BIOLOGICAL INVESTIGATION OF ETHYL ACETATE EXTRACT OF *WEDELIA TRILOBATA* LEAVES

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

Submitted by:

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DEDICATION

This Research paper is dedicated to

My beloved parents,

Who are my biggest Inspirations…

DECLARATION BY THE CANDIDATE

I, Shajib Kanti Dey, hereby declare that this dissertation, entitled "***Biological investigation of ethyl acetate extract 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 of fellowship.

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled "***Biological investigations of ethyl acetate of Wedelia trilobata leaves***" is a bonafide research work done by Shajib Kanti Dey (ID: 2011-1-70-062), in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

Dr. Chowdhury Faiz Hossain

Professor & Chairperson

Department of Pharmacy

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Aftabnagar, Dhaka

CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertation, entitled **"*Biological investigation of ethyl acetate extract of wedelia trilobata leaves*"** is a bonafide research work done, under my guidance and supervision by Shajib Kanti Dey (ID:2011-1-70-062), in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

Iftekhar Ahmed

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ABSTRACT

The study was designed for biological investigations of ethyl acetate 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 ethyl acetate, chloroform and ethyl acetate consecutively. And the aqueous fraction remaining at the end. The ethyl acetate fraction was remaining at the beaker was investigated for the total flavonoid content, brine shrimp lethality test and antimicrobial test. The fraction contained 228.824 mg AAE/g of total flavaniod content. Screening for cytotoxic properties using brine shrimp lethality bioassay with Tamoxifen (LC50 value of 13.38µg/ml) as positive control showed that the fraction have considerable cytotoxic potency exhibiting LC50 value 27.17 µg/ml. In antimicrobial activity investigation, the ethyl acetate fraction showed low antibacterial and antifungal activity against the tested organisms compared to Ciprofloxacin (30µg/disc) that was used as positive control. The ethyl acetate 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.

Chapter One

INTRODUCTION

1.1 Medicinal plants

Medicinal and aromatic plants play a significant role in the life of people and are present in innumerable forms. In Indian traditions, all the plants in this earth are considered as medicinal Hriday However, a simplest definition of the medicinal plant would be "Medicinal plants are those plants which are used in official and various traditional systems of medicines throughout the world". Other definition could be "Medicinal plants are plants that provide people with medicines - to prevent disease, maintain health or cure ailments". In one form or another, they benefit virtually everyone on earth. No exact definition of Medicinal Plant is possible. There are related issues, such as for nutrition, toiletry, body care, incense and ritual healing. Aromatic plants are a special class of plants used for their aroma and flavor. Many of them are exclusively used also for medicinal purposes in aromatherapy as well as in various systems of medicine. Similarly a number of medicinal plants also produce essential oils as well as being used for perfumery e.g. Petroselinum sativum, Daucus carota, Anethum graveolens and Pimpinella anisum, etc. In this chapter we shall deal these special classes of plants together as medicinal and aromatic plants. (Global journal of human social sciences, 2007)

1.2 Medicinal plants – History and context

Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. Written evidence of herbal remedies dates back over 5,000 years, to the Sumerians, who created lists of plants. A number of ancient cultures wrote on plants and their medical uses. In ancient Egypt, herbs are mentioned in Egyptian medical papyri, depicted in tomb illustrations, or on rare occasions found in medical jars containing trace amounts of herbs. The earliest known Greek herbals were those of Diocles of Carystus, written during the 3rd century B.C, and one by Krateuas from the 1st century B.C. Only a few fragments of these works have survived intact, but from what remains scholars have noted that there is a large amount of overlap with the Egyptian herbals. Seeds likely used for herbalism have been found in the archaeological sites of Bronze Age China dating from the Shang Dynasty. Over a hundred of the 224 drugs mentioned in the Huangdi Neijing, an early Chinese medical text, are herbs. Herbs

were also common in the medicine of ancient India, where the principal treatment for diseases was diet. De Materia Medica by Pedanius Dioscorides, a Roman physician, is a particularly important example of such writings. The documentation of herbs and their uses was a central part of both Western and Eastern medical scholarship through to the 1600s, and these works played an important role in the development of the science of botany.

Human beings have used plants for the treatment of diverse ailments for thousands of years. According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements, since they cannot afford the products of Western pharmaceutical industries, together with their side effects and lack of healthcare facilities. Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses.2 Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety margins and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell. Even with the advent of modern or allopathic medicine, Balick and Cox (1996) have noted that a number of important modern drugs have been derived from plants used by indigenous people. Traditional use of medicine is recognized as a way to learn about potential future medicines. Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. (Gupta and Maheshwari, 2007)

1.3 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 poison.

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

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

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

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

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

1.4 Cause of doing phytochemistry study of plants:

All plants produce chemical compounds as part of their normal metabolic activities. These phytochemicals 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. For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination. It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs—examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove. Toxic plants even have use in pharmaceutical development. (Williamson, 1996)

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. (Williamson, 1996)

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. (Williamson, 1996)

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 cyano glycosides in cherry pits that release toxins only when bitten by an herbivore. (Williamson, 1996)

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers, which are often strong smelling and thus may have had a protective function. They are the major components of resin, and of turpentine produced from resin. (The name "terpene" is derived from the word "turpentine"). Terpenes are major biosynthetic building blocks within nearly every living creature. Steroids, for example, are derivatives of the triterpene squalene. When terpenes are modified chemically, such as by oxidation or rearrangement of the carbon skeleton, the resulting compounds are generally referred to as terpenoids. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as natural flavor additives for food, as fragrances in perfumery, and in traditional and alternative medicines such as aromatherapy. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives. Vitamin A is an example of a terpene. The fragrance of rose and lavender is due to mono-terpenes. The carotenoids produce the reds, yellows and oranges of pumpkin, corn and tomatoes. (Williamson, 1996)

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:

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

b) 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.

c) To use agents as pharmacologic tools, e.g. lysergic acid diethylamide, mescaline and

d) To use the whole plant or part of it as an herbal remedy, e.g. cranberry, garlic etc.

