IN VITRO BIOLOGICAL INVESTIGATIONS OF PETROLEUM ETHER FRACTION OF *WEDELIA TRILOBATA* LEAVES

A dissertation submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Submitted By

Umme Jubaiya Azmee

ID: 2011-1-70-019

Department of Pharmacy

East West University

Dedication

This Research paper is dedicated

to My beloved parents,

Who are my biggest Inspirations…

DECLARATION BY THE CANDIDATE

I, Umme Jubaiya Azmee, thereby declare that this dissertation, entitled **"*In Vitro* Biological Investigations of Petroleum Ether of *Wedelia Trilobata* Leaves"** submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic research work carried out by me under the guidance of **Iftekhar Ahmed**, Lecturer, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or university for the award of any degree or diploma.

Umme Jubaiya Azmee

ID: 2011-1-70-019

Department of Pharmacy

East West University

Aftabnagar, Dhaka

CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertion entitled **"*In Vitro* Biological Investigations of Petroleum Ether Fraction of *Wedelia Trilobata* Leaves"** is a bonafide research work done, under my guidance and supervision by Umme Jubaiya Azmee (ID: 2011-1-70-019), in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

Iftekhar Ahmed
Lecturer & Supervisor
Department of Pharmacy
East West University
Aftabnagar, Dhaka

ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled **"*In Vitro* Biological Investigations of Petroleum Ether Fraction Of *Wedelia Trilobata* Leaves"** is a bonafide research work done by Umme Jubaiya Azmee (ID: 2011-1-70-019), in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Dr. Chowdhury Faiz Hossain

Professor & Chairperson

Department of Pharmacy

East West University

Aftabnagar, Dhaka

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ABSTRACT

The study was designed for biological investigations of petroleum ether fraction of methanolic extract of the leaves of *Wedelia trilobata* (Family: Asteraceae). The powdered leaves of *Wedelia trilobata* were extracted with methanol and then partitioned with petroleum ether, chloroform and ethyl acetate consecutively. And the aqueous fraction remaining at the end. The petroleum ether fraction was remaining at the beaker was investigated for the total flavonoid content, brine shrimp lethality test and antimicrobial test. The fraction contained 727 mg AAE/g of total flavaniod content. Screening for cytotoxic properties using brine shrimp lethality bioassay with tamoxifen (LC_{50} value of 13.38µg/ml) as positive control showed that the fraction have considerable cytotoxic potency exhibiting LC_{50} value 15.28 µg/ml. In antimicrobial activity investigation, the petroleum ether fraction showed low antibacterial and antifungal activity against the tested organisms compared to Ciprofloxacin (30µg/disc) that was used as positive control. The petroleum ether fraction showed strong cytotoxic activity, strong antioxidant activity and slight antimicrobial activity. Further investigations are needed for the proper identification and isolation of these bioactive compounds to produce safer drugs for treatment of harmful diseases.

Key words: *Wedelia trilobata*, Brine shrimp lethality bio-assay, flavonoid content, antimicrobial activity.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 MEDICINAL PLANTS

Many familiar medications of the twentieth century were developed from ancient healing traditions that treated health problems with specific plants. Today, science has isolated the medicinal properties of a large number of botanicals, and their healing components have been extracted and analyzed. Many plant components are now synthesized in large laboratories for use in pharmaceutical preparations.

"A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs." This definition of Medicinal Plant has been formulated by WHO (World Health Organization). The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal Plants". Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatile oils and contain minerals and vitamins, possess medicinal properties.

Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science or approved by regulatory agencies such as the United States Food and Drug administration or European Food Safety Authority to have medicinal effects. (Newman et al., 2003).

World Health Organization (WHO) has provided a definition of medicinal plants, that is "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 synthesis of useful drug". World Health Organization (WHO) reported that 80% of the world's population depends on medicinal plants for their primary health care. In the Plant Kingdom, Medicinal plants form the largest single grouping of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are trees. Plants are known to be the source of many chemical

compounds. Medicinal plants were used by people of ancient cultures without knowledge of their active ingredients. The common practice of taking crude extract orally is laden with hazards as the extracts may contain some toxic constituents. There is an ever increasing need to limit toxic clinical drugs. In modern times, the active ingredients and curative actions of medicinal plants were first investigated through the use of European Scientific methods. The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steroids, phenols glycosides and tannins. The information obtained from extracts of medicinal plants makes pharmacological studies possible. The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized.

Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow et al., 2003). Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo et al., 1996).

1.1.1 HISTORY OF PLANTS IN MEDICINE

The earliest known medical document is a 4000-year-old Sumerian clay tablet that recorded plant remedies for various illnesses. The ancient Egyptian Ebers papyrus from 3500 year ago lists hundreds of remedies. The Pun-tsao contains thousands of herbal cures attributed to Shen-nung, China‟s legendary emperor who lived 4500 years ago. In India, herbal medicine dates back several thousand years to the Rig-Veda, the collection of Hindu sacred verses. The Badianus Manuscript is an illustrated document that reports the traditional medical knowledge of the Aztecs. (Levetin et al., 2003).

Greek physician Hippocrates, who believed that disease had natural causes and used various herbal remedies in his treatments. Early Roman writings also influenced the development of western medicine, especially the works of Dioscorides, who compiled information on more than 600 species of plants with medicinal value in De Materia Medica. Many of the herbal remedies used by the Greeks and Romans were effective treatments that have become incorporated into modern medicine (e.g., willow bark tea, the precursor to aspirin). Dioscorides‟ work remained the standard medical reference in most of Europe for the next 1500 years. (Levetin et al., 2003).

The Renaissance saw a revival of herbalism in the identification of medicinally useful plants. This coupled with the invention of the printing press in 1450 ushered in the Age of Herbals. Many of the herbals were richly illustrated; all of them focused on the medicinal uses of plants, but also included much misinformation and superstition. The Doctrine of Signatures, for example, held that the medicinal use of plants could be ascertained by recognizing features of the plant that corresponded to human anatomy. For example, the red juice of bloodwort suggests that it should be used for blood disorders; the lobed appearance of liverworts suggests that it should be used to treat liver complaints; the "humanoid" form of mandrake root suggests that is should be used to promote male virility and ensure conception (Levetin et al., 2003).

1.1.2 RATIONALE AND OBJECTION OF THE PRESENT STUDY

"Herbalism is the oldest form of medicinal science known to mankind and our knowledge of the healing power of herbs has accumulated over thousands of years. People from every culture explored the natural world around them and used to furnish sustenance, shelter and medications…."- Norman Shealy.

Human struggle against the misery of three D's-Disease, Decay and Death is eternal. From the very inception of civilization, the inherent concern for getting as well as staying healthy has been instigating human venture for cure from his surroundings. Illness, physical

discomforts, injuries, wounds & fear of death had forced prehistoric man to use any natural substances that he could lay his hands on- "the green friends" PLANTs.

Plants have been used for thousands of years in many countries of the world that have formed the basis of traditional medicinal systems. The plant is a biosynthetic laboratory and the remedial phyto-elements produced through a cascade of biochemical reactions inside a plant significantly contribute to the traditional and modern medicines. These alluring active ingredients are nothing but the chemical defense against diseases which can hold back numerous pathological conditions and can reset physiological harmony. Approximately, 119 pure chemical substances extracted from higher plants are used as medicines throughout the world (Farnsworth et al., 1985).

The number of higher plant species (angiosperms and gymnosperms) on this planet has been estimated at 250,000 (Ayensu & DeFilipps, 1978), with a lower level at 215,000 (Cronquist, 1981; Cronquist, 1988) and an upper level as high as 500,000 (Tippo & Stern, 1977; Schultes, 1972). Of these, only about 6% plants have been screened for biological activity and a reported 15% has been evaluated phytochemically (Verpoorte, 2000).

Plant evolution that results in chemical diversity of secondary plant metabolites which may be equal or superior to that found in synthetic combinatorial libraries. It was estimated that in 1991 ,in the United States, for every 10,000 pure compounds (those based on synthesis) are biologically evaluated in vitro, of which 20 would be tested in animal models, 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration approval for marketing. The time required for this process was estimated as 10 years at a cost of $231 million (U.S.) (Vagelos, 1991). 1,000 or more substances can be screened by most large pharmaceutical manufacturers and some small biotechnology firms per week using high throughput in vitro assays. Some of these companies screen plant, microbial, and marine organisms in addition to synthetic compounds from their own programs.

A recent survey by the United Nations Commission for Trade & Development (UNCTAD) show that about 33% of drugs, produced in the developed countries, are derived from plants (UNCTAD,1974) and if microbes are added, 60% of medicinal products are of natural origin.

The large quantity of modern drugs comes from less than 15% of the plants, which are known to have been investigated pharmacologically, out of an estimated 2,50,000 to 5,00,000 species of higher plants growing on earth.(Farnsworth 1966).

World Health Organization has estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care (Schultes et al., 1972). Herbal medicine is a common element in Ayurvedic, homeopathic, naturopathic, traditional, 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 (WHO, 2002).

1.1.3 TRADITIONAL MEDICINE

Traditional medicine (also known as indigenous or folk medicine) comprises knowledge systems that developed over generations within various societies before the era of modern medicine.

The World Health Organization (WHO) defines traditional medicine as:

"Traditional medicine is the sum total of the knowledge, skills,indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

In some Asian and African countries, up to 80% of the population relies on traditional medicine for their primary health care needs. When adopted outside of its traditional culture, traditional medicine is often called complementary and alternative medicine.The WHO also notes, though, that "inappropriate use of traditional medicines or practices can have negative or dangerous effects" and that "further research is needed to ascertain the efficacy and safety" of several of the practices and medicinal plants used by traditional medicine systems. Core

disciplines which study traditional medicine include herbalism, ethnomedicine, ethnobotany, and medical anthropology.

Traditional medicine may include formalized aspects of folk medicine, i.e. longstanding remedies passed on and practiced by lay people. Practices known as traditional medicines include Ayurveda, Siddha medicine, Unani, ancient Iranian medicine, Irani, Islamic medicine, traditional Vietnamese medicine, traditional Chinese medicine, traditional Korean medicine, acupuncture, Muti, Ifá, traditional African medicine, and many other forms of healing practices.

In the written record, the study of herbs dates back 5,000 years to the ancient Sumerians, who described well-established medicinal uses for plants. Ancient Egyptian medicine of 1000 BC are known to have used various herbs for medicine. The Old Testament also mentions herb use and cultivation in regards to Kashrut. Many herbs and minerals used in Ayurveda were described by ancient Indian herbalists such as Charaka and Sushruta during the 1st millennium BC. The first Chinese herbal book was the Shennong Bencao Jing, compiled during the Han Dynasty but dating back to a much earlier date, which was later augmented as the Yaoxing Lun (Treatise on the Nature of Medicinal Herbs) during the Tang Dynasty. Early recognised Greek compilers of existing and current herbal knowledge include Pythagoreanism, Hippocrates, Aristotle, Theophrastus, Dioscorides and Galen.Roman writers included Pliny the Elder and Celsus.Pedanius Dioscorides included the writings of the herbalist Krateuas, physician to Mithridates VI King of Pontus from 120 to 63 BC in his De Materia Medica. De Materia Medica was translated into several languages and Turkish, Arabic and Hebrew names were added to it throughout the centuries. Latin manuscripts of De Materia Medica were combined with a Latin herbal by Apuleius Platonicus (Herbarium Apuleii Platonici) and were incorporated into the Anglo-Saxon codex Cotton Vitellius C.III.These early Greek and Roman compilations became the backbone of European medical theory and were translated by the Persian Avicenna (Ibn Sīnā, 980–1037), the Persian Rhazes (Rāzi, 865–925) and the Jewish Maimonides. Translations of Greek medical handbooks and manuscripts into Arabic took place in the eighth and ninth centuries.Arabic indigenous medicine developed from the conflict between the magic-based medicine of the Bedouins

