organic acids were identified as MAO inhibitors. The aqueous extracts showed least inhibitory activity, followed by the n-butanol fraction and the hexane extract whereas the ethyl acetate fraction exerted the highest inhibitory action. The active agents were identified as 1-methyl malate, 1-monomethyl citrate, 1,3-dimethylcitrate, and 1,2,3-trimethylcitrate. The purified components showed MAO-A inhibitory action with increasing number of methyl substituents, whilst the MAO-B inhibitory action was superior for 1-methylmalate compared to the mono- and dimethylcitrates. However, 1,2,3-trimethylcitrate exerted the strongest inhibition on both MAOs. When citrate was compared with its corresponding methyl derivatives, the methoxy moiety proved to be the effective moiety (Han et al., 2001).

## 2.15 Nutritional Importance

Cacti have long been considered an important nutritional source in Latin America (bread of the poor) among which *Opuntia* has gained highest economic importance worldwide. It is cultivated in several countries such as Mexico, Argentina, Brazil, Tunisia, Italy, Israel and China. Both fruit and stems have been regarded to be safe for food consumption. The constantly increasing demand for nutraceuticals is paralleled by a more pronounced request for natural ingredients and health-promoting foods. The multiple functional properties of cactus pear fit well this trend. Recent data revealed the high content of some chemical constituents, which can give added value to this fruit on a nutritional and technological functionality basis. High levels of betalains, taurine, calcium, magnesium, and antioxidants are noteworthy (Piga, 2004; Stintzing & Carle, 2005).

The *Opuntia* species cladodes and fruits serve as a source of varied number of phytoconstituents mainly sugar, phenolics and pigments. Total betalains are well reported with their qualitative and quantitative analytical methods. Though various analyticalmethods are reported, but still some focus is required towards HPTLC with marker's evidence. Although the reported evidences provide the effectiveness of *Opuntia* species, but active constituents, bioavailability, pharmacokinetics and physiological pathways for various biological actions are not well known with sufficient detail or confidence. Ethnopharmacological actions may be due to presence of phenolics and pigments. Still more attention is required towards the development of simple, feasible and cost effective pharmaceutical preparations of *Opuntia spp.* cladodes and fruit juice as well as the ethnopharmacological approach, if combined with mechanism of action, biochemical and physiological methods, would provide useful pharmacological leads.

# Chapter Three

# METHODS AND MATERIALS

## 3.1 Collection & Preparation of Plant Material

Plant sample (Leaves) of *Opuntia elatior* was collected from Noakhali on April 2015. Then proper identification of plant sample was done by an expert taxonomist. The leaves of the plant were sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried leaves was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Department of Pharmacy, East West University.

## 3.2 Extraction of the Plant Material

The fine powder of plants was soaked in 2 liter methanol and it was thoroughly shaken to dissolve the powder into the solvent. Then it was kept in a closely covered glass jar for 7 days and shaken several times during the process for more interaction between the powdered particles and the solvent. This process is termed as maceration. The cover of the jar was closed properly to prevent the entrance of air in the jar.

## 3.3 Filtration

After the extraction process the plant extract was filtered with sterilized cotton filter followed by Whatman No.1 filter paper. The filtrate was collected in a beaker. The filtration process was repeated three times by using cotton and filter paper. Then the filtrate was taken into a conical flask and covered with aluminiumfoil paper for later treatment with rotary evaporation.

## 3.4 Evaporation & Extract Preparation

For evaporating the solvent and collect for reuseHeidolphrotary evaporator machine was used featuring a vacuum pump which helped to reduce the pressure of the inside of glass tube coil, as well as the whole system. Reduction of pressure causes quick evaporation. The evaporation was done at 55 degree Celsius temperature. The number of rotation per minute was selected as 110 RPM. The pressure of the vacuum pumper machine (Biometra) was 6 bar. The water flow through the distillation chamber was also provided in a satisfactory flow rate.

The solvent that was evaporated flowed along the condenser where it was condensed back to liquid form. 70% of the solvent was collected and could be reused. The plant

extract was collected from the evaporating flask and the solvent is collected from the receiving flask. The extract is transferred into a 50 ml beaker and covered with aluminum foil.

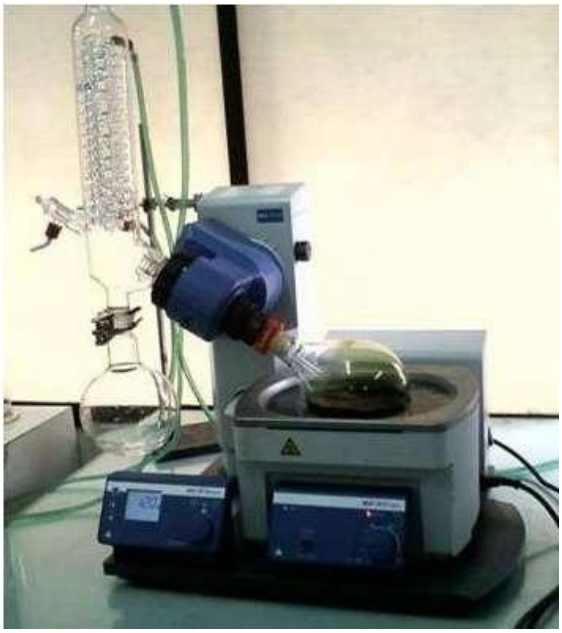


**Figure 3.1: Drying of extract using Rotary evaporator**

With a sharp utensil, the foil was penetrated to produce many pores so that it could be air dried into a concentrated solid residue.

## 3.5 Preparation of Mother Solution

5gm of methanol extract was triturated with 90ml of methanol containing 10ml of distilled water. The crude extract was dissolved completely. This is the mother solution.

## 3.6 Partition of mother solution

The mother solution was then partitioned off successively by three solvents of different polarity.

### 3.6.1 Partition with Pet-ether

The mother solution was taken in a separating funnel. 100ml of the pet-ether was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice (100ml X 3). The Petrolium- ether fraction was then air dried for solid residue.

### 3.6.2 Partition with Dichloromethane

To the mother solution left after partitioning with n-hexane, 12.5ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with dichloromethane (DCM). The process was repeated thrice (100ml X 3). The DCM fraction was then air dried for solid residue.

### 3.6.3 Partition with Ethyl Acetate

To the mother solution left after partitioning with n-hexane, 12.5ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with dichloromethane (DCM). The process was repeated thrice (100ml X 3). The DCM fraction was then air dried for solid residue.