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 ethno medical 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. (Daniel et al., 2001)

1.5 Medicinal plant use scenario in Bangladesh

In an estimate, the international market of medicinal plants related to trade stood at 60 billion US Dollar per year. The demand for medicinal plants based raw materials are growing at an approximate rate of 10-15% per year internationally. Medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of Bangladesh. In recent years, the growing demand for herbal product has led to a quantum jumping in volume of plants materials trade within and across the country. Bangladesh there is no systematic cultivation process or conservation strategies about medicinal plants. The local people conserve traditional knowledge through their experience and practice, which is handed down orally without any documentation. This knowledge is now under threat to extinction. This is a very

alarming situation with regard to natural growth of medicinal plants in the wilderness in this country. In this scenario, the survey on "Traditional and industrial use and market Scenario of Medicinal plants in Bangladesh." has been conducted by the DEBTEC researchers at Chakbazar, Dhaka, Bangladesh. We have found that there is worth of 11 million US dollars medicinal plant market in Bangladesh, which have been imported but not in the name of medicinal plants rather in the name of spices and other products. This research aimed at documenting the 'Present Status and Market Scenario of Medicinal Plants' in Bangladesh. Our research finding shows that 84.1% of the respondent use medicinal plants in health care. 18.3% of the villagers use Kabirazi in the disease in medium category.55.0% of our respondent's source of knowledge of using medicinal plant is family where 34.7% gained knowledge from neighbor. Only 14.3% of the respondents are involved with trading of medicinal plant. About 10.4% of the villagers are involved in cultivation, collection or business of medicinal plant. From the survey report it has been found that 46.6% industries are using above 60% of imported medicinal plants as their raw materials and 53.3% of the industries are using below 40%. The study revealed that 86.7% industries are importing Indian raw materials, 53.3% are importing the Pakistani one and very few of them are importing the raw materials from Nepal, Iran and Korea. According to the response of shop owners, the local raw materials of their products are mostly coming from 5 different areas of the country. Among those 90% are coming from Chittagong and again 76.6% from Tangail, 30% from Gazipur and another 30% from Khulna. In this scenario, appropriate steps must therefore be taken immediately in order to save this situation with regard to growth, conservation and supply of medicinal plants in the country. The best possible way of doing this is to bringing this more and more of these plants under planned cultivation. The cultivation of medicinal plants in Bangladesh will lead to the conservation and also protect the biodiversity. Ecological and biotic factors are suitable in Bangladesh for the cultivation of medicinal plants. We have been successful to sensitize the policy makers. In Bangladesh there is no facilities and skilled manpower for the processing of MPs. Our research is now aiming to develop processing unit and to train the garden owner for skilled manpower to value addition of MP, which will create the income generating women in rural areas. In Bangladesh, about 500 plant species have been identified as medicinal plants because of

their therapeutic properties, 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. (Bregum F, 2010)

1.6 *Wedelia trilobata*:

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 (Georg Wolfgang Wedel, 1645–1721). (Cabi.org, 2015)

1.6.1 Taxonomy of *Wedelia trilobata:*

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Asterales

Family: Asteraceae

Genus: Sphagneticola

Species: Sphagneticolatrilobata (Cabi.org, 2015)

Figure1.1: Flower and leaves of *Wedelia trilobata*

1.6.2 Botanical name:

Sphagneticola trilobata (L.) Pruski

1.6.3 Botany:

A creeping, succulent mat-forming perennial herb, with rounded stems rooting at the nodes. Leaves are opposite, ovate, dentate, shallowly 3-cleft, glossy and green. Flowers are 2 cm across and golden yellow. (Keyserver.lucidcentral.org, 2015)

Table 1.1 Different types of name of *Wedelia trilobata* in different countries: (Issg.org)

South Africa	Singapoer-madeliefie
Tonga	Ate
USA	Bay Biscayne creeping oxeye; yellow dots
Marshall Islands	ut mokadkad; ut telia
Jamaica	creeping oxeye
Brazil	arnica-do-mato, pseudo-arnica, vedelia
Cuba	Romero de playa
Germany	Wedelie, Goldstern-
Bahamas	Trailing wedelia
Chinese	Nan mei peng qi ju
French	Patte canard
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
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
Bengali	Mohavringaraj,Vringaraj
Puluwat	Atiat
Thailand	kra dum tong

1.6.4 Parts utilized

Leaves, flowers.

1.6.5 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. (Weeds of Australia, 2011)

1.6.6 Properties

• Study isolated Uses

• No reported folkloric medicinal use in the Philippines.

• In Trinidad and Tobago, used for reproductive problems, amenorrhea, dysmenorrhea.

• In South America, used to treat symptoms of colds and flu; for fevers and inflammations. (Floridata, 2015)

1.6.7 Botanical Description

It is a long lived (perennial) herb with a creeping or climbing habit. This matforming herb often creates a dense ground cover (usually 15-30 cm tall but occasionally upto 70 cm tall) that crowd out the growth of other species. It may also climb a short distance up trees or over other vegetation. The stems are rounded, green or reddish in color, and may be coarsely hairy. They grow up to 2 m long and regularly develop roots (adventitious roots) at their nodes. Short, semi-upright (ascending), flowering branches are produced of these creeping stems. The leaves are attractive, bright shiny green, somewhat fleshy oppositely arranged and simple, the blade obovate to elliptic or ovate and are stalkless (sessile) or borne on short stalks (petioles). These leaves 2-9 cm long and 2-5 cm wide, acute at the apex and winged and sessile at the base usually have three lobes (hence the name trilobata) and irregularly toothed (serrated) margins. They are glossy in appearance, mostly hairless (glabrous), and slightly fleshy (succulent) in nature. The single attractive brightyellow flower heads are daisy-like in appearance and are borne on the end of terminal and axillary stalks (peduncles) 2 to 9 cm long, with 2 to 4 series of bracts forming the involucre at the base of the flower. Each flower-head has 8-13 yellowish ‘petals’ (ray florets) that are 6-15 mm long with 1- to 3 finely toothed tips and are pistillate. In the centre of these flower-heads there are numerous tiny yellow tubular disc florets 4-5 mm long, and mixed with chaffy bracts. The ray and disc florets are both yellow. The base of each flower-head (capitulum) is enclosed in a row (involucre) of narrow (lanceolate) green bracts (about 1 cm long). Flowering occurs throughout the year, but is most common from spring to autumn. The fruit is a 2 to 4-angled achene, with short, narrow pappus scales on the top. The ‘seeds’ (i.e. achenes), when present, are 4-5 mm long and topped with a crown of short fringed scales. They are elongated in shape, brown in colour and have a rough surface texture. However, very few seeds reach maturity in cultivated or naturalised plants in Australia (Weeds of Australia, 2011)

1.6.8 Location

West Indies, Hawaii, south Florida, Central America, West Africa, especially at low elevations. (Stuartxchange.org, 2015)

1.6.9 Culture

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

Light: Sun to part shade.

Moisture: Moist to average.

Hardiness: USDA Zones 9 is northernmost boundary.

Propagation: Division.

1.6.10 Usage

Excellent ground cover in warm climates in its native range. Wedelia is especially good for soil retention and erosion control. Plantings are very attractive with nearly constant and prolific blooming. Wedelia may be mowed to keep low and manicured. The plant is no longer considered appropriate for any of these usages in Florida (and similar frostfree climates) where it has proven to be an invasive nuisance. (Stuartxchange.org, 2015)

1.6.11 Features

The plant has use in traditional medicine: crushed leaves are used as a poultice; tea is given to alleviate symptoms of colds and flu; and it is used in combination with other herbs to clear the placenta after birth.