and the Arabic translations of the Hellenic and Ayurvedic medical traditions. Spanish indigenous medicine was influenced by the Arabs from 711 to 1492. Islamic physicians and Muslim botanists such as al-Dinawari and Ibn al-Baitar significantly expanded on the earlier knowledge of materia medica. The most famous Arabic medical treatise was Avicenna's The Canon of Medicine, which was an early pharmacopoeia and introduced the method of clinical trials. The Canon was translated into Latin in the 12th century and remained a medical authority in Europe until the 17th century. The Unani system of traditional medicine is also based on the Canon.Translations of the early Roman-Greek compilations were made into German by Hieronymus Bock whose herbal published in 1546 was called Kreuter Buch. The book was translated into Dutch as Pemptades by Rembert Dodoens (1517–1585), and from Dutch into English by Carolus Clusius, (1526–1609), published by Henry Lyte in 1578 as A Nievve Herball. This became John Gerard's (1545–1612) Herball or General Hiftorie of Plantes.Each new work was a compilation of existing texts with new additions.Women's folk knowledge existed in undocumented parallel with these texts. Forty-four drugs, diluents, flavouring agents and emollients mentioned by Discorides are still listed in the official pharmacopoeias of Europe.The Puritans took Gerard's work to the United States where it influenced American Indigenous medicine.Francisco Hernández, physician to Philip II of Spain spent the years 1571–1577 gathering information in Mexico and then wrote Rerum Medicarum Novae Hispaniae Thesaurus, many versions of which have been published including one by Francisco Ximénez. Both Hernandez and Ximenez fitted Aztec ethnomedicinal information into the European concepts of disease such as "warm", "cold", and "moist", but it is not clear that the Aztec's used these categories.[12] Juan de Esteyneffer's Florilegio medicinal de todas las enfermedas compiled European texts and added 35 Mexican plants. Martín de la Cruz wrote an herbal in Nahuatl which was translated into Latin by Juan Badiano as Libellus de Medicinalibus Indorum Herbis or Codex Barberini, Latin 241 and given to King Carlos V of Spain in 1552.It was apparently written in haste and influenced by the European occupation of the previous 30 years. Fray Bernadino de Sahagún's used ethnographic methods to compile his codices that then became the Historia General de las Cosas de Nueva Espana , published in 1793. Castore Durante published his Herbario Nuovo

in 1585 describing medicinal plants from Europe and the East and West Indies. It was translated into German in 1609 and Italian editions were published for the next century.

1.1.4. SIGNIFICANCES OF MEDICINAL PLANTS TO HUMAN BEING

1. Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin.

2. Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine.

3. Many food crops have medicinal effects, for example garlic.

4. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species.

5. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons.

6. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants. (Chaudhary et al., 2010).

1.1.5 MEDICINAL PLANTS HAVE MANY CHARACTERISTICS WHEN USED AS A TREATMENT, AS FOLLOWS:

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

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

3. 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. (Hassan, 2015)

1.1.6 CAUSES OF DOING PHYTOCHEMISTRY STUDY OF PLANTS:

All plants produce chemical compounds as part of their normal metabolic activities. These chemicals are divided into (1) primary metabolites such as sugars and fats, which are found in all plants; and (2) secondary metabolites—compounds which are found in a smaller range of plants, serving a more specific function. [60] 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 insulin 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.

Plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs:

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 a 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 triterpenesqualene. 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.

A consortium of plant molecular researchers at Washington State University, the Donald Danforth Plant Science Center, the National Center for Genome Resources, and the University of Illinois at Chicago began an NIH-sponsored study of over thirty medicinal plant species late 2009. The initial work, to develop a sequence reference for the transcriptome of each, has led to the development of the Medicinal Plant Transcriptomics Database.

The goals of using plants as sources of therapeutic agents are

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

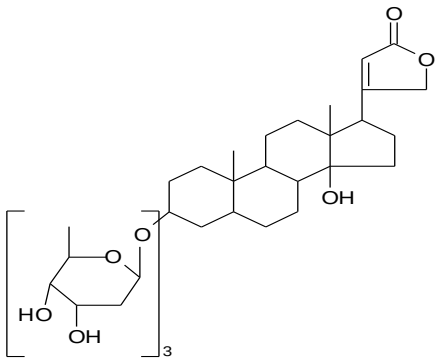

Figure-1.1 A: Digitoxin

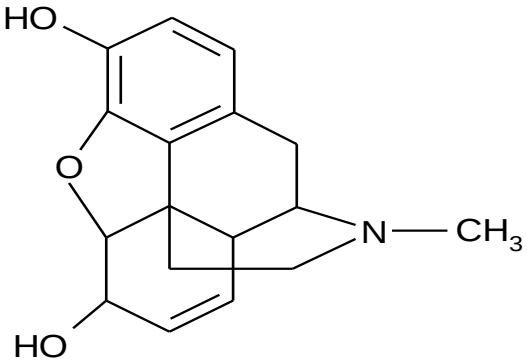

Figure-1.1 B: Morphine

2. To produce bioactive compounds of novel or known structures as lead compounds for semi synthesis 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;

3. To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline; and

4. To use the whole plant or part of it as a herbal remedy, e.g., cranberry, garlic etc. (Daniel et al., 2001)

There are several approaches for lead searching from plants and the isolated bioactive compounds are utilized in three basic ways

1. Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g.: digoxin, digitoxin, morphine.

2. Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g.: vincristine.

3. Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin. (Cox, P.A., 1994)

1.1.7 MEDICINAL PLANT USE SCENARIO IN BANGLADESH

In Bangladesh 5000 species of angiosperms are reported to occur (IUCN, 2003). The number of medicinal plants included in "Materia medica" of traditional medicine in this subcontinent at present stands as about 2,000. Since Bangladesh has an enormous resource of medicinal plants, majority of our population has to rely upon indigenous system of medication. The high cost of imported conventional drugs and inaccessibility to western health care facility, imply that traditional mode of health care is the only form of health care that is affordable and available to the rural people. On the other hand, even when western health facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective and as a result, traditional medicines usually exist side by side with western forms of health care.

Bioactive compounds deposited in medicinal plants can serve as important raw materials for pharmaceutical manufacturing. Therefore, well-judged and scientific investigation of this wealth can significantly contribute to the public health. Again, it was observed that developed countries mostly imports raw materials of valuable medicinal plants from developing countries. Where they are screened, analyzed and used in drug preparations, and returned as high priced medicines to developing countries. Thus, being available commodity of commerce, a country can also earn a good amount of foreign currency by exporting this natural wealth to other countries.

In Bangladesh, about 500 plant species have been identified as medicinal plants because of their therapeutic properties (Ghani, 2003). Approximately 400 herbal factories have been established in this country for producing Ayurvedic and Unani medicines. It has been estimated that Bangladesh has a market of about 100-core taka worth herbal products annually. The total size of the medicinal plant market at wholesale prices was estimated at some US$ 14 million per annum which corresponds to 17000 tons of products .It has been estimated that 12,500 tonnes of dried medicinal plant products are sold in Bangladesh that

have a worth of Tk 255 million to rural economy. At the factory level, 5000 tonnes of medicinal plants are imported annually that cost around 480 million taka (Alam et al., 1996). Although modern medicinal science has been developed to a great extent, many rural people of Bangladesh still depend on plant products and herbal remedies for treating their ailments.

The start of the new millennium has signaled the initiation of a new era of drug discovery. Pharmaceutical development is rapidly evolving due to changes in technology, a deeper understanding of diseases processes and, the highly publicized, decoding of the human genome sequence. Current drug development trends are shifting towards rationally designed drugs, which involve the identification of novel targets and the subsequent design of small molecule inhibitors examples include ricin, a toxin produced by the beans of Ricinus communis, has been found to be effectively couple to tumor targeted monoclonal antibiotics and has proved to be a very potent antitumor drug (Sharma, 1998). the tyrosine kinase inhibitors Glivec, Iressa and Herceptin and glycyrrhizin (from Glycyrrhizin species) etc. Bearing in mind these current trends, there is still a recess for natural products in present drug discovery efforts. The structural diversity in compounds found in nature far surpasses that which can be synthesized at the bench. More-over, natural products are generally small molecules (<1000 Daltons) with drug like properties. Though recombinant proteins and peptides account for increasing sales rates, the superiority of low-molecular mass compounds in human diseases therapy remains undisputed mainly due to more favourable compliance and bioavailability properties. Despite all the advancement in synthetic chemistry & biotechnology, plant kingdom is still one of the chief research areas of recent days.

1.1.8 POPULATION USING TRADITIONAL MEDICINE

In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in

2003-2004. In China, sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007 (Chaudhary et al., 2010).

1.1.9 MODERN MEDICINE FROM MEDICINAL PLANTS

Medicinal plants play a vital role for the development of new drugs. During 1950-1970 approximately 100 plants based new drugs were introduced in the USA drug market including deserpidine, reseinnamine, reserpine, vinblastine and vincristine which are derived from higher plants. From 1971 to 1990 new drugs such as ectoposide, E-guggulsterone, teniposide, nabilone, plaunotol, Z-guggulsterone, lectinan, artemisinin and ginkgolides appeared all over the world. 2% of drugs were introduced from 1991 to 1995 including paciltaxel, toptecan, gomishin, irinotecan etc. Plant based drugs provide outstanding contribution to modern therapeutics; for example: serpentine isolated from the root of Indian plant Rauwolfia serpentina in 1953, was a revolutionary event in the treatment of hypertension and lowering of blood pressure. Vinblastine isolated from the Catharanthus rosesus (Farnsworth and Blowster, 1967) is used for the treatment of Hodgkins, choriocarcinoma, non-non-hodgkins lymphomas, leukemia in children, testicular and neck cancer. (Alam et al., 1994). Vincristine is recommended for acute lymphocytic leukemia in childhood advanced stages of hodgkins, lymophosarcoma, small cell lung, cervical and breastcancer (Farnsworth, 1977). Phophyllotoxin is a constituent of Phodophyllum emodi currently used against testicular, small cell lung cancer and lymphomas. Indian indigenous tree of Nothapodytes nimmoniana (Mappia foetida) are mostly used in Japan for the treatment of cervical cancer. Plant derived drugs are used to cure mental illness, skin diseases, tuberculosis, diabetes, jaundice, hypertension and cancer. Medicinal plants play an important role in the development of potent therapeutic agents. Plant derived drugs came into use in the modern medicine through the uses of plant material as indigenous cure in folklore or traditional systems of medicine. More than 64 plants have been found to possess significant antibacterial properties; and more than 24 plants have been found to possess antidiabetic properties, antimicrobial studies of plants, plant for antiodotes activity- Daboia russellii and Naja kaouthia venom neutralization by lupeol acetate isolated from the root

extract of Indian sarsaparilla Hemidesmus indicus R.Br (Chatterjee et al., 2006). Which effectively neutralized Daboia russellii venom induced pathophysiological changes (Alam et al., 1994).

Moreover, unlike the rural communities who use fresh/dried plant material or their crude extracts, the industry lays importance on isolation of active principles or standardized fractions since crude extracts are not patentable. However, it is often seen that a crude extract is more active compared to the isolated active fractions e.g. Cirriformia tentaculata loses its activity upon fractionation with hexane (Kicklighter et al., 2003). It is generally believed that standardization of the plant material is not required when used by the rural communities for their primary health care. But, regardless of whether the medicinal plant is to be used by local communities or by industry, a systematic approach is required for a plant identified from traditional medicine, as is done in modern medicine. It is necessary to focus on all aspects of medicinal plant research: from cultivation, ethno-pharmacology, utilization, isolation and identification of active constituents to efficacy evaluation, pharmacology, safety, standardization, formulation and clinical evaluation. Artuso (1997) has outlined the entire process which includes formulating an appropriate strategy and he estimates that the entire process would take more than 10–20 years. This approach is very demanding since there is an estimated 250,000 species of higher plants present on this earth (Ayensu, 1978). However, this scenario would change with use of the high throughput advanced screening methods that are available today. Another approach than can prove to be a highly productive and cost effective in development of safe, effective and acceptable therapeutic agents is reverse pharmacology which is based on the documented therapeutic effects of plants in ancient texts. This paper will discuss the approaches that need to be considered while studying medicinal plants. It focuses on aspects of the medicinal plant research: from collection of plant material, to efficacy and safety evaluation through preclinical studies and phytochemical standardization. (Ayensu, 1978).

1.1.10 NATURAL PRODUCTS AS A RESOURCE FOR ESTABLISHED NEW DRUGS

Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products. Quinine, Theophyline, Penicillin G, Morphine, Digitoxine, Vincristine, Doxorubisin, Cyclosporin and vitamin A, all share two important characteristics: they are the cornerstones of modern pharmaceutical care and they are all natural products. The use of natural substance, particularly plants, to control diseases is centuries old practice that has lead to the discovery of more than half of all "Modern" pharmaceutical (Rahman et al ; 2007).