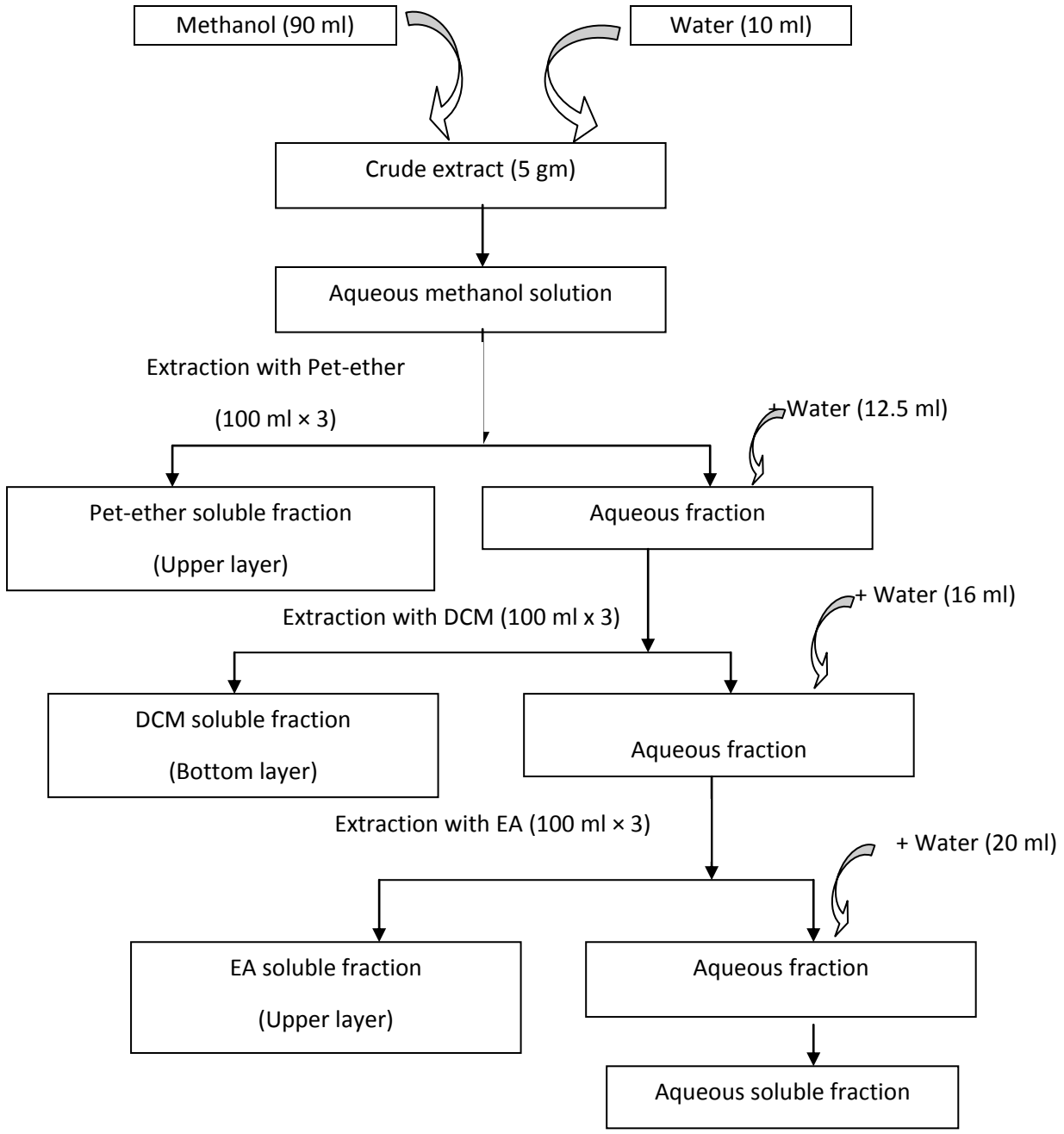



**Figure 3.2: Schematic representation of the partitioning of Methanolic crude *Opuntia elatior***

### 3.6.4 Collection of Dichloromethane Fraction

After partitioning the mother solution with the three different solvents the Dichloromethane fraction was collected and air dried. This ethyl acetate was further investigated for different pharmacological properties (antioxidant, cytotoxic and total reducing power).

## 3.7 Antioxidant activity

### 3.7.1 Total Phenolic Content

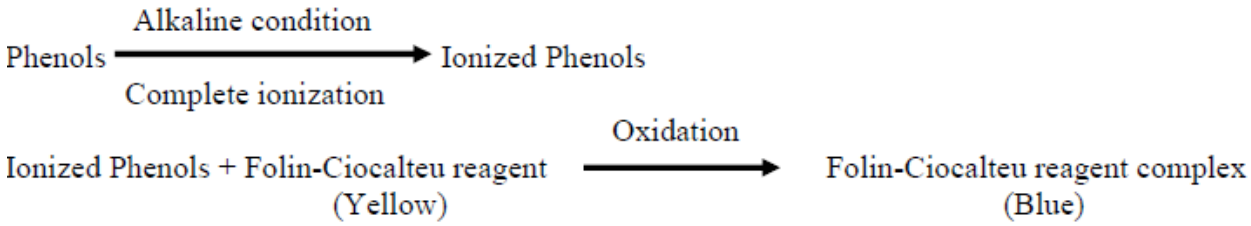
#### 3.7.1.1 Principle

The content of total phenolic compounds in plant methanolic extracts was determined by Folin– Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

**Table 3.1: Composition of 100mg Folin-Ciocalteu Reagent**

| Ingredient | Amount |
|---|---|
| **Water** | 57.5ml |
| **Lithium Sulfate** | 15.0mg |
| **Sodium Tungstate Dihydrate** | 10.0mg |
| **Hydrochloric Acid>=25%** | 10.0mg |
| **Phosphoric Acid 85% solution in water** | 5.0mg |
| **Molybdic Acid Sodium Dihydrate** | 2.5mg |

When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{-4}$.The intensity of the color change is measured in a spectrophotometer at 765 nm. The absorbance value will reflect the total phenolic content of the compound(Singleton *et.al,.*, 1999; Vinson *et.al,.*, 2005).




### 3.7.1.2 Apparatus & Reagents

**Table 3.2: Apparatus and reagents used for total phenolic content**

| | |
|---|---|
| **Folin-Ciocalteu reagent (10 fold diluted)** | UV-spectrophotometer |
| **Ascorbic acid** | Beaker (100 & 200ml) |
| **$Na_2CO_3$ solution (7.5%)** | Test tube |
| **Methanol** | Micropipette (50-200µl) |
| **Distilled water** | Cuvette |

### 3.7.1.3 Procedure

**Standard curve preparation:**

Ascorbic acid was used here as standard. Different ascorbic acid solutions were prepared having a concentration ranging from 120µg/ml to 80µg/ml. 5ml of FCR (diluted 10 times with water) and4ml of $Na_2CO_3$ (7.5%w/v) solution was added to ascorbic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 765nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

**Sample preparation:**

2mg of the*Ficusracemosa* ethyl acetate fraction was taken and dissolved in 1ml of distilled water to get a sample concentration of 2mg/ml.