Figure1.2: Flower and leaves of *Wedelia trilobata*

It was introduced to humans mostly as aground covering plant in Mexico, Central America (i.e. Belize, Costa Rica, Guatemala, Honduras, Nicaragua and Panama), the Caribbean and tropical South America (i.e. French Guiana, Guyana, Surinam, Venezuela, Brazil, Bolivia, Colombia, Ecuador and Peru).This species is widely naturalized in the coastal districts of northern and eastern Australia. It is most common in the coastal parts of south-eastern Queensland and north-eastern New South Wales.it is regarded as a significant environmental weed in Bangladesh, and a minor or potential environmental weed in New South Wales and Western Australia as well. Its whole plant and leaves are used to cure hair disease, jaundice, fevers, astringent, hemorrhages, toothache, asthma, bronchitis. (Stuartxchange.org, 2015)

1.6.12 Synonyms of *Wedelia trilobata*

Complaya trilobata (L.) Strother, Silphium trilobatum L., Thelechitonia trilobata (L.) H.Rob. & Cuatrec., Wedelia carnosa Rich. Wedelia paludosa DC. (Floridata) (Stuartxchange.org, 2015)

1.6.13 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.(Weeds of Australia, 2011)

1.6.13 Distinguishing Features:

1. A mat-forming groundcover, or occasionally a low-climbing plant, with hairy stems.

2. Its paired leaves are often three-lobed and have toothed margins.

3. These leaves are glossy in appearance and mostly hairless.

4. Its bright yellow daisy-like 'flowers' (20-30 mm across) are borne singly on stalks 3-15 cm long.

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

1.6.14 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. (Weeds of Australia, 2011)

1.6.16 Morphology of *wedelia trilobata:*

1.6.16.1 Stems and Leaves:

The stems are rounded, green or reddish in colour, and may be somewhat hairy (i.e. strigose or hirsute) to almost hairless (i.e. sub-glabrous). They grow up to 2 m long and regularly develop roots (i.e. adventitious roots) at their joints (i.e. nodes). Short, semi-upright (i.e. ascending), flowering branches are produced off these creeping (i.e. prostrate) stems. (Weeds of Australia, 2011)

The oppositely arranged leaves are stalkless (i.e. sessile) or borne on short stalks (i.e. petioles). These leaves (40-180 mm long and 15-80 mm wide) usually have three lobes and irregularly toothed (i.e. serrated) margins. They are glossy in appearance, mostly hairless (i.e. glabrous), and slightly fleshy (i.e. succulent) in nature. (Weeds of Australia, 2011)

Figure 1.3: Stems and leaves of *Wedelia trilobata*

1.6.16.2 Flowers and Fruit:

The bright yellow to orange-yellow flower-heads (i.e. capitula) are daisy-like in appearance and borne singly on upright stalks (i.e. erect peduncles) 3-15 cm long. Each flower-head (20-35 mm across) has 8-13 yellowish 'petals' (i.e. ray florets) that are 6-15 mm long with finely toothed tips. In the centre of these flower-heads there are numerous tiny yellow tubular flowers (i.e. tubular florets) 4-5 mm long. The base of each flower-head (i.e. capitulum) is enclosed in a row (i.e. involucre) of narrow (i.e. lanceolate) green bracts (about 1 cm long). Flowering occurs throughout the year, but is most common from spring through to autumn. (Weeds of Australia, 2011)

Figure 1.4: Flower of *Wedelia trilobata*

Figure 1.5: flower of *Wedelia trilobata*

1.6.16.3 Seeds:

The 'seeds' (i.e. achenes), when present, are 4-5 mm long and topped with a crown (i.e. pappus) of short fringed scales. They are elongated in shape, brown in color and have a rough (i.e. tuberculate) surface texture. However, very few seeds reach maturity in cultivated or naturalized. (Weeds of Australia, 2011)

Figure 1.6: Seeds of *Wedelia trilobata*

1.6.16.4 Roots:

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. This mat-forming (i.e. stoloniferous) plant often creates a dense ground cover (usually 15-30 cm tall but occasionally up to 70 cm tall) that crowds out the growth of other species. It may also climb a short distance up trees or over other vegetation. (Weeds of Australia, 2011)

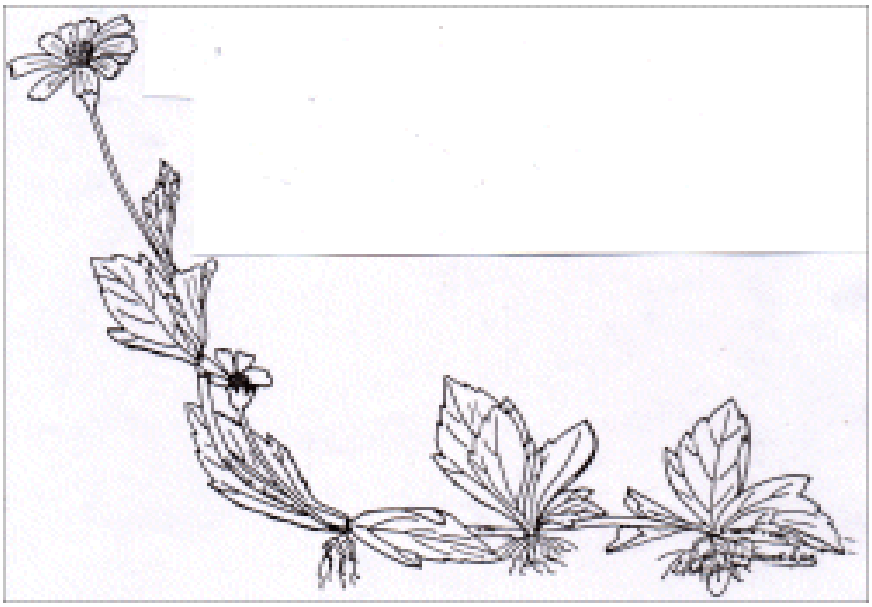

Figure 1.7: Roots of *Wedelia trilobata*

1.6.16.5 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 vegetative 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)

1.6.17 Local uses:

• After childbirth women drink a tea of W. trilobata, venvenn kawayib, to contract the uterus and stop hemorrhage.

• Chouvalyé wonzé (Portulaca pilosa) is sometimes added to it in making the tea.

• As a tisane, twef (Aristolochia constricta), go ponpon (Leonotis nepetaefolia) and hog plum bark (Spondias purpurea) are added to it. Also as a tisane, this plant is used for

cooling, sometimes with venvenn lache wat (Stachtarpheta spp.) and for inflammation when blood is passed.