1.1.10.1 CARDIOVASCULAR DRUGS

The cardiac glycosides, which include Digoxin, Digitoxin and Deslanoside, exert a powerful and selective positive inotropic action on the cardiac muscles.

1.1.10.2 ANTIMALARIAL AGENTS

Quinine is considered to be the drug of choice for severe chloroquine-resistant malaria due to P. falciparum. In the United States, the related alkaloid quinidine is recommended for this purpose because of its wide availability, there in its use as an antiarrhythmic agent.

1.1.10.3 ANTIBIOTICS

Today, new important anti-infectives are being discovered from microbial, plant and animal sources. For example, the antimalarial agent, Artemisinin, was isolated from the Chinese medicinal plant Artemesia annua.

1.1.10.4 CNS – DRUGS

One of the most cited examples of important natural product-derived drugs is the neuromuscular blocker, d-tubocurarine, which recently helped recognition of the possibility that a number of vastly different CNS and peripheral nervous system diseases may be

therapeutically controlled by selective nicotinic acetylcholine receptor (nAChR) agonists and has opened a new area of drugs design based on the Nicotine molecule.

1.1.10.5 CHOLESTEROL LOWERING AGENTS (HYPOLIPIDIMICS)

These drug acts by inhibition of 3-hydroxy–3– methylgutaryl coenzyme- A reeducate (HMG-CoA reductase), an enzyme critical in the biosynthesis of cholesterol. The first of the HMG-CoA reductase inhibitors were isolated from Penicillium sp.

1.1.10.6 ANTIHYPERGLYCEMICS

The major antihyperglycemic drugs are natural products derived from popular plant such as Momordica charantia (Karela); Tinospora cordifolia (Guduchi); Gymnema sylevestre (Gurmar); Azadirachta indica (Neem); Ficus benghalensis (Indian banyan tree); Aegle marmelos (bel or bilva); Aloe vera; Allium sativum – Garlic etc.

1.1.10.7 IMMUNOMODULATORS

The immunomodulator Cyclosporin was originally isolated from a soil fungus, Trichoderma polysporum. This compound was a major breakthrough for organ transplantation.

1.1.10.8 HEPATOPROTECTIVES

Numbers of plants are used for the effective treatment of liver disorder. Some of these plants are – Tinospora cordifolea (Guduchi or Gulvel), Astracaulta longifolea, Cleoma viscose, Bauhania varieaata, Alstoma scholaris etc.

1.2 PLANT PROFILE

Wedelia Trilobata is one kind of species of the Asteraceae family commonly known as Bhringraj (local name in Bangladesh), Singapore Daisy, Rabbits Paw, Trailing Daisy, Bay Biscayne creeping-oxeye, Creeping oxeye, Trailing daisy. It would be hard to find another groundcover better suited to hot, dry conditions than wedelia (Fig. 1). Attractive, glossy, dark green, lobed leaves, rapidly spreading growth habit, and a continuous display of small, bright yellow, daisy-like blooms create a much-favored landscape plant.

Figure-1.2: Leaves of *Wedelia Trilobata*

1.2.1 PREFERRED SCIENTIFIC NAME

Wedelia trilobata (L.) A.S. Hitchc.

1.2.2 TAXONOMICAL CLASSIFICATION

Division: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: Wedelia
Species: trilobata

Scientific Name: Wedelia trilobata

Common Name: wedelia

Plant type: perennial; herbaceous. (Taxonomy of Vascular Plants in Botanical Garden, 2015)

1.2.3 OTHER SCIENTIFIC NAMES

- Acmella brasiliensis Spreng
- Acmella spilanthoides Cass.
- Buphthalmum procumbens Desf. Ex Steud
- Buphthalmum repens Lam.
- Complaya trilobata (L.) Strother
- Sereneum trilobata (L.) Kuntze
- Silphium trilobatum L.
- Sphagneticola ulei O. Hoffm.
- Stemmodontia carnosa (Rich.) O.F. Cook & G.N. Collins
- Thelechitonia trilobata (L.) H. Rob & Cautrec.
- Verbesina carnosa (Rich.) M. Gómez
- Verbesina tridentata Sprengel
- Wedelia brasiliensis (Spreng.) S.F. Blake
- Wedelia carnosa Rich. ex Pers.
- Wedelina paludosa DC.
- Sphagneticola trilobata (L.) Pruski

1.2.4 INTERNATIONAL COMMON NAMES

- English: Bay Biscayne creeping-oxeye; creeping daisy; creeping ox-eye; creeping wedelia; gold-cup; rabbit's paw; Singapore daisy; trailing daisy; water zinnia; wild marigold; yellow dots.
- Spanish: clavelín de playa; clavelito de muerto; clavellin (Panama); manzanilla; manzanilla de playa; margarita amarilla; margarita de pasto; romerillo; romerillo; saladillo macho; yerba buena cimarrona

- French: patte canard
- Chinese: nan mei peng qi ju (Cabi.org, 2015)

1.2.5 LOCAL COMMON NAMES

- Bahamas: trailing wedelia
- Brazil: insulin; vedélia
- Cuba: romero de playa
- Germany: Wedelie, Goldstern-
- Jamaica: creeping oxeye
- Lesser Antilles: bobena; carpet daisy; graveyard daisy; graveyard grass; herb soleil; lad love; pa a kanna; pasture sage; piss weed; venvenn kawayib; verven carib; vin vin caribe; yellow marigold; zeb a fan
- Marshall Islands: ut mokadkad; ut telia
- Micronesia, Federated states of: atiat; dihpw ongohng; dihpwoangoahng suwed (Pohnpei); rostangrang; tuhke ongohng
- Palau: ngesil ra ngebard
- Saint Lucia: venvenn kawayib
- South Africa: Singapoer-madeliefie
- Tonga: ate
- USA: Bay Biscayne creeping oxeye; yellow dots (Cabi.org, 2015).

1.2.6 DESCRIPTION

Height: .5 to 1 feet

Spread: depends upon supporting structure

Plant habit: upright

Plant density: dense

Growth rate: fast

Texture: medium

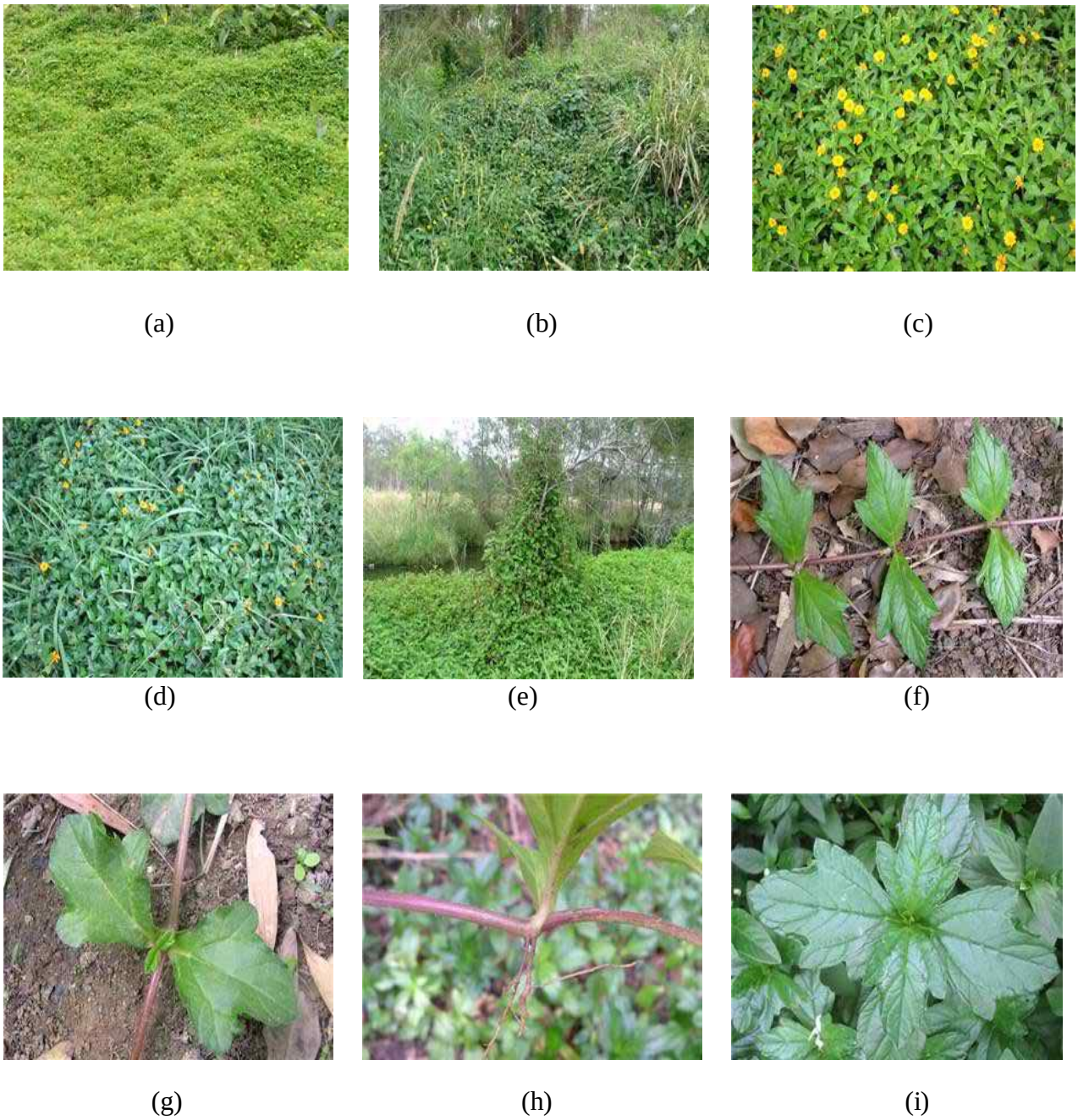

Figure-1.3 : (a) dense infestation, (b) infestation, (c) habit, (d)scrambling habit, (e) climbing habit, (f) creeping stem with paired leaves, (g) close-up stem and lobed leaves, (h) close-up of stem showing rooting at the joints, (i) lobed leaves of *Wedelia Trilobata*.

(j)

(k)

(l)

(m)

(n)

(o)

Figure-1.4 : (j) bright yellow flower-head, (k) close-up of sterile, unfilled, 'seeds ', (l) seedling, (m) close-up of flower-head with several 'petals', (n) flower-head with immature fruit, (o) mature 'seeds' of *Wedelia Trilobata* .

1.2.6.1 FOLIAGE

Leaf arrangement: opposite/sub-opposite

Leaf type: simple

Leaf margin: serrate; lobed

Leaf shape: obovate

Leaf venation: bowed; brachidodrome

Leaf type and persistence: evergreen

Leaf blade length: 2 to 4 inches

Leaf color: green

Fall color: no fall color change

Fall characteristic: not showy (Gilman, 2015)

1.2.6.2 FLOWER

Flower color: yellow

Flower characteristic: year-round flowering (Gilman, 2015)

1.2.6.3 FRUIT

Fruit shape: elongated

Fruit length: less than .5 inch

Fruit cover: dry or hard

Fruit color: brown

Fruit characteristic: inconspicuous and not showy (Gilman, 2015)

1.2.6.4 TRUNK AND BRANCHES

Trunk/bark/branches: not applicable

Current year stem/twig color: green

Current year stem/twig thickness: medium (Gilman, 2015)

1.2.6.5 CULTURE

Grows best in moist, well-drained, fertile soil, but does fine in poor soil as well. Quite adaptable in tropical climates

Light requirement: plant grows in part shade/part sun

Soil tolerances: extended flooding; alkaline; clay; sand; acidic; loam

Drought tolerance: moderate

Soil salt tolerances: good

Plant spacing: 18 to 24 inches

Moisture: Moist to average.

Hardiness: USDA Zones 9 is northernmost boundary.

Propagation: Division (Gilman, 2015)

1.2.6.6 OTHER

Roots: not applicable

Winter interest: no special winter interest

Outstanding plant: not particularly outstanding

Invasive potential: potentially invasive

Pest resistance: long-term health usually not affected by pests. (Gilman, 2015)

1.2.7 CONSTITUENTS

• Study isolated main bioactive sesquiterpene lactones, trilobolid-6-O-isobutyrates A and B.
• From the flower, the structure of trilobolide-6-O-isobytyrate shows a eudesmanolide sesquiterpene skeleton.