**Determination of total phenol content:**

- 1.0ml of plant extract (200µg/ml) of different concentrations (120µg/ml, 110µg/ml, 100µg/ml, 90µg/ml and 80µg/ml) was taken in test tubes.

- 5ml of Folin–ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- 4ml of Sodium carbonate solution was added into the test tube.
- The test tubes containing the samples were incubated for 1hr at the room temperature to complete the reaction.
- Then the absorbance of the solution was measured at 765nm using a spectrophotometer against blank.
- A typical blank solution containing methanol was taken.

## 3.7.2 Total Flavonoid Content

### 3.7.2.1 Principle

Aluminium chloride ($AlCl_3$) colorimetric method is incorporated to determine the total flavonoid contents of the crude plant extract. The basic principle of the assay method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols of the crude extract. In addition aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the Aor B-ring of flavonoids. The formed flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride has an absorptivity maximum at 510nm (Chang *et.al,.*, 2002). Therefore, the amount of flavonoid in the crude extract can be quantified by measuring the absorbance of reaction mixture at 510nm using a UV-visible spectrophotometer against a blank containing all reagents except the extracts. Quercetin at various concentrations was used as standard (Chang *et.al,*.2002).

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex ($\lambda$ max 510nm).

### 3.7.2.2 Apparatus &Reagents

**Table 3.3: Apparatus and reagents used for total flavonoid content**

| | |
|---|---|
| **Aluminium chloride** | Spatula |
| **Methanol** | Analytical balance |
| **Ascorbic acid** | Pipette and pumper |
| **Sodium hydroxide** | Aqueous fraction |
| **Sodium nitrite** | Test tubes and beaker |

### 3.7.2.3 Procedure

**Preparation of 10% Aluminium Chloride ($AlCl_3$) Solution:** 10mg of $AlCl_3$ was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

**Preparation of 4% NaOH Solution:** 4mg of NaOH was taken into a 100ml volumetric flask and the volume was adjusted by distilled water.

**Preparation of 5% (W/V) $NaNO_2$ Solution:** 5mg of $NaNO_2$was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

**Preparation of Standard Solution:** The stock solution was prepared by taking 0.025gm of ascorbic acid and dissolved into 5ml of ethanol. The concentration of this solution was 5µg/µl of ascorbic acid. The experimental concentrations from this stock solution were prepared by the following manner.

**Table 3.4: Preparation of standard solution**

| Concentration (µg/ml) | Solution taken from stock solution (µl) | Volume adjusted by ethanol (ml) | Final volume (ml) |
|---|---|---|---|
| **250** | 250 | 4.75 | 5 |
| **200** | 200 | 4.80 | 5 |
| **150** | 150 | 4.85 | 5 |
| **100** | 100 | 4.90 | 5 |
| **50** | 50 | 4.95 | 5 |

**Preparation of Extract Solution:**5ml of each plant extracts were taken and dissolved into 5ml of methanol. The concentration of the solution was 1mg/ml of plant extracts. Then the following steps were carried out. 1.5ml extract was taken in a test tube and then 6ml of distilled water was added. Then 5% of $NaNO_2$ was added and incubated for 6 minutes. 10% $AlCl_3$ was added and incubated for 6 minutes. 4% NaOH and 0.6ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 1.5ml methanol was taken and the same procedure was repeated. Then the absorbance of the solution was measured at 510nm using a spectrophotometer against blank.

### 3.7.3 Determination of total Flavonoid content

1.5ml extract was taken in a test tube and then 6ml of distilled water was added. Then 5% of $NaNO_2$ was added and incubated for 6 minutes. 10% $AlCl_3$was added and incubated for 6minutes. 4% NaOH and 0.6ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 1.5ml methanol was taken and the same procedure was repeated. Then the absorbance of the solution was measured at 510nm using a spectrophotometer against blank.