• 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 (Issg.org, 2015)

1.6.18 Ethno pharmacological uses:

The aerial parts of this plant are used in traditional medicine in the Caribbean and Central America against bronchitis, colds, abdominal pains, dysmenorrheal, and even as a fertility enhancer. In folk medicine, it is employed to treat backache, muscle cramps, rheumatism, stubborn wounds, sores and swellings, and arthritic painful joints. The Miskito Indians of eastern Nicaragua use leaves for treatment of kidney dysfunction, cold, stingray wounds, snakebite, purge and amenorrhea. Coe and Anderson (1996) reported that fruits, leaves and stem are used in childbirth and in the treatment of bites and stings, fever and infection. W. trilobata, was utilized in Hong Kong as a substitute for W. chinensis, a traditional Chinese medicine used for the treatment of the common cold, hepatitis, indigestion and infections. In Trinidad and Tobago, used for reproductive problems, amenorrhea, dysmenorrheal. It is used for the treatment of fever and malaria in Vietnam .Unpublished reports indicate that aqueous infusion has been employed locally and empirically in Southern part of Brazil in the management of diabetes. In fact, it is popularly referred to as insulina due to its observed antidiabetic properties. Flowers and leaf part of the plant were used in the ladies for the purpose of amenorrhea, childbirth, abortion and to clear the placenta after birth. The literature review reveals that the fresh entire plant is used as molluscicidal activity, antibacterial and antimycobacterial activity. Suriname's traditional medicine uses the stem, leaves, and flower boiled in water for hepatitis, indigestion due to sluggish liver, white stools, burning in the urine and stopping of urine and for infections. Boiled fresh stems and leaves were used for bathe those suffering from backache, muscle cramps, rheumatism, or swellings. Used for painful joints of arthritis, fresh leaves and stems are mashed and spread on a cloth and applied to area, wrapped securely with a warm covering in South America. Wedelia species is used in lower Thailand for headache and fever (Issg.org, 2015)

1.6.19 General impacts:

If *wedelia trilobata* becomes established in plantations, it will compete with crops for nutrients, light and water, and reduce crop yields. It rapidly escapes from gardens to roadsides and plantations, where it can overgrow plants and develop into a thick cover .Forms a dense ground cover, crowding out or preventing regeneration of other species. (Issg.org, 2015)

Chapter Two

LITERATURE REVIEW

2.1 Phytochemical constituents:

The plant, *Wedelia trilobata* L. was analyzed in a research for its chemical composition. Chemical analysis showed that the plant is rich in nutrients, especially antioxidant compounds such as total phenol, vitamin C, grandiflorenic acid and β-carotene. Phytochemical screening showed that the methanolic extract contains the bioactive constituents such as tannins, saponin, alkaloids, essential oils, flavonoids, tannins, terpenoids, and phenolic compounds. The flower-heads contained essential oil as phellandrene; limonene; terpinene; trans-caryophyllene and pinene.The aerial parts contained two eudesmanolide sesquiterpene lactones and an ent-kaurenic derivative as presented below sesquiterpene lacton, deterpene, from the leaves of this plant we isolated friedelan-3-ol-amyrine acetate and 3-tigloyloxykaur-16-en-19-oic acid.

Studies on phytochemical constituents, quantification of total phenol, alkaloid content and In-vitro anti-oxidant activity of *Wedelia trilobata.*

In a study extracted dried plant of *Wedelia trilobata* in petether, chloroform, ethyl acetate, and methanol was collected and these extracts were checked for their phytochemical constituents. The whole plant of *Wedelia trilobata* revealed the presence of steroids, triterpenoids, glycosides, saponins, tannins, alkaloids, saponins, phenols and carbohydrates. (Khatun et al., 2012)

2.2 Pharmacological Activities:

Pharmacological reports revealed that this plant has antioxidant, analgesic, anti-inflammatory, antimicrobial, wound healing, larvicidal, trypanocidal, uterinecontraction, 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. (Khatun et al., 2012)

2.2.1 Antidiabetic Activity:

Male albino rats with diabetes induced by the administration of streptozotocin(45 mg/kg, i.v.) were treated with oral administration of *W. trilobata* (50 mg/kg).It was found to reduce blood glucose levels and improved weight gained which was accompanied by a marked restoration of decreased vitamin C and reduced glutathione in liver and kidney tissues of STZ-treated rats. In vitro data revealed that *W. trilobata* caused an inhibition of lipid peroxidation under Fe2+ or sodium nitro prusside assaults. Conversely, *W. trilobata* also caused a reduction in the high.

Levels of thiobarbituric acid reactive substances (TBARS) observed in the liver, kidney, and testes as well as high serum triglyceride, ALT and AST of diabetic rats. (Rungprom et al., 2010) demonstrated that the methanolic extract of *W. trilobata* was found to be the potent alpha-glucosidase inhibitor comparable to the authentic drug, Acarbose® (Khatun et al., 2012)

2.2.2 Central nervous system (CNS) depressantactivity

The petroleum ether, chloroform, ethyl acetate and methanol extract of leaves of *W. trilobata* (30mg/kg, i.p.) were evaluated for CNS depressant activity using pentobarbitone induced sleeping time, and locomotor activity in mice. The petroleum ether extracts potentiated pentobarbitone sodium induced sleeping time in mice than other extracts. Theanimal treated with petroleum ether extract showed reduction in the locomotor activity scores was significantly higher than that of standard drug diazepam and other extract. The petroleum ether extract represented good CNS depressant activity. (Toppo et al., 2012)

2.2.3 Antileishmaniasis activity

Kaurenic acid (ent-kaur-16-in-19-oic), isolated from the Venezuelan plant *W. trilobata* was evaluated on Leishmania (V) braziliensis both in vivo and in vitro. The compound had a lethal effect on axenic amastigotes and promastigotes with LD_{50} of 0.25 and 0.78 g/ml, respectively, in 24 h. additionally, a 70% reduction was observed in the size of the skin lesions in Balb/c mice with no evident toxic effect. The results indicated that this compound has a potent leishmanicidal effect on L. (V.) braziliensis. (Khatun et al., 2012)

2.2.4 Antimicrobial activity

A biological screening of activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi of crude extracts from *W. trilobata* (10 g/ml) was reported. Then-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 Shigellasonnei* (Gram-negative bacteria). The ethyl acetate extract was active only against *Salmonellagroup 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,Mucorsp., Trichophytonrubrum*).Ethanol extract of leaf, stem and flowerof W. trilobata(10 g/ml) was assessed forits antimicrobial efficacy using disc methodagainst different fungi (*A. flavus, A. niger,A. nidulans, A. flaviceps, Alternaria carthami, Aternariahe lianthi, Cercospora carthami, Fusarium solani, Fusarium oxysporum, Fusarium verticilloides and Nigro sporaoryzae*)and bacteria (*B. subtilis, Pseudomonas fluorescens, Clavi bacter Michigan ensissubsp. michiganensis, Xanthomonasoryzaepv oryzae, Xanthomonasaxanopodispv. malvacearumand* strains of S. *aureus, E. coli,P. aeruginosa and K. pneumoniae*). Theethanolic stem extract significantly inhibited the growth of almost all the bacteria isolates but did not show any significant effect onfungal isolates. The leaf extract showed morepotent against *P. aeruginosa, K. pneumoniae,P. fluorescens, X. oryzaepv. oryzae, X.axanopodispv.*

Malvacearum.W. trilobata flowers, leaves and stems were extracted with ten times of ethyl alcohol. The extract was then partitioned by N hexane, ethyl acetate, N-butyl alcohol and water to evaluate its antimicrobial activity. The result showed that most extracts had antimicrobial activities except the water extracts from flower. The ethyl acetate extract was the most effective among all the extracts.The methanolic flower extract of *W.trilobata* was screened for antibacterial activity by disc diffusion method against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus and Shigellaflexneri*. The extract showed a moderate inhibitory activity against all bacterial species with zones of inhibition of 10-16 mm in comparison with chloramphenicol which showed zones of inhibition 12-24 mm. (Khatun et al., 2012)