• Contains the diterpene (kaurenoic acid), eudesmanolide lactones and luteolin (in leaves and stems. (Stuartxchange.com, 2015)

1.2.8 PROPERTIES

• Study isolated

1.2.9 LOCATION

West Indies, Hawaii, south Florida, Central America, West Africa, especially at low elevations.

1.2.10 GEOGRAPHICAL DISTRIBUTION

Native to Mexico, Central America (i.e. Belize, Costa Rica, Guatemala, Honduras, Nicaragua and Panama), the and throughout the Caribbean, where it is noted as a weed in Trinidad, Puerto SGuiana, Guyana, Surinam, Venezuela, Brazil, Bolivia, Colombia, Ecuador and Peru). Naturalized in South Africa, Florida, Louisiana, Hawaii, Puerto Rico, and the Virgin Islands. Escaped in many tropical regions of the world, including Australia (South-eastern Queensland and north-eastern New South Wales), the Pacific Islands (i.e. American Samoa, the Cook Islands, Fiji, French Polynesia, Guam, Kiribati, the Marshall Islands, Nauru, Niue, New Caledonia, Palau, Western Samoa, Tonga and Hawaii), Malaysia, Indonesia, Thailand, India, Papua New Guinea.

1.2.11 DISTINGUISHING FEATURES

- A mat-forming groundcover, or occasionally a low-climbing plant, with hairy stems.
- Its paired leaves are often three-lobed and have toothed margins.
- These leaves are glossy in appearance and mostly hairless.
- Its bright yellow daisy-like 'flowers' (20-30 mm across) are borne singly on stalks 3-15 cm long.

Each flower has 8-13 yellowish 'petals' (6-15 mm long) with finely toothed tips. (Weeds of Australia, 2011)

1.2.12 HABITAT

A weed of urban bushland, closeditatforests, forest margins, open woodlands, waterways, lake margins, wetlands, roadsides, disturbed sites, waste areas, vacant lots, and coastal sand dunes in tropical and sub-tropical regions. It may also encroach into lawns, footpaths and parks from nearby gardens. (Google.com.bd, 2015)

1.2.13 REPRODUCTION AND DISPERSAL

Stems from new plants where they touch the ground and pieces readily take root. Plants usually develop few fertile seeds. Commonly spread by dumping of garden waste .This plant usually reproduces vegetatively by stem fragments, while viable seeds are rarely produced. Stem fragments readily take root where they come into contact with the ground and can develop into new plants. Such segments are commonly spread in dumped garden waste, by mowing and slashing, and during floods. (Keyserver.lucidcentral.org, 2015)

1.2.14 LOCAL USES

1. After childbirth women drink a tea of W. trilobata, venvennkawayib, to contract the uterus and stop hemorrhage.

2. Chouvalyéwonzé (Portulacapilosa) is sometimes added to it in making the tea.

3. As a tisane, twef (Aristolochiaconstricta), go ponpon (Leonotisnepetaefolia) and hog plum bark (Spondiaspurpurea) are added to it . Also as a tisane, this plant is used for cooling, sometimes with venvennlachewat (Stachtarpheta spp.), and for inflammation when blood is passed.

4. When a nerve is pinched and unable to straighten arm, a good bit of W. trilobata is pounded, mixed with a spoon of castor oil and applied. (Graveson, 2012)

1.2.15 MEDICINAL USES

1. *Wedelia trilobata* is a medicinal plant is used to treat hepatitis, infections

2. Used to clear the placenta after birth

3. Used for menstrual pain and

4. Unspecified female complaints. **(**Flowersofindia.net, 2015)

1.2.16 TRADITIONAL USES

1. In Trinidad and Tobago, used for reproductive problems, amenorrhea, dysmenorrhea.
2. In South America, used to treat symptoms of colds and flu; for fevers and inflammations.

3. Studies:

i. Anti inflammatory: An investigation of four herbal drugs, including Sphagneticola trilobata, on the anti-inflammatory activity of Central American plants used in traditional medicine, showed all the extracts reduced croton oil-induced ear dermatitis. Results suggest the lipophilic extracts to be potential sources of anti inflammatory activity.

ii. Antimicrobial: A study of the n-hexane extract of Wedelia trilobata showed antibacterial activity against Bacilus subtilis, Mycobacterium smegmatis, Pseudomonas aeruginosa, Salmonella group C, S paratyphi and Shigella sonnei.

iii. Analgesic: Study in mice on the analgesic activity of the ethanol extracts of W. trilobata, W bilofra and E alba showed dose-dependent blocking of writhing response.

1.2.17 TOXICITY

1.2.17.1 HOSTS / SPECIES AFFECTED

S. trilobata is a threatening invasive species in agricultural and forestry land, urban areas and roadsides. It forms a dense ground cover, crowding or preventing regeneration of other species (HEAR, 2008). It has been reported as a weed of taro (Colocasiaesculenta) in Fiji (Invasive Species Compendium, 2015).

1.2.17.2 ECONOMIC IMPACT

There are costs parasitic weed (Invasive Species Compendium, 2015).

1.2.17.3 IMPACT ON HABITATS

W. trilobata is an aggressive weed which forms a dense ground cover. This plant will spread rapidly excluding other ground cover vegetation. It has a vine-like habit and grows up into shrubs and trees thus retarding their growth. Batian off and Franks (1997) reported that W. trilobata invaded the sandy beach fronts along the east coast of Queensland in Australia. Competition with other vegetation may be enhanced by allopathic effects and the same allopathic substances may also damage brine shrimps. (Invasive Species Compendium, 2015)

1.2.17.4 IMPACT ON BIODIVERSITY

W. trilobata has been ranked as a threatening invasive species that has invaded and affected the native biota of many areas in Hawaii, many other Pacific islands, the West Indies and southern Florida, USA, Central America, West Africa and Asia. In addition, it will also invade rainforest margins. The regeneration of native species in invaded areas can be inhibited due to rapid growth of S. trilobata and this can lead to reduction of biodiversity in invaded areas. (Invasive Species Compendium, 2015)

1.2.17.5 SOCIAL IMPACT

W. trilobata has an ornamental value. Subramanium (1996) reported that it could be maintained on fences, arches, compounds, building walls, trees, pillars and roofs to provide aesthetic appeal.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANTIMICROBIAL ACTIVITY OF SPHAGNETICOLA TRILOBATA (L.) PRUSKI, AGAINST SOME HUMAN PATHOGENIC BACTERIA AND FUNGI, INDIA

The side effect and quick microbial adaptation to resist synthetic antibiotic has compelled researcher to find out compound from natural sources are free from side effect and resistancy. In this connection the present study has been carried out for assessment of antimicrobial activities of methanolic and aqueous extracts of leaf, stem, root and flower of Sphagneticola trilobata (L.) Pruski, against bacteria namely Pseudomonas aeruginosa (MTCC -7296), Staphylococcus aureus, (MTCC- 7443), Salmonella typhi (MTCC- 733), Mycobacterium tuberculosis (MTCC-300) and fungal organisms namely Microsporum canis (MTCC –2820), Epidermophyton floccossum (MTCC-613), Trichophyton rubrum (MTCC-296) and Aspergillus candidus (MTCC-1989). The zone of inhibition (ZOI) for the methanolic extract of leaf of S. trilobata was found 8.99±0.46mm, 16.92 ±0.58mm and 12.93±0.28mm against S. aureus, S. typhi and P. aeruginosa respectively. The ZOI for The methanolic extract of flower was found 23.79±0.27mm, 19.66 ±0.94 mm and 23.60±0.92mm against S. aureus, S. typhi and P. aeruginosa respectively. Besides, the ZOI for methanolic extract of both root and stem was found 09.19±0.34 and 08.66±0.43mm against S. aureus only. The highest zone of inhibition (23.79mm) was found in the methanolic extract of flower against S. aureus. The ZOI for methanolic and aqueous extract of leaf and methanolic extract of root was found 17.73± 0.46mm, 15.66±0.63mm and 16.19±0.33mm respectively against Epidermophyton floccossum. The ZOI for methanolic extract of leaf was found17.33±0.34mm against Trichophyton rubrum while the ZOI for aqueous extract of leaf was found 13.73±0.49 mm against Microsporum canis. The highest zone of inhibition (17.73mm) was found in the methanolic extract of leaf against Epidermophyton floccossum. Above findings may be exploited for application against respective pathogenic microorganism and modern drug formulation. (TOPPO et al., 2012)

2.2 WOUND-HEALING POTENTIAL OF GRADIFLORENIA ACID FROM WEDELIA TRILOBATA (L) LEAVES ,THAILAND

The ethyl acetate fraction from ethanolic extract of Wedelia trilobata (L.) leaves displayed wound healing properties. The ethyl acetate fraction was further subjected to bioassay-guided fractionation which afforded isolation of grandiflorenic acid which requiresfurther investigation toproveitswound healing potential. The grandiflorenicacidfromleavesofWedelia trilobata was assessed for its possible activity on BJ human fibroblast and HaCaT keratinocytes proliferation, and effect on in vitro scratch assay, collagen content, TGF- β2 levels, and nitric oxide, TNF -□ □and IL- 1β -determination using Raw 264.7 cells. Grandiflorenicacid (2.5µg/mL) produced percentage viability of BJhuman fibroblast, and HaCaTkeratinocytes116, and 106% respectively. Grandiflorenic acid (2.5 µg/mL) induced a 100% migration rate in the in vitro scratch assay and the collagen contentwasincreased to171.2µg/mLcomparedtothecontrol (61.1µg/mL) with BJhuman fibroblast. Grandiflorenic acid (2.5µg/mL) neither produced any significant increase in TGF- β2 levels of HaCaT keratinocytes cells nor induced migration of HaCaT cells in the in vitro scratch assay. The present study provides scientific evidence that grandiflorenic acid has potential wound healing activity due to combination of fibroblast stimulation and inhibiting prolonging inflammatory phaseofwound healing evidentbyreduced levels of inflammatory cytokines from macrophage Raw 264.7cells. (Balekar et al., 2013)

2.3 IN VITRO PROPAGATION OF WEDELIA TRILOBATA (L) USING PHORMIDIUM SUBINCRUSTATUM EXTRACTS: A NOBEL APPROACH, SOUTH INDIA

Most micropropagation involves the proliferation of callus, shoot and root tissue using MS medium supplemented with commercial growth hormones. While such media have arguably been too successful in terms of multiplication yields, it has become increasingly important to improve productivity and reduce the time taken to multiply commercially important material.