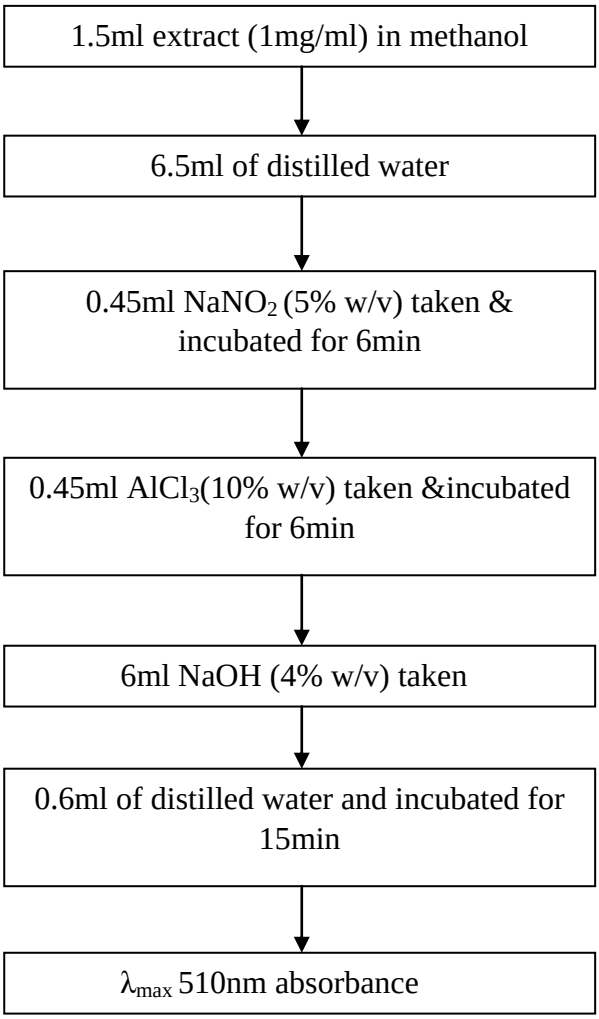



**Figure 3.3: Schematic diagram of preparation of extract solution**

**Preparation of blank solution:**

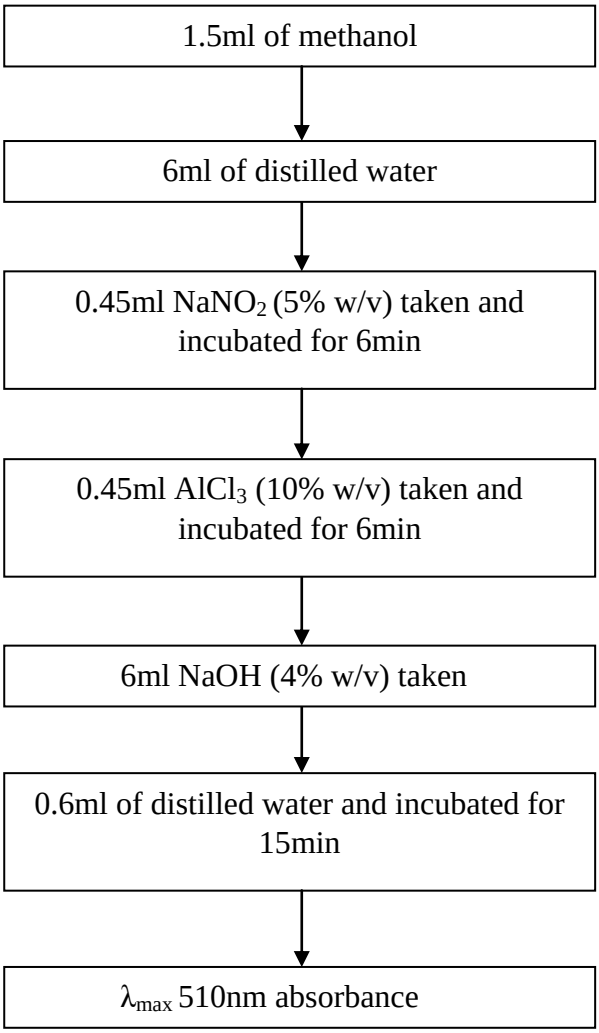



**Figure 3.4: Schematic diagram of preparation of blank solution**

# 3.8 Reducing power Assay

## 3.8.1 Principle

The reducing power of petroleum ether extract of *Opuntia elatior* was determined by the method of Oyaizu [12]. Substances, which have reduction potential, react with potassium ferricyanide ($Fe^{3+}$) to form potassium ferrocyanide ($Fe^{2+}$), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

Potassium ferricyanide + Ferric chloride $\xrightarrow{\text{Antioxidant}}$ Potassium ferrocyanide + ferrous chloride

## 3.8.2 Apparatus and Reagents

**Table 3.5: Apparatus and reagents for Reducing power assay**

| | |
|---|---|
| Potassium ferricyanide | Analytical balance |
| Phosphate buffer | Pipette |
| Trichloro acetic acid | Pumper |
| Ferric chloride | Aqueous fraction |
| Ascorbic acid | Beakers |
| Methanol | Test tubes |
| Spatula | Water |

## 3.8.3 Procedure

**Phosphate buffer (0.2 M, pH 6.6) preparation**

Dibasic sodium phosphate (18.75 ml of 0.2M) is mixed with 31.25 ml monobasic sodium phosphate and diluted to 100 ml with water.

**Potassium ferricyanide (1% w/v) preparation**

1mg of potassium ferricyanide ($K_3[Fe(CN)_6]$) was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

**Trichloro acetic acid (10%) preparation**

10mg of trichloro acetic acid ($CCl_3COOH$) was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

**Ferric chloride (0.1%) preparation**

0.1mg of ferric chloride ($FeCl_3$) was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

**Standard solution preparation**

The stock solution was prepared by taking 0.025gm of ascorbic acid and dissolved into 5ml of methanol. The concentration of this solution was 5µg/µl of ascorbic acid. The experimental concentrations from this stock solution were prepared by the following manner.

**Table 3.6: Different concentrations of ascorbic acid solution preparation**

| Concentration (µg/ml) | Solution taken from stock solution (µl) | Volume adjusted by methanol (ml) | Final volume (ml) |
|---|---|---|---|
| 250 | 250 | 4.75 | 5 |
| 200 | 200 | 4.80 | 5 |
| 150 | 150 | 4.85 | 5 |
| 100 | 100 | 4.90 | 5 |
| 50 | 50 | 4.95 | 5 |

**Extract solution preparation**

5mg of plant extract was taken and dissolved into 5ml of methanol. The concentration of the solution was 1mg/ml of plant extract.

## 3.8.4 Determination of reducing power

2.0 ml plant extract solution and ascorbic acid in different concentrations were taken in test tubes and mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml) and incubated at 500°C for 20 min. 2.5 ml Trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml upper layer (supernatant solution) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. A blank was prepared without adding extract.

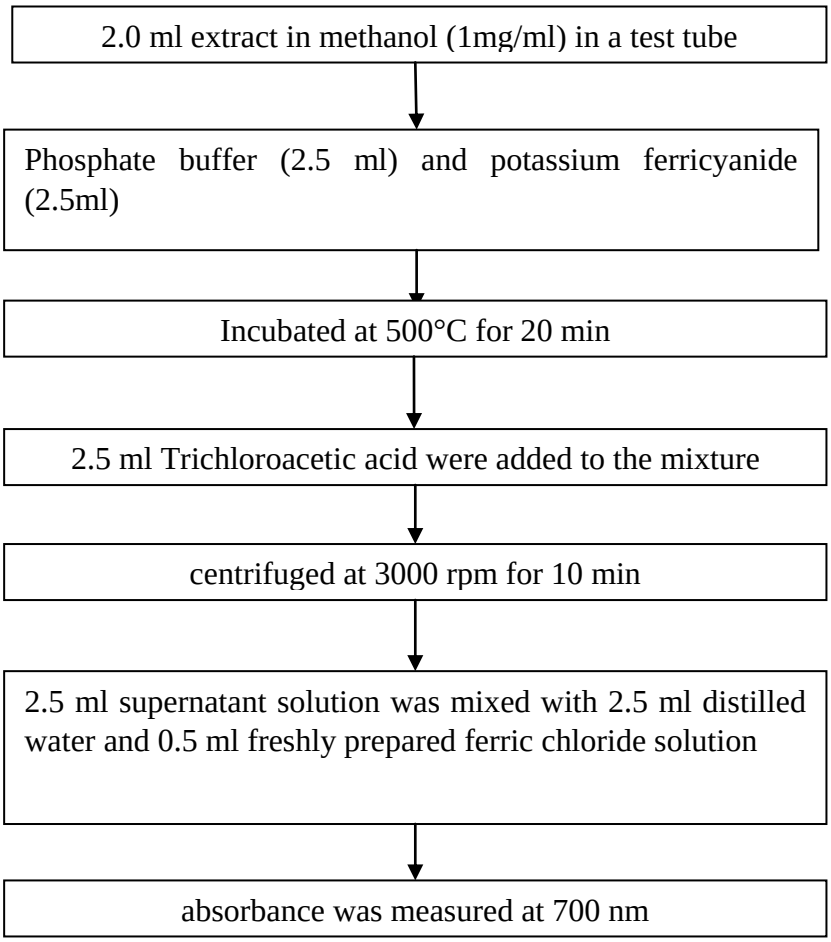



**Figure 3.5: Schematic diagram of preparation of extract solution**

$$\%\,\text{increase in Reducing Power} = \left(\frac{A_{test}}{A_{blank}} - 1\right) \times 100\%$$

Where $A_{test}$ is absorbance of test solution; $A_{blank}$ is absorbance of blank. Increased absorbance of the reaction mixture indicates increase in reducing power (Oyaizu, 1980).