2.2.5Antioxidant activity

Ethanol extract of leaf, stem and flower(0.5 mg/ml) of *W. trilobata* was evaluated for its antioxidant activity by measuring the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the ferric reducing antioxidant power (FRAP)assay. It was observed that ethanol extract of the leaf of *W. trilobata* offered higher activity than that of stem and flower. At a concentration of 0.1 mg/ml, the scavenging activity of ethanol extract of the stem and flower and leaves reached 82.64, 55.41 and86.17% respectively but less than those of ascorbic acid (98%) and BHT (97.8%) at 0.1mg/ml, the study showed that the extracts have the proton donating ability and could serve as free radical inhibitors or scavenging, acting possibly as primary antioxidants. The FRAP values for the ethanol extract of leaf and stem were significantly lower than that of ascorbic acid but higher than that of BHT .The methanolic extract of *W. trilobata* flower showed good antioxidant activity (IC50 = 90 g/ml) in DPPH method. Reference standard ascorbic acid showedIC50 of 60 g/ml. The extract of *W. trilobata* flower exhibited higher ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity with IC50of 80 g/ml whereas gallic acid showedIC50 of 30 g/ml. (Toppo et al., 2012)

2.2.6 Anti-inflammatory activity

Ethanol extract of leaf, stem and flower (0.5 mg/ml) of *W. trilobata* was evaluated for its in vitro anti-inflammatory using albumin denaturation, membrane stabilization assay and proteinase inhibitory assay. All the three extracts were effective in inhibiting heat induced albumin denaturation. Maximum inhibition 87.14% was observed from leaf extract followed by stem (86.76%) and flower (61.63%). All the extracts were effective in inhibiting the heat induced hemolysis. The extracts inhibited the heat

Induced hemolysis of RBCs to varying degree. The maximum inhibitions 78.11%from the leaf extract followed by the stem (74.17%) and flower (58.74%).The *W. trilobata* ethanolic extract exhibited significant antiproteinase activity from different parts. The maximum inhibition was observed from leaf ethanolic extract (84.19%), in decreasing order was stem (81.84%) and flower ethanolic extract (67.17%).The ethyl alcohol and water extracts of *W. trilobata* flowers were used to treat RAW 264.7 macrophage, which induced inflammation by LPS. In the nitric oxide assay, the extracts of *W. trilobata* flower had better inhibitory ability against LPS induced inflammation. (Toppo et al., 2012)

2.2.7 Wound healing activity

An ethanolic extract of *W. trilobata* leaves was subjected to column chromatography. Hexane, ethyl acetate (WEA) and chloroform: methanol (50:50) (WCM) fractions was obtained. The fractions were tested using relevant in vitro wound healing assays. WEA (3 g/mL) promoted fibroblastL929 viability up to more than 90% before and more than 85% after hydrogen peroxide induced oxidative stress. WEA (3g/mL) induced a 70% migration rate in the in vitro scratch assay and the collagen content was increased to 261 g/mL compared to the control (57.5 g/mL) [47].The ent-kaura-9(11), 16-dien-19-oic acid isolated from *W. trilobata* leaves offered wound healing activity due to a combination of antimicrobial, stimulation of fibroblast growth and protection of the cells from hydrogen peroxide-induced injury, all of which could play some role in its effect on tissue repair. It showed promising antibacterial activity with MIC value of15.62 g/mL against S. aureus

and 7.81g/mL against S. epidermidis. Theent-kaura-9(11), 16-dien-19-oic acid (2.5-0.08 g/mL) produced an increase in the percentage viability of mouse fibroblastL929 cells from 97-117% and protection of the fibroblast L929 cells against oxidative stress induced by hydrogen peroxide(94-80%)..(Keerthiga et al., 2012)

2.2.8 Cytotoxic activity

In transient transfection assay the N-hexane and ethyl alcohol extracts of *W. trilobata* flower could activate PPAR. In MTT assay of SK-Hep-1, extract of *W. trilobata* flower had the best inhibitory ability. The ethyl alcohol extracts of *W. trilobata* had the best ability to diminish the expression of matrix metalloproteinase (MMP)-9 and MMP-2. The ethyl alcohol extracts of flower had good anti-migration and anti-invasion ability especially on 80 g/mL dose. .(Keerthiga et al., 2012)

2.2.9 Analgesic activity

Comparative study in mice on the analgesic activity of the ethanol extracts of *W. trilobata,* W. bi flora and E. Alba was evaluated by acetic acid induced writhing method. It was found that *W. trilobata* extract showed dose-dependent blocking of writhing response. Dose of 500 mg/kg of *W. trilobata* extract and aspirin (500 mg/kg) block the writhing response by 49.17%and 68.68%.Kaurenoic acid (10 mg/kg) obtained from *Wedelia trilobata* kaurenoic acid inhibited overt nociception like behavior induced by phenyl-p-benzoquinone, complete Freund's adjuvant (CFA) and formalin. Kaurenoic acid (1-10 mg/kg p.o.) also inhibited acute carrageenin and PGE2 induced and chronic CFA induced mechanical hyperalgesia. . (Keerthiga et al., 2012)

2.2.10 In the treatment of reproductive problems

Previous research has shown that Caribbean women and Creoles have always used bitter herbs to control their fertility. A study was conducted focused on the plants used for reproductive purposes in

Chiang Mai J. Sci. 2014; 41(3) 601Trinidad and Tobago. The plants used to address women's reproductive problems were used mainly for infertility, menstrual pain and childbirth. Results showed that *W. trilobata* was used for menstrual pain and for the female complaints. The non-experimental validation method can be used to advise the public on which plants are safe, effective and useful, and which are not; pending clinical trials. This is important since few clinical trials were conducted on Caribbean plants. .(Keerthiga et al., 2012).

2.3 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*, *Staphylococcus aureus*, (MTCC- 7443), *Salmonella typhi*, *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)

2.4 Wound-healing potential of gradif lorenia acid from wedelia trilobata (l) leaves ,thailand

The ethyl acetate fraction from ethanolic extract of *Wedelia trilobata* leaves displayed wound healing properties. The ethyl acetate fraction was further subjected to bioassay-guided fractionation which afforded isolation of grand iflorenic acid which requires urther investigation to prove its wound healing potential. The grand iflorenic acid from leaves of *Wedelia 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. Grand iflorenicacid (2.5µg/mL) produced percentage viability of B J human fibroblast, and HaCaTkeratinocytes116, and 106% respectively. Grand iflorenic acid (2.5µg/mL) induced a 100% migration rate in the in vitro scratch assay and the collagen content was increased to171.2µg/mL compared to the control (61.1µg/mL) with BJ human fibroblast. Grand iflorenic 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 grand if lorenic acid has potential wound healing activity due to combination of fibroblast stimulation and inhibiting prolonging inflammatory phase of wound healing evident by

reduced levels of inflammatory cytokines from macrophage raw 264.7cells. (Balekar et al., 2013)

2.5 In vitro propagation of wedelia trilobata (l) using phormidium sub incrustatum extracts: a nobel approach, south india