The present study reveals the potential effect of extracellular products (EP) and biomass water extracts (BWE) of Phormidium subincrustatum on regeneration of Wedelia trilobata. The growth parameters of plantlets (11cm shoot length and 12 leaves per shoot) were proliferated from the nodal explants when cultured on basal MS media supplemented with 10% cyanobacterial extracts as in the positive control. Initiation of callus growth was observed on the cut surfaces of the leaf sections within 10-15 days of culture with MS medium with BWE, compared to the control. Tremendous increase in shoot length and callus volume over a short period indicates that MS media with added cyanobacterial extracellular product can be used as a better alternative to other chemically synthesized growth regulators in MS media for callus and shoot induction. (Keerthiga et al., 2012)

2.4 LIGHT LIMITATION AND LITTER OF AN INVASIVE CLONAL PLANT, WEDELIA TRILOBATA INHIBIT IT'S SEEDING RECRUITMENT, CHINA

Wedelia trilobata blooms profusely and produces copious viable seeds in the field. However, seedlings of W. trilobata were not detected under mother ramets and few emerged seedlings were found in the bare ground near to populations. In laboratory experiments, low light significantly inhibited seed germination. Leaf extracts also decreased seed germination and inhibited seedling growth, and significant interactions were found between low light and leaf extracts on seed germination. However, seeds were found to germinate in an invaded field after removal of the W. trilobata plant canopy. The results indicate that lack of light and the presence of its own litter might be two major factors responsible for the low numbers of W. trilobata seedlings found in the field. New populations will establish from seeds once the limitating factors are eliminated, and seeds can be the agents of long-distance dispersal; therefore, prevention of seed production remains an important component in controlling the spread of this invasive clonal plant. (Qi et al., 2014)

2.5 ANTIMICROBIAL, ANTIOXIDANT AND IN VITRO ANTI-INFLAMMATORY ACTIVITY AND PHYTOCHEMICAL SCREENING OF WATER EXTRACT OF WEDELIA TRILOBATA (L) HITCHC, INDIA

The aim of the study was to evaluate antimicrobial, antioxidant and anti-inflammatory activity of dry and fresh parts of leaf, stem and flower from the water extract of Wedelia trilobata. The antimicrobial activity of water extracts of fresh and dry parts against 9 different strains of bacteria and 11 different species of fungi were determined using standard method (paper disc method). The fresh parts water extracts showed that, leaf and flower extracts were most potent inhibiting all isolates of with different zones of inhibition but did not inhibited the growth of fungi tested. All the extracts have only moderately inhibited the all fungi. The minimum microbial concentration (MMC) of the active extract was observed from fresh part extracts of leaf, flower and stem ranged from 0.4 to 5.0 mg/ml for the sensitive bacteria. In case of fungi, the minimum inhibitory concentration (MIC) of the active extracts ranged from 2.4 to 6.0 mg/ml. Together, these data suggest that the W. trilobata fresh parts extracts analyzed are potential antimicrobial candidates with a broad range of activity. Phytochemical screening of extracts showed the presence of tannins, cardiac glycosides, flavonoids, terpenoids, phenols, saponins and coumarins. Leaf and flower water have showed highest total phenolic content. In 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and ferric ion reducing antioxidant power (FRAP) method, the leaf and flower had showed free radical inhibition of 86, 83 and 1623.21, 1611.26, respectively and they also showed in vitro anti-inflammatory activity by inhibiting the heat induced albumin denaturation and red blood cells membrane stabilization with 89.61 and 86.81 and 78.82, 76.65 g/ml, respectively. Proteinase activity was also significantly inhibited by the leaf (83.91 g/ml) and flower (81.17 g/ml). From the result, it is concluded that phytochemicals present in the W. trilobata extract may be responsible and can be used as antimicrobial, antioxidant and anti-inflammatory agent. (M. et al., 2011)

2.6 ACCLIMATION OF PHOTOSYSTEM II TO HIGH TEMPERATURE IN TWO WEDELIA SPECIES FROM DIFFERENT GEOGRAPHICAL ORIGINS: IMPLICATION FOR BIOLOGICAL INVASIONS UPON GLOBAL WARMING, BANGLADESH

More intense, more frequent, and longer heat waves are expected in the future due to global warming, which could have dramatic ecological impacts. However, few studies have involved invasive species. The aims of this study were to examine the effect of extreme heating (40/35 °C for 30 d) on the growth and photosynthesis of an alien invasive species *Wedelia trilobata* and its indigenous congener (*Wedelia chinensis*) in South China, and to determine the development of this invasive species and its potential adaptive mechanism. In comparison with *W. chinensis*, *W. trilobata* suffered less inhibition of the relative growth rate (RGR) and biomass production due to high temperature, which was consistent with the changes of photosystem II (PSII) activity and net photosynthetic rate (P_n). High temperature caused a partial inhibition of PSII, but the adverse effect was more severe in *W. chinensis*. Measurement of the minimum fluorescence (F_o) versus temperature curves showed that *W. trilobata* had a higher inflexion temperature of F_o (T_i), indicating greater thermostability of the photosynthetic apparatus. Moreover, comparisons of absorbed light energy partitioning revealed that *W. trilobata* increased xanthophyll-dependent thermal dissipation (Φ_{NPQ}) under high temperature, while retaining the higher fraction of absorbed light allocated to photochemistry (Φ_{PSII}) relative to *W. chinensis*. The results suggest that the invasive *W. trilobata* has a high thermostability of its photosynthetic apparatus and an effective regulating mechanism in energy partitioning of PSII complexes to minimize potential damage and to retain greater capability for carbon assimilation. These factors confer greater heat stress tolerance compared with the native species. Therefore, the invasive *W. trilobata* may become more aggressive with the increasingly extreme heat climates. (Song et al., 2015)

2.7 IN VITRO PROPAGATION OF SPHAGNETICOLA TRILOBATA (L.) PRUSKI, KOREA

An efficient protocol for in vitro propagation of Sphagneticola trilobata is described through adventitious shoot regeneration and axillary shoot multiplication. Direct adventitious shoot buds were initiated from leaf explants after three weeks of culture on MS medium containing different concentrations of cytokinins (BA, Kinetin and 2iP). Among the growth regulators tested, BAP induced maximum number of shoots at 1.0 mg l-1 and the corresponding percentage of shoot induction was 74%. Axillary shoot multiplication was achieved by culturing shoot tip and nodal explants on MS medium containing different concentrations and combinations of plant growth regulators. The highest number of shoot buds was achieved when nodal explants cultured on MS medium fortified with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1}IAA and 2.0 mg l^{-1}GA3 with an average of 42 shoots per explant. Maximum rooting (100 %) was obtained on half-strength MS medium fortified with 2.0 mg l^{-1}IBA. The plantlets were successfully acclimatized after four weeks. This protocol could be utilized for in vitro clonal propagation of this economically important plant. (Sivanesan et al ., 2000)

2.8 WEDELIA TRILOBATA L.: A PHYTOCHEMICAL AND PHARMACOLOGICAL REVIEW

Studies on the traditional use of medicines are recognized as a way to learn about potential future medicines. *Wedelia* is an extensive genus of the family Asteraceae, comprising about 60 different species. *Wedelia trilobata* Linn. has long been used as traditional herbal medicine in South America, China, Japan, India and for the treatment of a variety of ailments. The aim of this review was to collect all available scientific literature published and combine it into this review. The present review comprises the ethnopharmacological, phytochemical and therapeutic potential of *W. trilobata*. An exhaustive survey of literature revealed that tannin, saponins, flavonoids, phenol, terpenoids constitute major classes of phytoconstituents of this plant. Pharmacological reports revealed that this plant has

antioxidant, analgesic, anti-inflammatory, antimicrobial, wound healing, larvicidal, trypanocidal, uterine contraction, antitumor, hepatoprotective, and in the treatment of diabetes, menstrual pain and reproductive problems in women. *W. trilobata* seems to hold great potential for in-depth investigation for various biological activities, especially their effects on inflammation, bacterial infections, and reproductive system. Through this review, the authors hope to attract the attention of natural product researchers throughout the world to focus on the unexplored potential of *W. trilobata*, and it may be useful in developing new formulations with more therapeutic value. (Balekar et al., 4014)

2.9 ANTIOXIDANT, ANTIBACTERIAL AND DNA PROTECTING ACTIVITY OF SELECTED MEDICINALLY IMPORTANT *ASTERACEAE* PLANTS, INDIA

Asteraceae is the largest family of flowering plants, traditionally known for its medicinal properties. In the present study antioxidant properties of 10 selected *Asteraceae* species were assessed by DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS (2,2'-azino-bis(3-thylbenzthiazoline-6-sulphonic acid) method. The plants were extracted sequentially in soxhlet apparatus with petroleum ether, hexane, ethyl acetate, chloroform, methanol and water in the increasing order of polarity. These extracts were subjected to find its antioxidant activity and total phenolic contents. Antibacterial activity against some human pathogenic bacteria was tested by agar disk diffusion method. Among all the organic solvent extracts, methanol extracts had very good antioxidant and antibacterial activity. The extracts showed inhibition of human pathogenic bacteria in the order: *Escherichia coli* > *Klebsiella pneumonia* > *Shigella flexneri* > *Staphylococcus aureus* > *Bacillus subtilis* > *Bacillus cereus*. Minimum inhibitory concentration (MIC) was 100 µg/100 µl for many plant extracts, whereas MIC of *G. bosvallea* and *W. trilobata* was 70 µg/100 µl for *Bacillus subtilis*, *Klebsiella pneumonia* and *Shigella flexneri*. The extracts were tested for pTZ57R/T plasmid DNA protection against hydroxyl radicals as evidenced by DNA fragmentation assay. Significant and positive linear correlations (R2 = 0.9294) were found between total antioxidant capacities and phenolic contents indicating that phenolics were the dominant

antioxidant constituents in tested medicinal plants which are discussed in this manuscript. Our study clearly demonstrated that the selected plants have good antioxidant, antibacterial and DNA protecting properties. (Prakash et al., 2012)

2.10 ANTIMICROBIAL ACTIVITY OF WEDELIA TRILOBATA CRUDE EXTRACTS

A biological screening of activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi of crude extracts from Wedelia trilobata is reported. The n-hexane extract showed antibacterial activity against Bacillus subtilis, Mycobacterium smegmatis, Staphylococcus aureus, and Staphylococcus epidermidis (Gram-positive bacteria); along with Proteus vulgaris, Pseudomonas aeruginosa, Salmonella group C, Salmonella paratyphi, and Shigella sonnei (Gram-negative bacteria). The ethyl acetate extract was active only against Salmonella group C; and the aqueous extract was inactive against the tested bacteria. None of the tested extracts showed biological activity against the yeasts (Candida albicans, Candida tropicalis, Rhodotorula rubra) or the fungi (Aspergillus flavus, Aspergillus niger, Mucor sp., Trichophyton rubrum). (Rosas-Romero et al., 1999)

2.11 IMPACT OF SPHAGNETICOLA TRILOBATA ON PLANT DIVERSITY IN SOIL IN SOUTH-EAST VITI LEVU, FIJI

Wedelia (*Sphagneticola trilobata* (L.) Pruski) has become one of the most dominant invasive plant species in Fiji. However, the soil seed bank of its monospecific stand and its ability to reproduce by seed is relatively unknown. A soil seed bank study was undertaken in a monospecific stand of *S. trilobata* in Sawani, Natavea and Wainivesi in south-east Viti Levu, Fiji in March 2012. The soil samples were collected from within 1.0 m2 quadrat taken at 10 spots in each of the study areas and spread thinly over a base of Yates Thrive Premium potting mix in seedling trays and placed in a glasshouse at Koronivia Research Station, Fiji. A total of 23, 26 and 33 plant species were found in the soil seed bank in Wainivesi, Sawani and Natavea respectively which may have succumbed to *S. trilobata* invasiability. There

were ca. 3,800 (17%), 2,100 (11%) and 2,600 (6%) germinable *S. trilobata* seeds·m-2 in the soil seed bank in Wainivesi, Sawani and Natavea areas respectively. This study has demonstrated that *S. trilobata* seeds may have a role in the spread of the invasive species in Fiji and movement of soil to *S. trilobata* free areas should be restricted. (Macanawai 2013)

2.12 ESSENTIAL OIL COMPOSITION OF *SPHAGNETICOLA TRILOBATA* (L.) PRUSKI, INDIA

This study aimed to explore the potential of an invasive alien species, *Sphagneticola trilobata* (L.) Pruski, that grows in foothill region of northern India. The volatile oil from the aerial parts of *S. trilobata* was isolated by hydrodistillation method and analysed using capillary gas chromatography–flame ionization detector (GC–FID) and GC–mass spectrometry (GC–MS) during different seasons. Volatile oil yield varied from 0.18 to 0.25% in different seasons, with the maximum in winter season. Altogether, 43 constituents, representing 96.1–97.3% of the total oil composition were identified. Major constituents of the oils were α-pinene (78.6–83.3%), α-phellandrene (1.3–4.1%), sabinene (1.4–1.9%), limonene (1.2–1.9%), β-pinene (1.0–1.6%), camphene (0.7–2.0%), 10-nor-calamenen-10-one (<0.05–1.5%), germacrene D (0.1–1.4%) and γ-amorphene (<0.05–1.3%). The comparative results showed no big differences in the oil composition of this plant due to season of collection. It is concluded that the *S. trilobata* population grown in this region could be utilized as a potential source of industrial molecule, α-pinene. (Verma et al., 2014)