# 3.9 Brine Shrimp Lethality Bioassay

## 3.9.1 Principle

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, and pharmacological activities of natural products etc. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or

toxicology is simply pharmacology at a higher dose. Thus (*in-vivo*) lethality, a simple zoological organism, (Brine shrimp napulii- *Artemia salina*) can be used as a convenient monitoring for screening and fractionation in the discovery of new bioactive natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. Brine shrimp is the English name of the genus Artemia of aquatic crustaceans. Artemia is the only genus in the family Artemiidae (Olowa andNuneza, 2013; Rishikesh*et.al,*.2013).

## 3.9.2 Apparatus & Reagents

**Table 3.7: Apparatus and reagents for Brine shrimp lethality bioassay**

| | |
|---|---|
| *Artemia salina* leach (brine shrimp eggs) | Pipettes & Micropipette |
| Sea salt (NaCl) | Glass vials |
| Small tank with perforated dividing dam to hatch the shrimp | Magnifying glass |
| Lamp to attract shrimps | Test samples |

## 3.9.3 Procedure

### 3.9.3.1 Preparation of Sea Water

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000ml by distilled water in a 1000ml beaker for *Artemia salina* hatching. 1-2 drops of NaOH solution of 1N was added with a dropper to obtain the pH 8.4 as sea water.

A rectangular tank was divided in to two unequal compartments by a porous separator. The larger compartment was darkened while the smaller one was kept illuminated. Then a dry preserved egg of *Artemia salina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. The eggs of *Artemia salina* were hatched at room temperature (25-30ºC) for 18-24hr. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. 10 living shrimps were then collected by a

pipette and then added to each of the test tubes containing 5ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay hatching of brine shrimp.

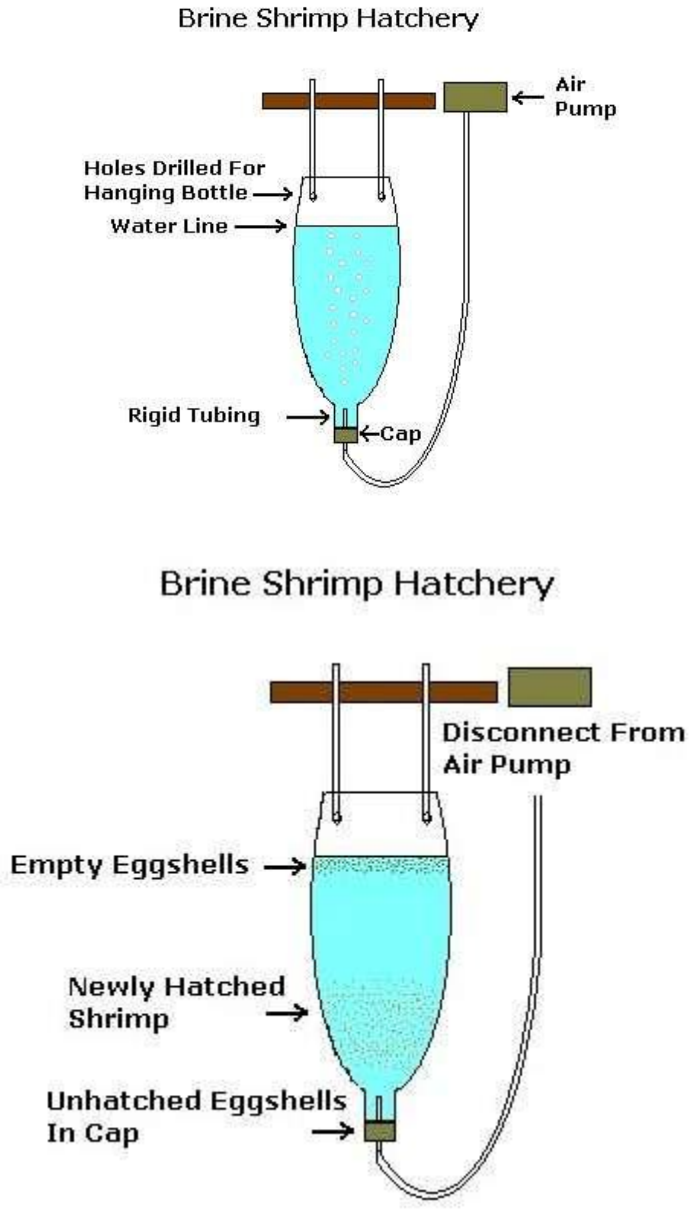



**Figure 3.6: Brine shrimp Hatchery**

### 3.9.3.2 Preparation of Test Solutions

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes

were taken for standard drug tamoxifen for ten concentrations of it and another one test tube for control test.

All the test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

### 3.9.3.3 Preparation of the Test Samples of Experimental Plant

All the test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

### 3.9.3.4 Preparation of the Positive Control Group

In the present study tamoxifen is used as the positive control. Measured amount of the tamoxifen is dissolved in DMSO to get an initial concentration of 20µg/ml. From that stock solution serial dilutions are made using DMSO to get 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml. Then ten living brine shrimp nauplii in 5ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

### 3.9.3.5 Preparation ofthe Negative Control Group

100µl of DMSO was added to the pre-marked test tube containing 5ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

### 3.9.3.6 Counting of Nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

# Chapter Four

# Result & Discussion

# 4.1 Antioxidant Test Results

Antioxidant tests are classified by various methods. Samples were subjected to various standard methods to determine various scavenging capacity and amount that is equivalent to the standard like ascorbic acids. Antioxidant property of the aqueous fraction of *Opuntia elatior* extract was determined by following methods-

- ❖ Determination of total phenolic content
- ❖ Determination of total flavonoids content

## 4.1.1 Result of Total Phenolic content

The dichloromethane extract of leaves and the aqueous fractions of the ethanol extract of *Opuntia elatior* were subjected to determine total phenolic content. Ascorbic acid was used as reference standard.

### 4.1.1.1 Preparation of Standard Curve

**Table 4.1: Total phenol content of Ascorbic acid**

| Concentration (μg/ml) | Absorbance (at 756 nm) | Regression line | $R^2$value |
|---|---|---|---|
| 80 | 2.406 | | |
| 90 | 2.473 | | |
| 100 | 2.767 | y = 0.193x+ 2.177 | 0.937 |
| 110 | 3.057 | | |
| 120 | 3.080 | | |

A linear relationship was observed when the absorbances were plotted against concentrations, asshown in Figure 4.1. This linear curve was considered as a standard curve.