Most micro propagation 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 sub-incrustatum 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% cyano bacterial 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 cyano bacterial 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)

2.6 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)

2.7 Antimicrobial, antioxidant and in vitro anti-inflammatory activity and phytochemical screening of water extract of wedelia trilobata (l) hit

Antimicrobial, antioxidant and in vitro anti-inflammatory activity and phytochemical screening of water extract of *Wedelia trilobata* Hitchc of 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)

Acclimation of photosystem II to high temperature in two Wedelia species from different geographical origins: implications for biological invasions upon global warming of 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 (Pn). High temperature caused a partial inhibition of PSII, but the adverse effect was more severe in W. chinensis. Measurement of the minimum fluorescence (Fo) versus temperature curves showed that *W. trilobata* had a higher inflexion temperature of Fo (Ti), indicating greater thermostability of the photosynthetic apparatus. Moreover, comparisons of absorbed light energy partitioning revealed that *W. trilobata* increased xanthophyll-dependent thermal dissipation (ΦNPQ) under high temperature, while retaining the higher fraction of absorbed light allocated to photochemistry (ΦPSII) 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)

2.8 Discussion

The present review emphasizes the phytochemical, traditional, pharmacological and, clinical reports on *W. trilobata.* Tannin, saponins, flavonoids, phenolic, terpenoids constitute major classes of phyto constituents of this plant. The plant contains a range of phytochemical substances credited with various pharmacological properties. Recent research carried out indicates its uses such as antioxidant, anti-inflammatory, antimicrobial, wound healing, antidiabetic activity. In recent years, the search for phytochemicals possessing antioxidant, antimicrobial and anti-inflammatory properties have been on the rise due to their potential use in the therapy of various chronic and infectious diseases. Epidemiology and experimental studies have implicated oxidative cellular damage arising from an imbalance between free radical generating and scavenging systems as the primary cause of cardio-vascular, diseases, cancer, aging etc. Due to risk of adverse effects encountered with the use of synthetic antibiotics, medicinal plants may offer an alternative source for antimicrobial agent with significant activity against pathogenic and infective microorganisms. In addition, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance (Chiang Mai J. Sci. 2014) genes. Strong antioxidants, antimicrobial and anti-inflammatory activities specifically in the ethanolic leaf and stem extract of *W. Trilobata* were observed. These activities may be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids, phenols and saponins . The authors are involved in evaluating the wound healing effect of W. trilobata with a view to isolating bioactive phytoconstituent(s). One of the phytoconstituent isolated and evaluated for wound healing potential is grand iflorenic acid .The presence of a wide range of chemical compounds indicates that the plant could lead the way for the

development of novel agents having good biological activity. Exploration of the chemical compounds of the plant will provide the basis for developing such a lead. The phyto medicines can be developed as an alternative and are relatively inexpensive than modern drugs. One of the reasons is their use, preparation, and safety is already understood in traditional systems of medicines. Many chemical compounds are present in the plant but isolation of active constituents can be carried out using different extraction methods such as microwave extraction, isolation and by using various appropriate chromatographic techniques. Despite a long tradition of use of *W. trilobata* for treatment of various ailments, it still remains unexplored pharmacologically to prove its traditional claims. Thus it can be considered as a valuable plant in both traditional and modern drug development areas for its versatile medicinal uses. Emphasis must be laid on the pharmacological activity of the phyto constituent to unravel the hidden medicinal qualities of this plant as well as the local knowledge system should be globalize which would increase the benefits obtained to wider population. There are no clinical data available that would provide evidence of efficacy of *W. trilobata* in humans. Extracts and constituents of *W. trilobata* may have considerable clinical potential in humans and need to be studied further in in vivo models and ultimately in clinical studies.

Table 2.1 Biological and pharmacological activities (in vitro and in vivo) of *W. trilobata*.

Extract/compound	Pharmacological activity
n-hexane extract of aerial part without flower	ANTIBACTERIAL ACTIVITY Inhibitory effect on Gram positive bacteria, *Bacillus cereus, Bacillus subtilis, Mycobacterium megmatis, Staphylococcus aureus, Staphylococcus epidermidis and Gram negative bacteria, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella group C, Salmonella paratyphi, Shigellasonnei*
Kaurenoic acid (ent-kaur-16-in-19-oic), isolated from the Venezuelan plant *W. trilobata*	ANTILEISHMANIASIS ACTIVITY Potent leishmanicidal effect on L. (V.) braziliensis
Ethyl acetate extract of aerial part without flower	Inhibitory effect on Gram negative bacteria, Salmonella group C
Aqueous extract of leaves	ANTIDIABETIC ACTIVITY Reduction in blood glucose level in streptozotocin induced diabetes
Methanolic extract of aerial parts	α-glucosidase inhibitor
Ethanol extract of leaves	ANTIBACTERIAL ACTIVITY Strong inhibitory effect on *P. aeruginosa, K. pneumoniae, P. fluorescens, X. oryzaepv. oryzae, X. axanopodispv. malvacearum, moderately inhibited the E.coli, C. michiganensissub sp. michiganensisbut less activity was observed on S. aureus.*

Ethanol extract of stem	ANTIBACTERIAL ACTIVITY Inhibitory effect *on Bacillus subtilis, Pseudomonas fluorescens, Clavibactermichiganensissub sp. michiganensis, Xanthomonasoryzaepv. oryzae, Xanthomonasaxanopodispv. malvacearumand strains of Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumonia*
Ethanol extract of flower	Strong inhibitory effect on *Staphylococcus aureus, X. oryzaepv. oryzaemoderately inhibited the K.pneumoniae, P. fluorescens, X. axanopodispv. malvacearumbut less activity was observed on E.coli, P. aeruginosa, Clavibactermichiganensis sub sp. Michiganensis*
Methanol extract of flower	Moderate inhibitory activity against Bacillus cereus, *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus and Shigellaflexneri*
The petroleum ether, chloroform, ethyl acetate and methanol extract of leaves	CNS DEPRESSANT ACTIVITY The petroleum ether extract represented good CNS depressant activity
Ethanol extract of stem, leaves and flower	ANTIFUNGAL ACTIVITY Weak inhibition against *Aspergillus flavus A. niger, A. nidulans, A. Flaviceps, Fusariumsolani, F. oxysporum, F. verticilloides*
Ethanol extracts	ANALGESIC ACTIVITY Blocked the writhing response by 49.17%

Kaurenoic acid, a diterpene obtained from *Wedelia trilobata*	Exhibited analgesic effect by inhibiting cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP sensitive potassium channel signaling pathway.
Ethanol extract of leaf, stem and flower	ANTIOXIDANT ACTIVITY DPPH radical scavenging activity was more for leaves than stem and flower
n-hexane and ethyl alcohol extracts	CYTOTOXIC ACTIVITY The ethyl alcohol extracts of flower had good anti-migration and anti-invasion ability especially on 80 μg/mL dose
Ethyl acetate fraction of *Wedelia trilobata*	DPPH radical scavenging activity
Ethyl acetate (WEA) and The WEA chloroform: methanol (50:50) WCM) fractions from ethanolic extract of *W. trilobata* leaves	WOUND HEALING ACTIVITY The WEA displayed antibacterial and fibroblast stimulatory activities while WCM exhibited antioxidant activity
ent-kaura-9(11), 16-dien-19-oic acid isolated from *W. trilobata* leaves	Offered wound healing activity due to a combination of antimicrobial, stimulation of fibroblast growth
Methanol extract of flower	DPPH radical scavenging activity