2.13 EFFECTS OF SIMULATED ACID RAIN ON THE ALLELOPATHIC POTENTIAL OF INVASIVE WEED *WEDELIA TRILOBATA*

Acid rain poses a major threat to natural ecosystems in rapidly-developing industrialized regions like southern China. Despite the significant environmental impact of acid rain, little is known about its effects on important aspects of ecosystem dynamics such as plant-plant allelopathic interactions. The major invasive weed *Wedelia trilobata* in southern China, was used in this study to examine the possible effects of acid rain on the allelopathic potential of invasive plant species. The phytotoxicities of aqueous leachates and dried leaf litter of field-

grown *W. trilobata* plants exposed to simulated acid rain [(SAR) of pH 2.5, 4.0, 5.6, 7.0 water control] were determined in *in-vitro* assays on two receptor species: *Brassica campestris* and *Raphanus sativus*. Substantial increases in the phytotoxicity of the leachates as well as leaf litter were observed as a function of decreasing SAR pH. Additionally, glasshouse experiments were done to determine the effects of various SAR-treatments on *W. trilobata* biomass accumulation and shoot height, both parameters showed modest increases at SAR pH 4.0 and decreases at SAR pH 2.5 than control (pH 7.0) plants. These data indicated that acidic conditions increased the allelopathic potential of *W. trilobata*, suggesting that acid rain exposure may increase the invasiveness of this weed. (WANG et al., 2012)

2.14 ANTIBACTERIAL ACTIVITY OF FLOWER HEADS OF *WEDELIA TRILOBATA* (L.) A. S. HITCHC

Present study is conducted to analyse the antibacterial activity of flower heads of W*edelia trilobata* against pathogenic bacterial strains and to analyse phytochemical background of various extracts. Fresh flower heads collected from Kerala, India. Air dried flower heads were extracted successively in petroleum ether, chloroform, acetone, ethanol and water. Preliminary antibacterial activity was analysed by disc-diffusion method and further confirmed by MIC and MBC. Preliminary detection of phytochemicals was done. Among the ten bacterial strains nine showed considerable inhibition of growth towards acetone extract. Acetone extract selected for detailed antibacterial evaluation tests like minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC). The MIC and MBC of floral extracts tested against one of the most sensitive strains, *Streptococcus haemolyticus*. MIC and MBC values were 25 mg/ml and 50 mg/ml respectively. Preliminary phytochemical evaluation revealed the presence of flavonoids, phenolics and terpenoids in active acetone extract. The present investigation showed the effectiveness of crude extract of this plant against tested bacterial strains. The presence of potential phytochemicals like phenolics, flavonoids and terpenoids in active acetone extract might be one of the reasons for its antibacterial property. (Shankar and Thomas 2014)

2.15 CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF ESSENTIAL OIL FROM WEDELIA PROSTRATA

This study deals with the chemical composition, antioxidant and antimicrobial activity of essential oils of *Wedelia prostrata* and their main constituents in vitro. A total of 70 components representing 99.26 % of the total oil were identified. The main compounds in the oil were limonene (11.38 %) and α-pinene (10.74 %). Antioxidant assays (1,1-diphenyl-2-picrylhydrazyl, superoxide anion radical, and reducing power test) demonstrate moderate activities for the essential oil and its main components (limonene and α-pinene). The essential oil (1000 μg/disc) exhibited promising antimicrobial activity against 10 strains of test microorganisms as a diameter of zones of inhibition (20.8 to 22.2 mm) and MIC values (125 to 250 μg/ml). The activities of limonene and α-pinene were also determined as main components of the oil. α-Pinene showed higher antimicrobial activity than the essential oil with a diameter of zones of inhibition (20.7 to 22.3 mm) and MIC values (62.5 to 125 μg/ml). The antioxidant and antimicrobial properties of the essential oil may be attributed to the synergistic effects of its diverse major and minor components. (Dai et al., 2013)

CHAPTER THREE

MATERIALS & METHODS

3.1 COLLECTION AND PREPARATION OF PLANT MATERIAL

Plant sample (leaves) of *Wedelia trilobata* was collected from Aftabnagar, Badda. 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 24hrs 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

About 650gm of the powdered material was taken in separate clean, round bottomed flask (5 liters) and soaked in 3.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39°C with a rotary evaporation.

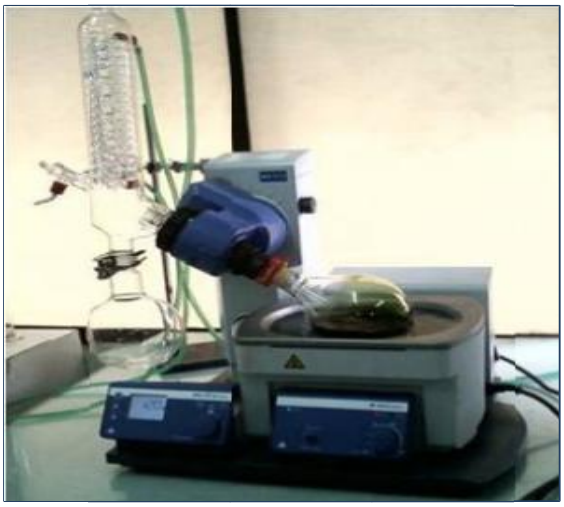

Figure 3.1: Drying of extract using rotary evaporator

The concentrated extract was then air dried to solid residue. The weight of the crude methanol extract obtained from the powdered whole plant was 25gm respectively.

3.3 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.

3.4 PARTITION OF MOTHER SOLUTION

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

3.4.1 PARTITION WITH PETROLIUM ETHER

To the mother solution was taken in a separating funnel. 100 ml of petroleum ether was added to it and the funnel was shaken and kept undistributed. The organic portion was collected. The process was repeated thrice (100ml × 3). The petroleum ether fraction was dried for solid residue.

3.4.2 PARTITION WITH CHLOROFORM

To the mother solution that was left after partitioning with petroleum ether, 12.5 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with $CHCl_3$ (100ml × 3). The $CHCl_3$ soluble fractions were collected together and air dried.

3.4.3 PARTITION WITH ETHYL ACETATE

To the mother solution that after washing with petroleum ether, $CHCl_3$, 16 ml of distilled water was added and mixed uniformly. Then the mother solution was taken in a separating funnel and extracted with ethyl acetate (100 × 3). The ethyl acetate soluble fractions were collected together and air dried.

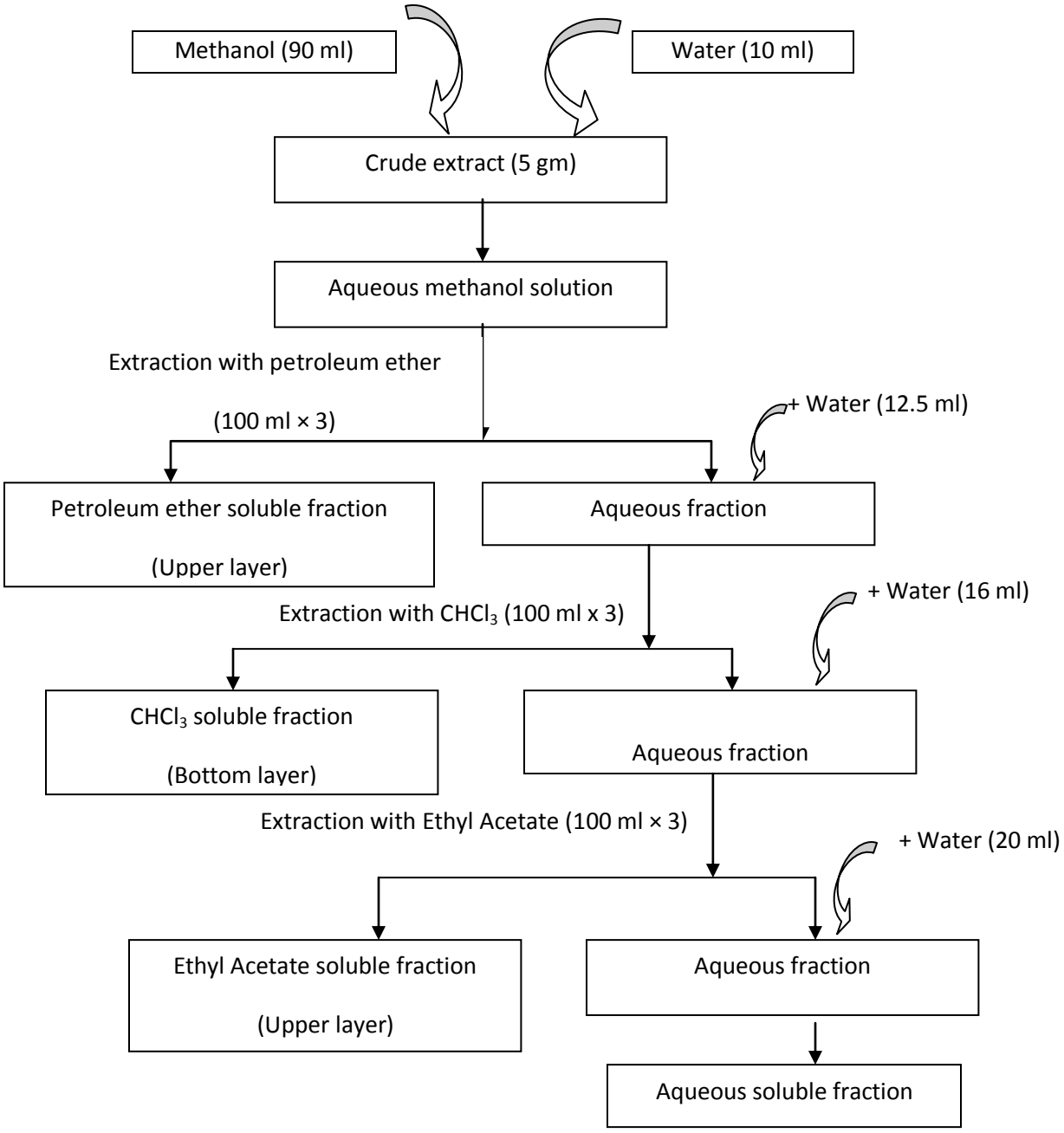

Figure 3.2: Schematic representation of the partitioning of methanolic crude extract of *Wedelia Trilobata* leaves

3.4.4 COLLECTION OF AQUEOUS FRACTION

After partitioning the mother solution with the three different solvents the aqueous fraction remaining at the end was collected and air dried. This aqueous fraction was further investigated for different pharmacological properties (antioxidant, cytotoxic and antibacterial).

3.5 ANTIOXIDANT ACTIVITY

3.5.1 TOTAL FLAVONOID CONTENT

3.5.1.1 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 A or B-ring of flavonoids. The formed flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride has an absorptivity maximum at 510nm. 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. Quercetinat various concentrations was used as standard (Chang et al., 2002).

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex (λmax510nm)

3.5.1.2 APPARATUS AND REAGENTS

- Aluminum chloride
- Methanol
- Ascorbic acid
- Sodium hydroxide
- Sodium nitrite
- Spatula
- Analytical balance
- Pipette and pumper
- Petroleum ether fraction
- Test tubes and beaker

3.5.1.2 PROCEDURE

Aluminium chloride (10%) solution preparation

10mg of aluminium chloride (AlCl3) was taken into a 100ml of a volumetric flask and the volume was adjusted by distilled water.

NaOH (4%) solution preparation

4mg of sodium hydroxide (NaOH) was taken into a 100ml volumetric flask and the volume was adjusted by distilled water.

NaNO2 (5%) solution preparation

5mg of sodium nitrite NaNO2 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 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 3.1: 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.

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 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.

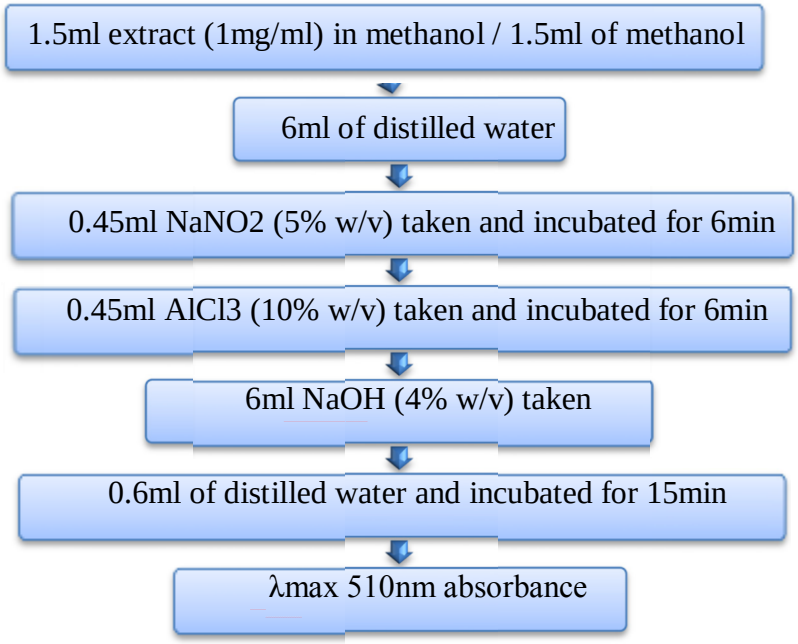

Figure 3.3: Schematic diagram of flavonoid content test

3.6 BRINE SHRIMP LETHALITY BIOASSAY

3.6.1 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 and Nuñeza, 2013; Rishikesh et al., 2013).