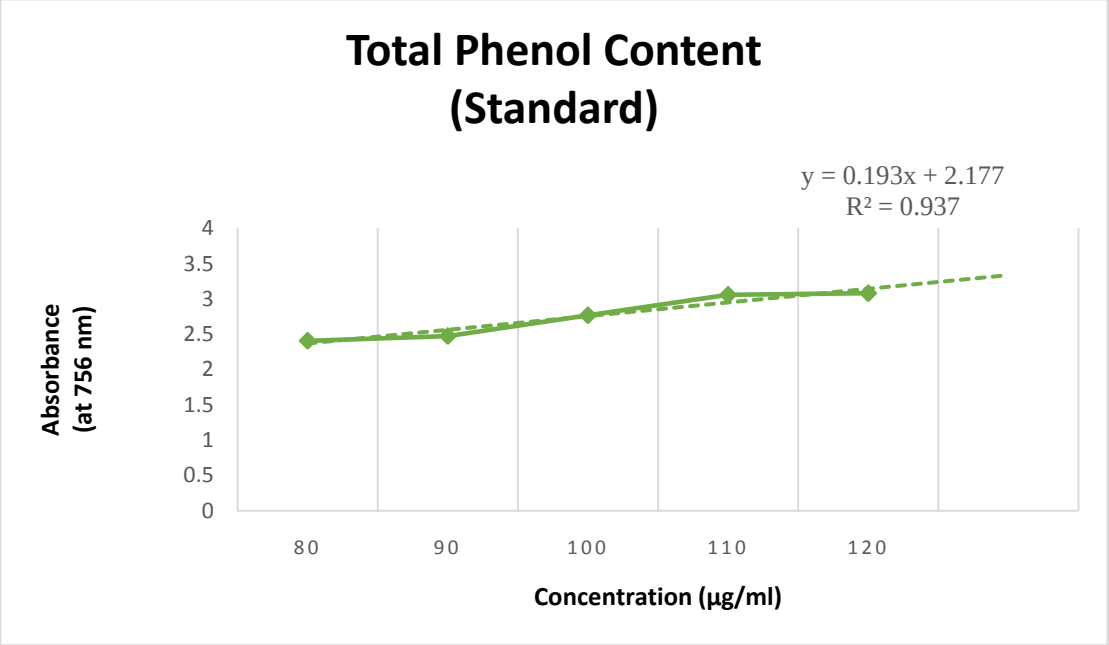



**Figure 4.1: Graphical representation of assay of phenolic content of ascorbic acid**

### 4.1.1.2 Total Phenol content present in Extract

Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of ascorbic acid equivalents (AAE), the total phenolic content present in the extract is calculated and given in the table below.

**Table 4.2: Total phenolic content of Dichloromethane fraction of *Opuntia elatior***

| Sample | Concentration (mg/ml) | Absorbance | Total phenolic content (mg of AAE/g of dried extract) |
|---|---|---|---|
| Dichloromethane fraction of *Opuntia elatior* | 2 | 1.45 | 16.47 |

### 4.1.1.3Discussion

The Total Phenolic content of *Opuntia elatior* was found. The plant extract contains very poor phenolic content. The total phenolic content of Dichloromethane fraction is 16.47 mg AAE/g. Further research is needed on the determination of the correlation between the antioxidant capacity and the chemical components of the plant.

## 4.1.2Result of Total Flavonoid Content

The dicholoromethane fractions of *Opuntia elatior* were subjected to determine total flavonoid content. Ascorbic acid was used as reference standard.

### 4.1.2.1Preparation of Standard Curve

**Table 4.3: Total Flavonoid content of Ascorbic acid**

| Concentration (µg/ml) | Absorbance (at 510 nm) | Regression line | $R^2$value |
|---|---|---|---|
| 50 | 0.05 | | |
| 100 | 0.13 | | |
| 150 | 0.19 | y = 0.0017x-0.042 | 0.991 |
| 200 | 0.29 | | |
| 250 | 0.39 | | |

After absorbance were taken of different solution of ascorbic acid of concentrations ranging from 50µg/µl to 250µg/µl, a linear relationship was observed when the absorbance were plotted against concentrations, as shown in Figure 4.2 This linear curve was considered as a standard curve.

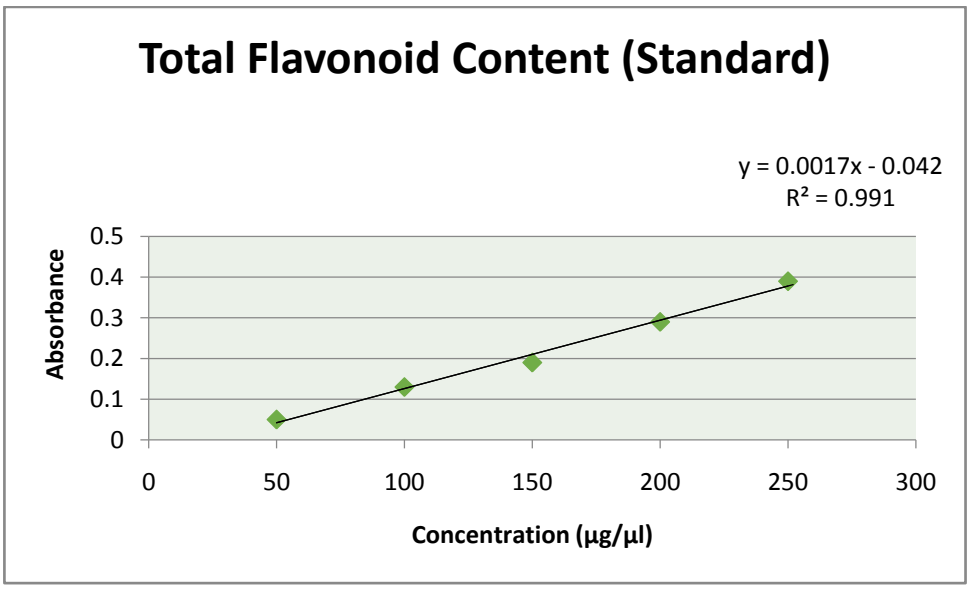



**Figure 4.2: Graphical representation of assay of flavonoid content of ascorbic acid**

#### 4.1.2.2Total Flavonoid Content Present in Extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.4.

**Table 4.4: Total Flavonoid content of dichloromethane fraction of *Opuntia elatior* extract**

| Sample | Concentration (mg/ml) | Absorbance | Total Flavonoid content (mg of AAE/g of dried extract) |
|---|---|---|---|
| Dichloromethane fraction of *Opuntia elatior* | 1 | 0.019 | 35.88 |

#### 4.1.2.3Discussion

To determine the total flavonoid content of the test samples the standard curve was used. For 1mg/ml concentration of dichloromethane fraction of *Opuntia elatior* 35.88 mg of AAE/gm of dried extract of flavonoid content was found. So it can be said that, the extract contains poor antioxidative compounds.

## 4.2 Result of Total Reducing Power

The dicholoromethane fractions of *Opuntia elatior* were subjected to determine total reducing power content. Ascorbic acid was used as reference standard.