Ethanol extract of leaf,	ANTIINFLAMMATORY ACTIVITY All the three extract was effective in inhibiting heat 27 Stem and flower induced albumin denaturation. Maximum inhibition 87.14% was observed from leaf extract followed by Stem (86.76%) and flower (61.63%).
	The extracts inhibited the heat induced hemolysis of 27 RBCs to varying degree. The maximum inhibitions 78.11% from leaf extract followed by stem (74.17%) and flower (58.74%).
	The ethanolic extract exhibited significant 27 antiproteinase activity from different parts. The maximum inhibition was observed from leaf ethanolic extract (84.19%), in decreasing order was stem (81.84%) and flower ethanolic extract (67.17%).

Chapter Three

METHOD AND MATERIALS

3.1 Collection and preparation of plant material

Plant sample (leaves) of Wedelia trilobata was collected from Hatirjheel, Aftabnagar. 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.

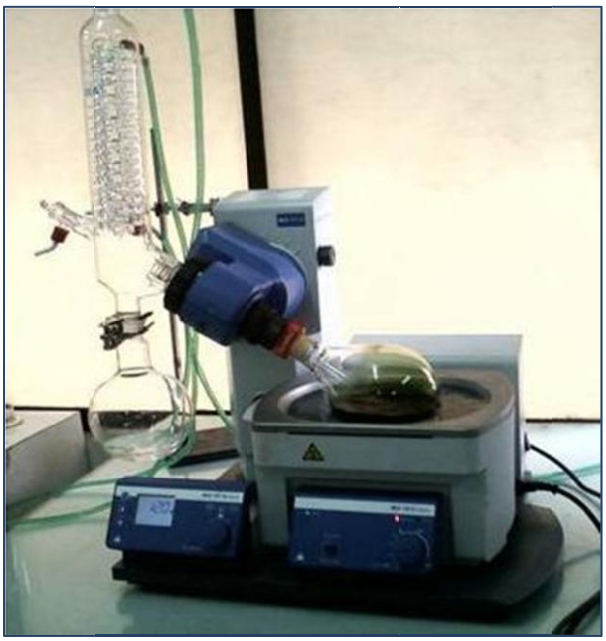

Figure 3.1: Drying of extract using rotary evaporator

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 four solvents of different polarity.

3.4.1 Partition with petroleum ether

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

3.4.2 Partition with chloroform

To the mother solution that was left after partitioning with petroleum ether 16ml 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 left after washing with petroleum ether and $CHCl_3$, was then taken in a separating funnel and extracted with Ethyl acetate (100ml × 3). The Ethyl acetate soluble fractions were collected together and air dried.

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3.4.4 Collection of ethyl acetate fraction

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

3.5 Antioxidant Activity

3.5.1. 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. Quercetin at various concentrations was used as standard (Chang, 2002).

Flavonoid (Extract) + $AlCl_3$ (reagent)= Formation of flavonoid-aluminium complex (λ_{max}510nm)

3.5.2 Apparatus and reagents

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

3.5.3 Procedure

Aluminium chloride (10%) solution preparation

10mg of aluminium chloride ($AlCl_3$) 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.

$NaNO_2$ (5%) solution preparation

5mg of sodium nitrite $NaNO_2$ 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.

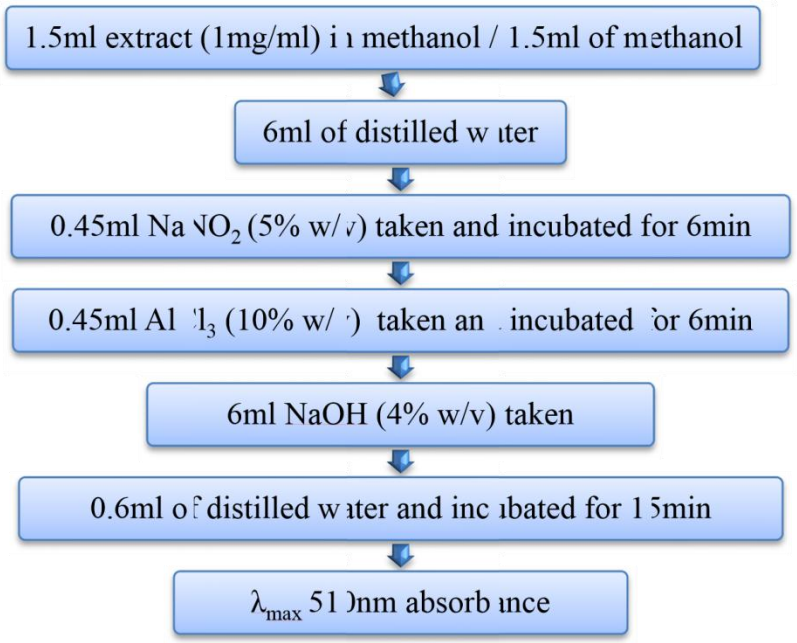

Figure 3.2: Schematic diagram of flavonoid content test

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- *Artemiasalina*) 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

- *Artemiasalina* leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- 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 *Artemiasalina* hatching. 12 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 *Artemiasalina* Leach were 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 *Artemiasalina* 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 apipette and then added to each of the test tubes containing 5ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay.

Fig 3.3: *Artemiasalina* 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 test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tubeand fresh 100µl DMSO was added to vial. Thus the

concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

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/ml. Then ten living brine shrimp nauplii in 5ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

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 mortalityrate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

3.6.3.5 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	➢Screw cap test tubes
➢Petri dishes	➢Nose mask and Hand gloves

➢Inoculating loop	➢Laminar air flow hood
➢Sterile cotton	➢Autoclave
➢Sterile forceps	➢Incubator
➢Spirit burner	➢Ethanol
➢Micropipette	➢Nutrient Agar Medium

3.7.2.2 Test sample of *Wedelia trilobata*

Ethyl acetate 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.

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.