3.6.2 APPARATUS AND REAGENTS

- Artemia salina leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank perfotated dividing dam to hatch the shrimp
- Lamp to attract
- Pipettes & Micropipette
- Glass vials
- Magnifying glass
- Test samples
- Dimethyl sulfoxide (DMSO)

3.6.3 PROCEDURE

3.6.3.1 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 0.1N was added with a dropper to obtain the pH 8.4 as sea water.

3.6.3.2 HATCHING OF BRINE SHRIMP

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 dry preserved eggs 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 18-24hrs. 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.

Figure 3.4: Artemia salina 24 hours old

3.6.3.3 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.

Preparation of test samples of experimental plant

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

Preparation of 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 which 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/mll. 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.

Preparation of 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.

3.6.3.4 COUNTING OF NAUPLII

After 24hrs, 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.

3.7 ANTIMICROBIAL ACTIVITY BY DISC DIFFUSION METHOD

3.7.1 PRINCIPLE

The disk diffusion susceptibility method is simple and well-standardized. Bacterial inoculums are applied to the surface of a large agar plate. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. Plates are incubated for 16–24hrs at 35°C prior to determination of results. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The zones of growth inhibition are measured to the nearest millimeter around each of the antibiotic disks. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium (Barry, 1976).

3.7.2 APPARATUS AND REAGENTS

3.7.2.1 MATERIALS

Filter paper discs	Laminar air flow hood
Petri dishes	Autoclave
Inoculating loop	Incubator
Sterile cotton	Ethanol
Sterile forceps	Nutrient Agar Medium
Spirit burner	Screw cap test tubes
Micropipette	Nose mask and Hand gloves

3.7.2.2 TEST SAMPLE OF WEDELIA TRILOBATA

Petroleum Ether fraction of methanolic extract of *Wedelia trilobata* leaves were taken as test sample.

3.7.2.3 TEST ORGANISMS

The bacterial strains used for the experiment were collected as pure cultures from the East West University microbiology laboratory. Gram positive bacteria, Gram-negative bacteria and fungi organisms were taken for the test and they are listed in the following table.

Table 3.2: List of microorganisms

Type of microorganism	Name of microorganism
Gram +ve bacteria	Beta-hemolytic streptococcus Bacillus subtilis
Gram –ve bacteria	Escherichia coli Salmonella paratyphi Pseudomonas aeruginosa
Fungi	Saccharomyces cerevisiae

3.7.3 PROCEDURE

3.7.3.1 PREPARATION OF THE MEDIUM

To prepare required volume of this medium, 5.6gm of agar medium was taken in a bottle with a cap and distilled water was added to it to make 200ml volume. The contents were then autoclaved to make a clear solution.

Figure 3.5: Autoclave machine

3.7.3.2 STERILIZATION PROCEDURE

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in laminar hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the laminar hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of 121℃ and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

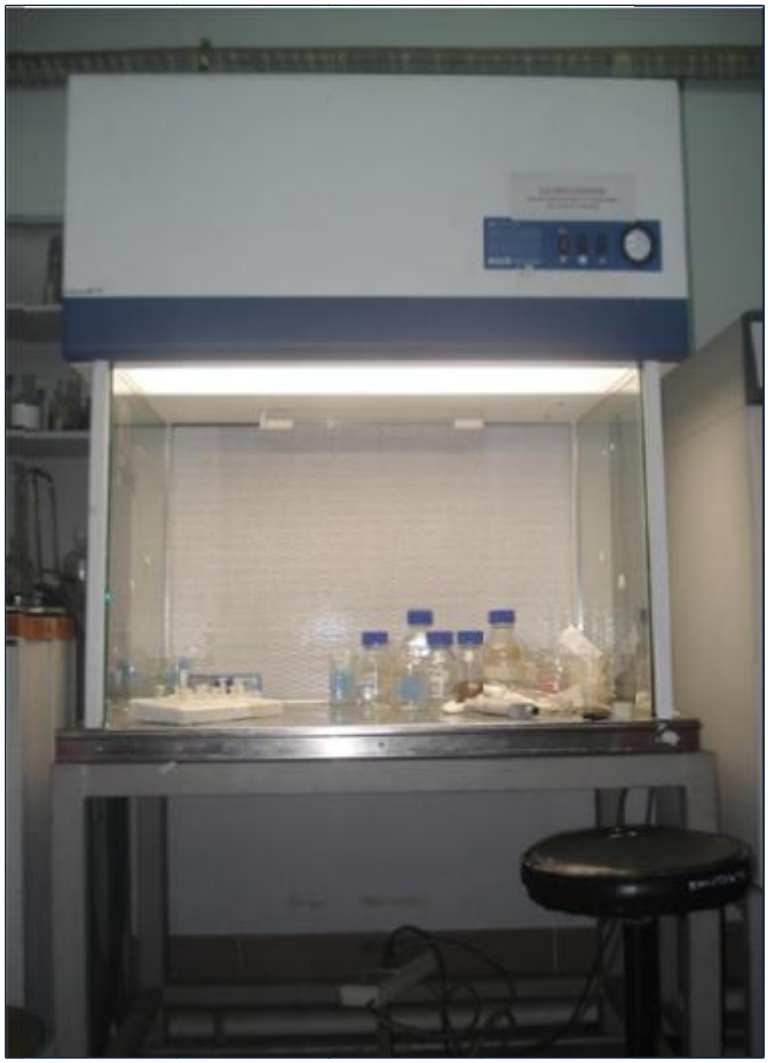

Figure 3.6: Laminar hood

3.7.3.3 PREPARATION OF TEST PLATE

The test organisms were transferred from the subculture to petridish containing about 10ml of melted and sterilized agar medium. The bacterial and fungal suspensions were taken by a loop mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

3.7.3.4 PREPARATION OF DISCS

Three types of discs were used for antimicrobial screening.

1. Standard discs: These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Ciprofloxacin disc was used as the reference.
2. Blank discs: These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.
3. Sample discs: These discs were soaked with solutions of test samples of known concentration, dried and used to determine the anti-activity of the samples.

3.7.3.5 PREPARATION OF TEST SAMPLE

Measured amount of test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

3.7.3.6 APPLICATION OF TEST SAMPLES

Standard Ciprofloxacin discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Methanol discs were used as negative controls which ensure that the residual solvents (left over the discs even after air- drying) and the filter paper were not active themselves.

3.7.3.7 DIFFUSION AND INCUBATION

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then inverted and kept in an incubator at 37°C for 24hrs.

Figure 3.7: Incubator

3.7.3.8 DETERMINATION OF ANTIMICROBIAL ACTIVITY BY MEASURING THE ZONE OF INHIBITION

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

CHAPTER FOUR

RESULTS & 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 Petroleum Ether fraction of methanolic extract of *Wedelia trilobata* (leaves) was determined by following methods-

- Determination of DPPH radical scavenging assay
- Determination of total phenolic content
- Determination of total flavonoids content

4.1.1 TOTAL FLAVONOID CONTENT RESULT

The Pet. ether fractions of *Wedelia trilobata* leaves were subjected to determine total flavonoid content present. Here, ascorbic acid (AA) was used as reference standard.

4.1.1.1 PREPARATION OF STANDARD CURVE

Table 4.1: Total flavonoid content of ascorbic acid

Concentration	Absorbance	Regression line	R^2 value
50	0.05	y =0.0017x-0.042	0.991
100	0.13		
150	0.19		
200	0.29		
250	0.39		

After absorbances were taken of different solution of ascorbic acid of concentrations ranging from 50µg/ml to 250µg/ml, a linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.1. This linear curve was considered as a standard curve. Regression analysis is calculated in Microsoft Office Excel 2010.

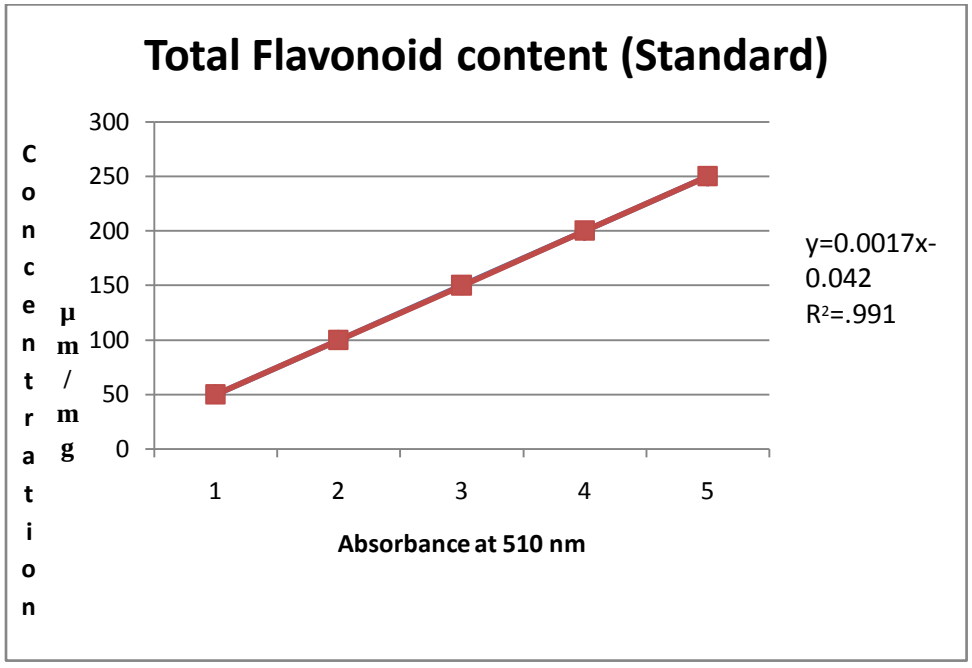

Figure - 4.1: Graphical representation of assay of flavonoid content of ascorbic acid

4.1.1.2 TOTAL FLAVONOID PRESENT IN PETROLEUM ETHER FRACTION

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 the Table 4.2

Table 4.2: Total flavonoid content of Petroleum ether fraction of leaves of *Wedelia trilobata*

Sample	Concentration (mg/ml)	Absorbance	Total flavonoid content (mg of AAE/g of dried extract)
Pet. ether fraction of *Wedelia tilobata*	1	1.195	727.647

4.1.1.3 DISCUSSION

To determine the total flavonoid content of the test samples the standard curve was used. In 1mg/ml concentration of Pet. Ether fraction of *Wedelia trilobata* (leaves) 727.647 mg of AAE/gm of dried extract of flavonoid content was found. So this extract contains antioxidative compounds.

4.2 BRINE SHRIMP LETHALITY BIO-ASSAY RESULT

The Pet. ether fraction of the methanolic extract of *Wedelia trilobata* leaves 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 aqueous 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:

$$\% \text{ mortality} = (\text{number of dead nauplii/total number}) \times 100$$

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. The concentration-% mortality data were analyzed by using Microsoft Office Excel 2010.

4.2.1 PREPARATION OF STANDARD CURVE

Tamoxifen was used as positive control.