### 4.2.1 Preparation of Standard Curve

**Table 4.5: Total Reducing power of Ascorbic acid**

| Concentration (μg/ml) | Absorbance (at 756 nm) | Regression line | $R^2$value |
|---|---|---|---|
| 50 | 0.355 | | |
| 100 | 1.603 | | |
| 150 | 2.284 | y= 0.010 x+0.266 | 0.821 |
| 200 | 2.126 | | |
| 250 | 2.657 | | |

A linear relationship was observed when the absorbances were plotted against concentrations, asshown in Figure 4.3. This linear curve was considered as a standard curve.

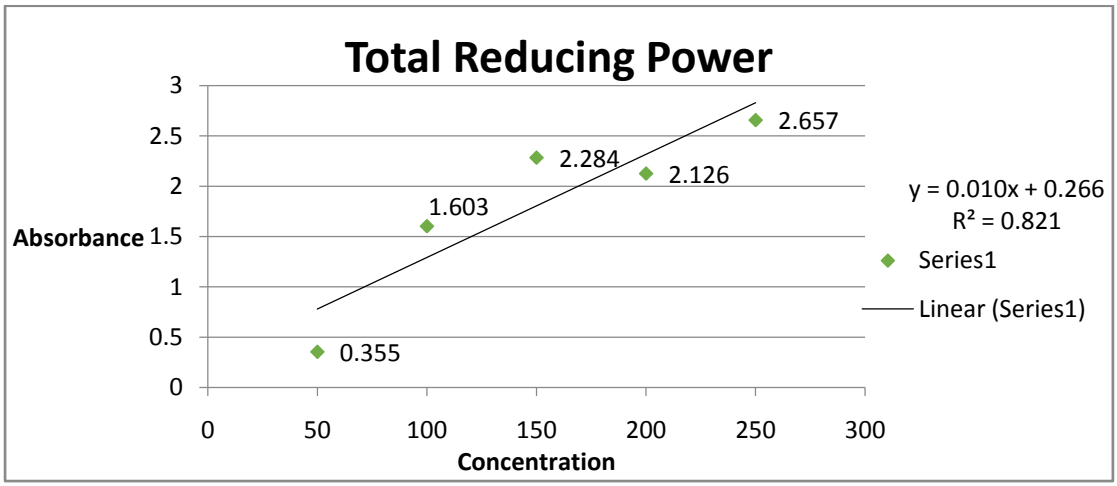



**Figure 4.3: Graphical representation of assay of total reducing power of Ascorbic acid**

### 4.2.1.1 Total Reducing power assay in Extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.8.

**Table 4.6: Total reducing power of Dichloromethane fraction of *Opuntia elatior* extract**

| Sample | Concentration (mg/ml) | Absorbance | Total Reducing power content (mg of AAE/g of dried extract) |
|---|---|---|---|
| Dichloromethane fraction of *Opuntia elatior* | 1 | 0.947 | 68.1 |

### 4.2.1.2 Discussion

To determine the total Total reducing power content of the test samples the standard curve was used. For 1mg/ml concentration of dichloromethane fraction of *Opuntiaelatior* 1.33 mg of AAE/gm of dried extract of total reducing power content

was found. So it can be said that, the extract contains poortotal reducing power content.

## 4.3Result of Brine Shrimp Lethality Bio-Assay

The dichloromethane fraction of the *Opuntia elatior* extract were subjected to brine shrimp lethality bioassay following the procedure (Meyer *et.al,*, 1982). After 24hrs, the test tubes were inspected using a magnifying glass and the number of survivors counted. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a median Lethal Concentration ($LC_{50}$) value. This represents the concentration of the standard or ethyl acetate extract that produces death in half of the test subjects after a certain period. The percentage mortality at each concentration was determined using the following formula:

$$\%\ \text{Mortality} = \frac{(\text{Number of dead nauplii}) \times 100}{\text{Total number of nauplii}}$$

The $LC_{50}$of the test samples was obtained by a plot of percentage of the shrimps died (% Mortality) against the logarithm of the sample concentration (Log C) and the best-fit line was obtained from the curve data by means of regression analysis.

### 4.3.1 Preparation of Curve for Standard

Here, Tamoxifen was used as reference standard

**Table 4.7: Results of the bioassay of Tamoxifen (standard)**

| Test tube no. | Concentration (C) (µg/ml) | Log C | Number of Nauplii alive | Number of Nauplii dead | % Mortality | $LC_{50}$ (µg/ml) |
|---|---|---|---|---|---|---|
| 1 | 400 | 2.602 | 0 | 10 | 100 | |
| 2 | 200 | 2.301 | 1 | 9 | 90 | |
| 3 | 100 | 2.000 | 2 | 8 | 80 | |
| 4 | 50 | 1.699 | 3 | 7 | 70 | |
| 5 | 25 | 1.398 | 4 | 5 | 50 | 25.00 |
| 6 | 12.5 | 1.097 | 5 | 5 | 50 | |
| 7 | 6.25 | 0.796 | 6 | 4 | 40 | |
| 8 | 3.125 | 0.495 | 7 | 3 | 30 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | 1.5625 | 0.194 | 8 | 2 | 20 | |
| 10 | 0.78125 | -0.107 | 9 | 1 | 10 | |

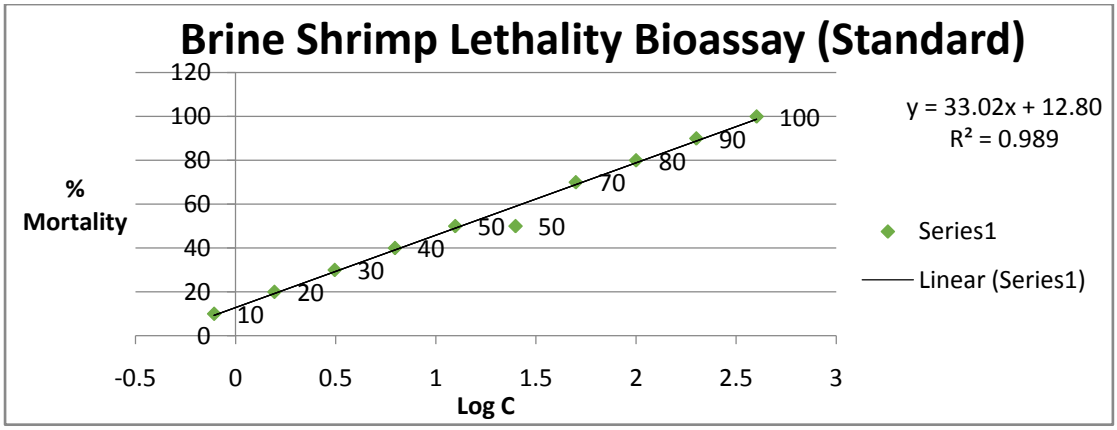



**Figure 4.4: % mortality and predicted regression line of Tamoxifen (standard)**

## 4.3.2 Preparation of Dichloromethane Fraction Curve

**Table 4.8: Results of the bioassay of dichloromethane fraction (extract)**

| Test tube no. | Concentration (C) (µg/ml) | LogC | Number of nauplii alive | Number of Naupliidead | % Mortality | $LC_{50}$ (µg/ml) |
|---|---|---|---|---|---|---|
| 1 | 400 | 2.602 | 0 | 8 | 80 | |
| 2 | 200 | 2.301 | 0 | 7 | 70 | |
| 3 | 100 | 2.000 | 1 | 7 | 70 | |
| 4 | 50 | 1.699 | 3 | 6 | 60 | |
| 5 | 25 | 1.398 | 4 | 6 | 60 | 12.5 |
| 6 | 12.5 | 1.097 | 6 | 5 | 50 | |
| 7 | 6.25 | 0.796 | 6 | 5 | 50 | |
| 8 | 3.125 | 0.495 | 7 | 3 | 30 | |
| 9 | 1.5625 | 0.194 | 8 | 2 | 20 | |
| 10 | .078125 | -0.107 | 8 | 2 | 20 | |

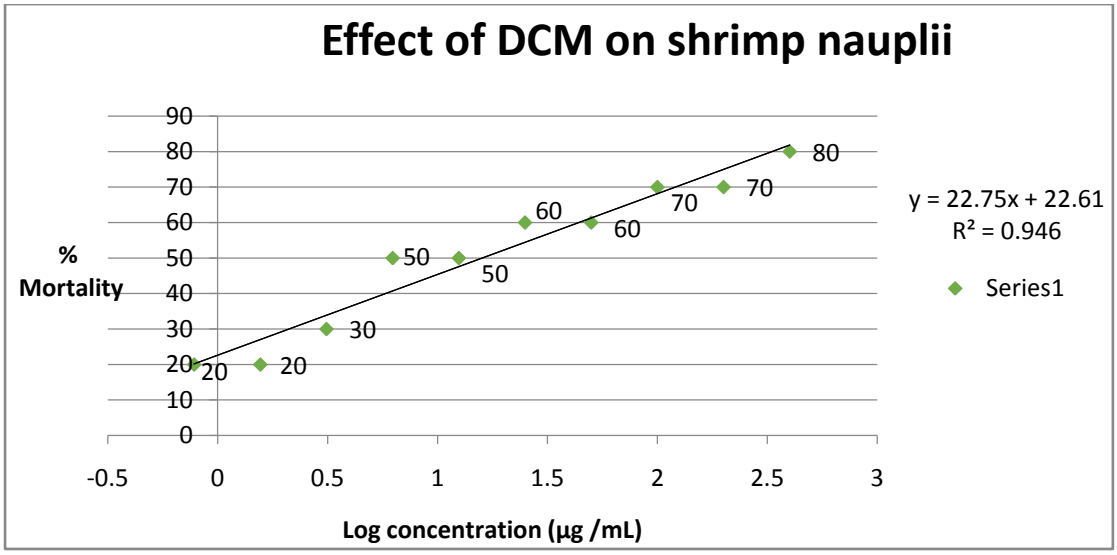



**Figure 4.5: % mortality and predicted regression line of Dichloromethane fraction (extract).**

## 4.3.3 Discussion

In Brine Shrimp Lethality bioassay, varying degree of lethality was observed with exposure to different concentrations of the test samples. The degree of lethality was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and dichloromethane fraction samples. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400µg/ml, whereas the least mortalities at lowest concentration 0.78125µg/ml as shown in Table 4.5 and Table 4.6.

**Table 4.9: Cytotoxic activity of Tamoxifen and dichloromethane fraction of *Opuntia elatior***

| Sample | Linear regression equation | $R^2$ value | $LC_{50}$ (µg/ml, 24hr) |
|---|---|---|---|
| Standard (Tamoxifen) | y = 33.02x + 12.80 | 0.989 | 25.00 |
| Extract (DCM fraction) | y = 22.75x + 22.61 | 0.946 | 12.50 |

In this investigation, standard and dichloromethane fraction exhibited cytotoxic activities with the $LC_{50}$ values 13.38µg/ml and 17.64µg/ml respectively as shown in

Table 4.9. For dichloromethane fraction $R^2$ value is less than the standard which indicates that the extract has less potent activity than standard against brine shrimp nauplii.

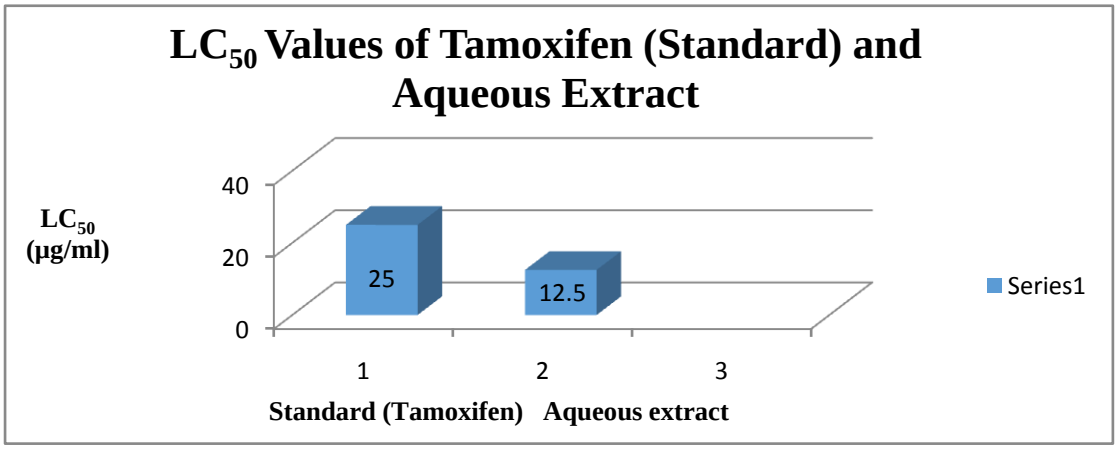



**Figure 4.6: Comparison between $LC_{50}$ values of standard and extract**

From the above figure it can be concluded that for dichloromethane fraction the lethal concentration required to kill 50% of the sample population is higher than the standard. So the extract is less potent than Tamoxifen (Standard) at lower concentration.

# Chapter Five

# Conclusion

## 5.1 Conclusion

As the literature review suggests, the presence of several phytochemical compounds in *Opuntia elatior*makes the plant pharmacologically active. The present study showed that it has very poor antioxidant activity that could not make it a potent drug against free radical mediated diseases.

The dichloromethane extract possesses cytotoxic activity that could be a better treatment in tumor as well as cancer. The study also showed that, the extract showed low to moderate antimicrobial activity that could be a better treatment in antimicrobial infections. However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant, cytotoxic and antimicrobial agent.

The medicinal values of the leaves of this plant may be related to their phytochemical constituent. So, further investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be tested. It will help in the development of new novel and safe drugs for the treatment of various diseases.

# Chapter Six

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