Table 4.3: 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	13.38
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	5	5	50	
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	

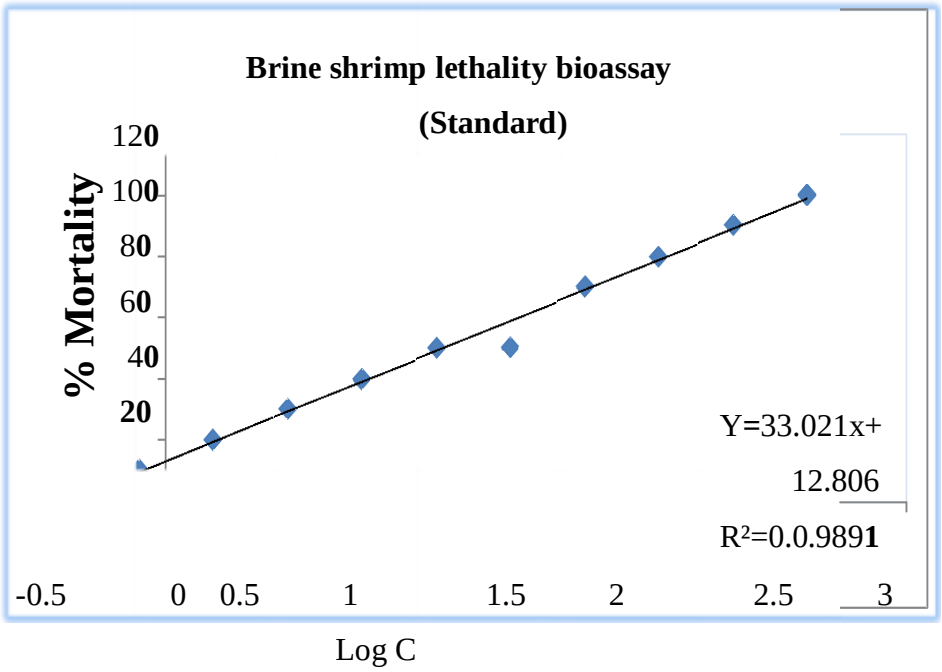

Figure 4.2: Plot of % mortality and predicted regression line of Tamoxifen (standard)

4.2.2 PREPARATION OF PET.ETHER FRACTION CURVE

Table 4.4: Results of the bioassay of petroleum ether fraction (extract)

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	15.28
2	200	2.301	2	8	80	
3	100	2.000	2	8	80	
4	50	1.699	4	6	60	
5	25	1.398	5	5	50	
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	

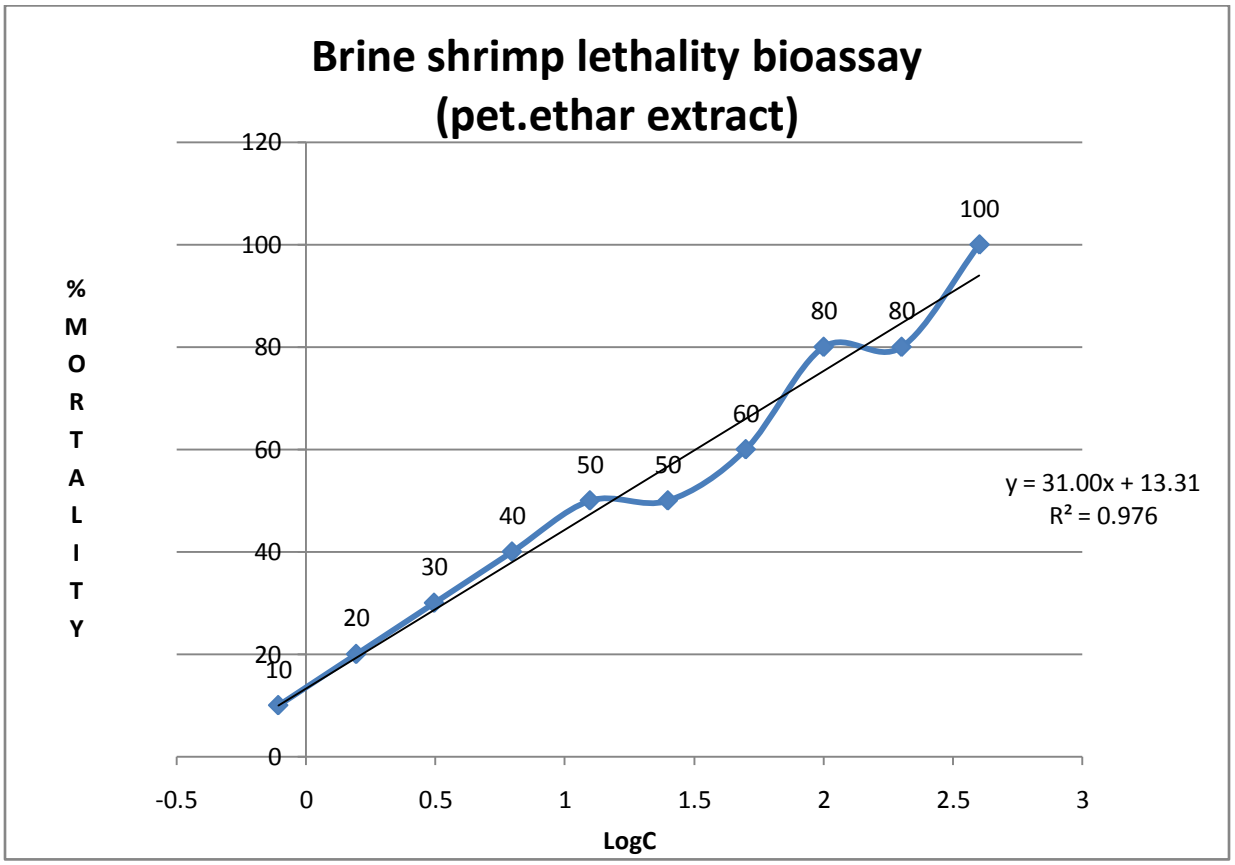

Figure 4.3: Plot of % mortality and predicted regression line of petroleum ether (extract)

4.2.3 DISCUSSION

In the Brine Shrimp lethality bioassay, varying degree of lethality was observed with exposure to different concentration of the test samples. The degree of the lethality was found to be proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and Petroleum Ether fraction samples. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400 µm/ml, whereas the least mortalities at lowest concentration 0.78125 µm/ml as shown in the Table 4.4.

Table 4.5: Cytotoxic activity of Tamoxifen and Pet. Ether fraction of *Wedelia trilobata* leaves

Sample	Linear regression	R^2 value	LC_{50} (µg/ml, 24hr)
Standard (Tamoxifen)	y = 33.021x + 12.806	0.9891	13.38
Extract (Pet. ether fraction)	y = 29.19x + 18.57	0.976	15.28

In this investigation, standard and Petroleum Ether fraction exhibited cytotoxic activities with the LC_{50} values 13.38 µg/ml and 15.28 µg/ml respectively as shown in Table 4.5. For both standard and Petroleum Ether fraction the R^2 values is closer to one which indicates that the extract has potent activity against brine shrimp nauplii comparable to the standard (Tamoxifen)

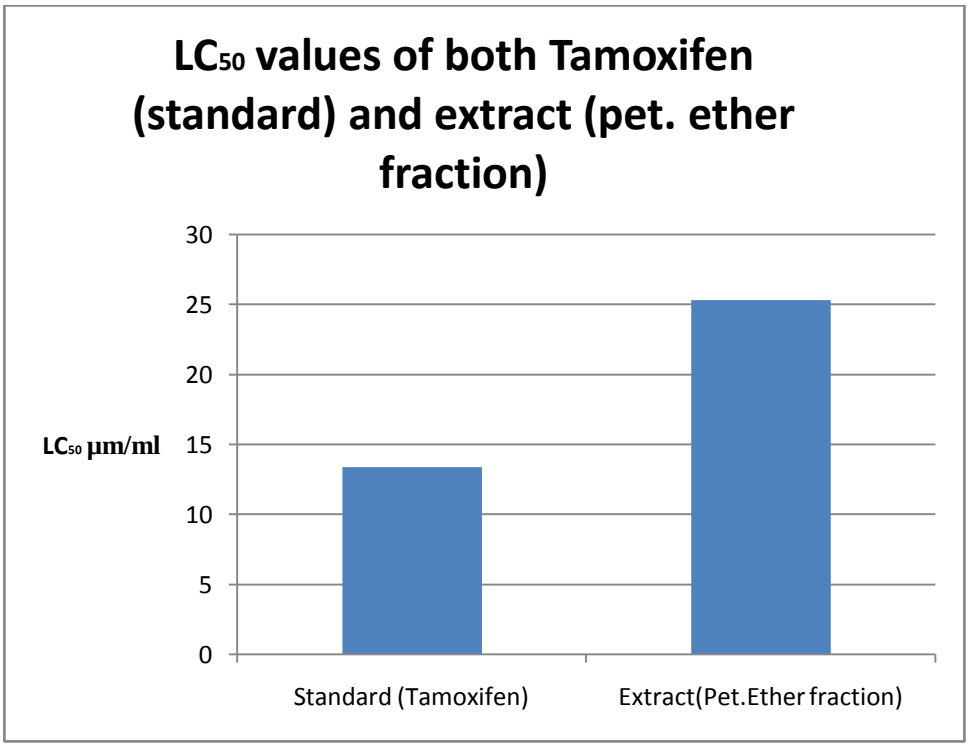

Figure 4.4: Comparison between LC50 values of standard and extract

From the above figure it can be concluded that for aqueous fraction the lethal concentration required to kill 50% of the sample population is lower than the standard. So the extract is more potent than ascorbic acid at lower concentration.

4.3 ANTIMICROBIAL TEST RESULTS

The antimicrobial activities of Pet. ether fraction of methanolic extract of *Wedelia trilobata* leaves were examined in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The Pet. Ether fraction was subjected to the various bacterial and fungal cultures and from that zones of inhibition were measured. Here Ciprofloxacin was used as standard reference.

4.3.1 ZONE OF INHIBITION OF STANDARD AND PET. ETHER FRACTION

Table 4.6: Antimicrobial activity of standard sample (Ciprofloxacin) and Pet. ether fraction

Type of microorganism		Zone of inhibition (mm)	
		Standard sample	Pet. ether fraction
Gram positive bacteria	*Bacillus cereus*	38	8
	Bacillus megaterium	38	7
	Bacillus subtilis	40	6
	Staphylococcus aureus	40	8
	Sarcina lutea	37	6
Gram negative	*Salmonella paratyphi*	38	6
	Salmonella typhi	36	7
	Vibrio parahemolyticus	40	7
	Escherichia coli	36	5
	Vibrio mimicus	35	8
	Shigella dysenteriae	38	8
	Pseudomonas aeruginosa	38	6
	Shigella boydil	48	6
Fungi	*Sacharomyces cerevacae*	35	6
	Candida albicans	26	6
	Aspergillus niger	30	8

4.3.2 DISCUSSION

Petroleum Ether of methanolic extract of *Wedelia trilobata* showed low antimicrobial activity when compared to Ciprofloxacin. None of the zone of inhibition of Petroleum Ether fraction is equal to Ciprofloxacin against any bacteria or fungi as shown in the Figure 4.6. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against Aspergillus niger (8mm) comparable to the standard (30mm).

CHAPTER FIVE

CONCLUSION

CONCLUSION

For the plant physiologist, work on medicinal plants opens up a wide range of research possibilities, and plant physiological studies would indeed have a major role to play in this burgeoning field. With only a few exceptions, many widely used medicinal plants have not received the extensive plant physiological characterization received by food crops or model plant systems. Although active phytochemicals may have been identified, in general, many pathways for the biosynthesis of specific medicinal compounds and the factors (biotic and abiotic) regulating their production remain unclear. At present, a major concern with the use of phytomedicines regards the maintenance of consistent medicinal quality in botanical medicines.

Therefore, plant materials can be potential sources of chemically interesting and biologically important drug entrant. And for this purpose the plant can be further screened against various diseases in order to find out its unexplored efficacy with a gaze to the future with a great deal of disease. It can be antibacterial, anticonvulsant, antinociceptive, cardiotonic, antipyretic, arsenic induced toxicity, antifungal, synergism and antagonism with antibiotics. In my experiment it shows very positive result for Anti-oxidant activity, Brine shrimp lethality test and Anti-diabetic test. There are some established research reports regarding the phytochemical and pharmacological properties of this plant. Still there are plenty of scopes to establish a variety of properties which are significantly beneficial to mankind.

The results obtained in this study indicate that the Petroleum Ether fraction of the leaves of *Wedelia trilobata* have significant cytotoxic activity. Experimental evaluation showed that the leaves of this plant also possess antimicrobial and antioxidant properties. Investigations performed on the aqueous extract proved that the leaves contain flavonoid compounds. Since *W.Trilobata* leaves exhibited potent cytotoxic activity, so the leaves can be further evaluated for anticancer, pesticidal and antitumor properties. Detailed investigations can be carried out to isolate and identify the active compounds present in the leaf extract that are responsible for such kind of pharmacological activity for development of novel and safe drugs. Further tests can be performed to evaluate whether the leaves possess some other potent pharmacological activities.

CHAPTER SIX

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REFERENCES

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