Fig 3.4: 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°C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

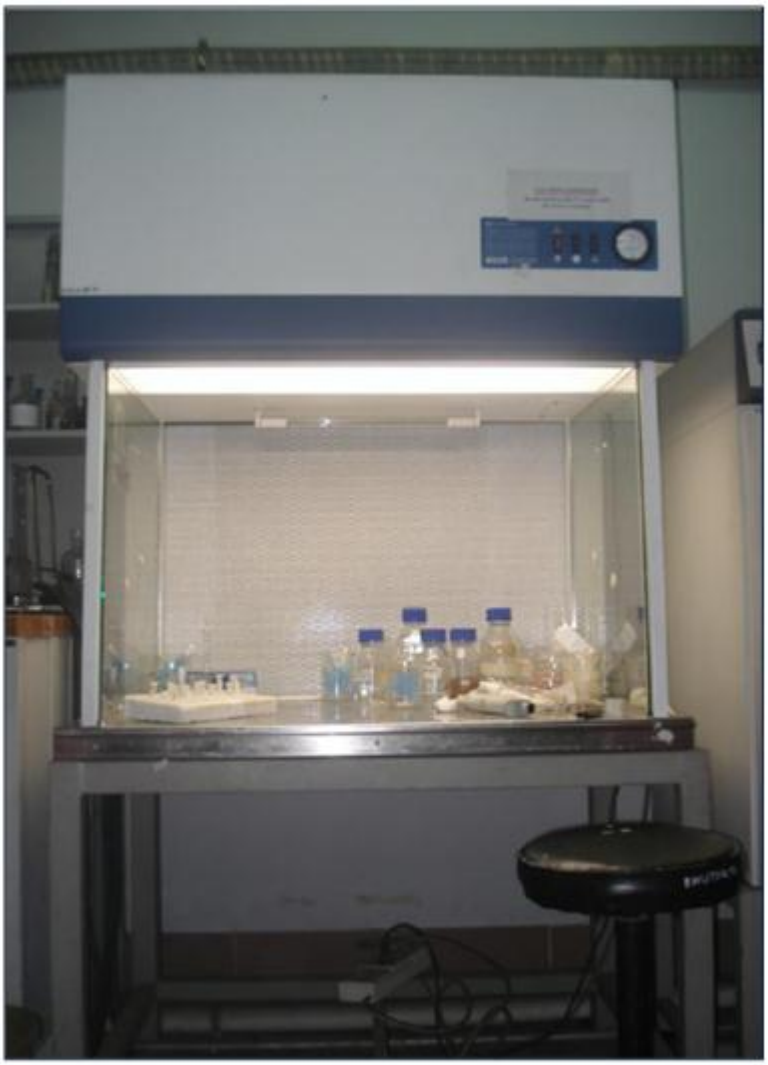

Fig 3.5: Laminar Hood

3.7.3.3 Preparation of test plate

The test organisms were transferred from the subculture to petri dish 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 petri dish with the help of this cotton bud.

3.7.3.4 Preparation of discs

Three types of discs were used for antimicrobial screening.

- **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.
- **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.
- **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 petri dish 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.

Fig 3.6: 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

RESULT AND 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 ethyl acetate extract of *Wedilia trilobata* (leaves) was determined by following methods:

Determination of total flavonoids content

4.1.1 Total flavonoid content result

The ethyl acetate 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 value	R2 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.

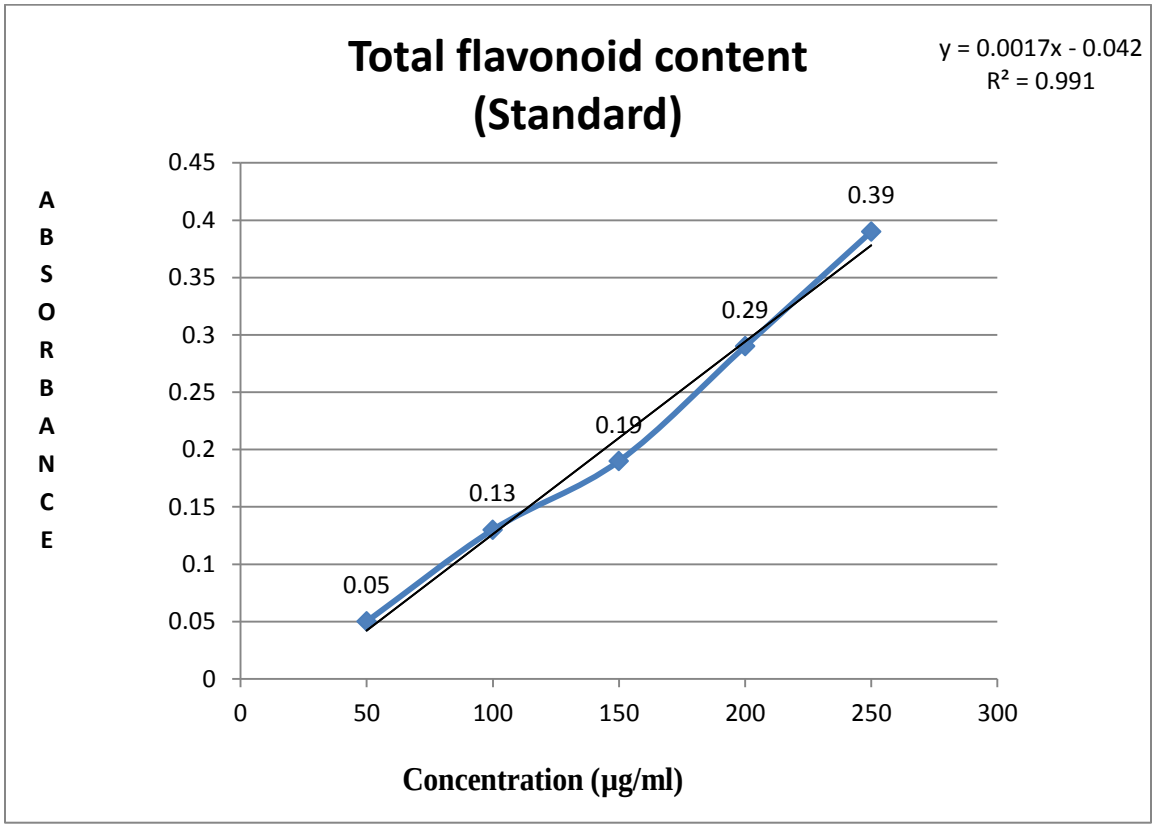

Figure 4.1: Graphical representation of assay of flavonoid content of ascorbic acid

4.1.1.2 Total flavonoid content present in ethyl acetate extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract s calculated and is given in Table 4.2.

Table 4.2: Total flavonoid content of ethyl acetate fraction of leaves of *Wedelia trilobata*

Sample	Concentration (mg/ml)	Absorbance	Total flavonoid content(mg of AAE/g of dried extract)
Ethyl acetate fraction of *Wedelia trilobata*	1	0.347	228.824

4.1.1.3 Discussion

To determine the total flavonoid content of the test samples the standard curve was used. In1mg/ml concentration of ethyl acetate fraction of *Wedelia trilobata* (leaves) 228.824 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 ethyl acetate extract of *Wedelia trilobata* leaves were subjected to brine shrimp lethality bioassay (Meyer et al., 1982). After 24hrs, the test tubes were inspected using a magnifying glass and the number of survivors counted. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a median Lethal Concentration (LC_{50}) value. This represents the concentration of the standard or ethyl acetate extract that produces death in half of the test subjects after a certain period. The percentage mortality at each concentration was determined using the following formula:

% mortality = (number of dead nauplii / total number) × 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	LC50 (µg/ml)
1	400	2.602	0	10	100	
2	200	2.301	1	9	90	
3	100	2.000	2	8	80	
4	50	1.699	3	7	70	
5	25	1.398	5	5	50	13.38
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	

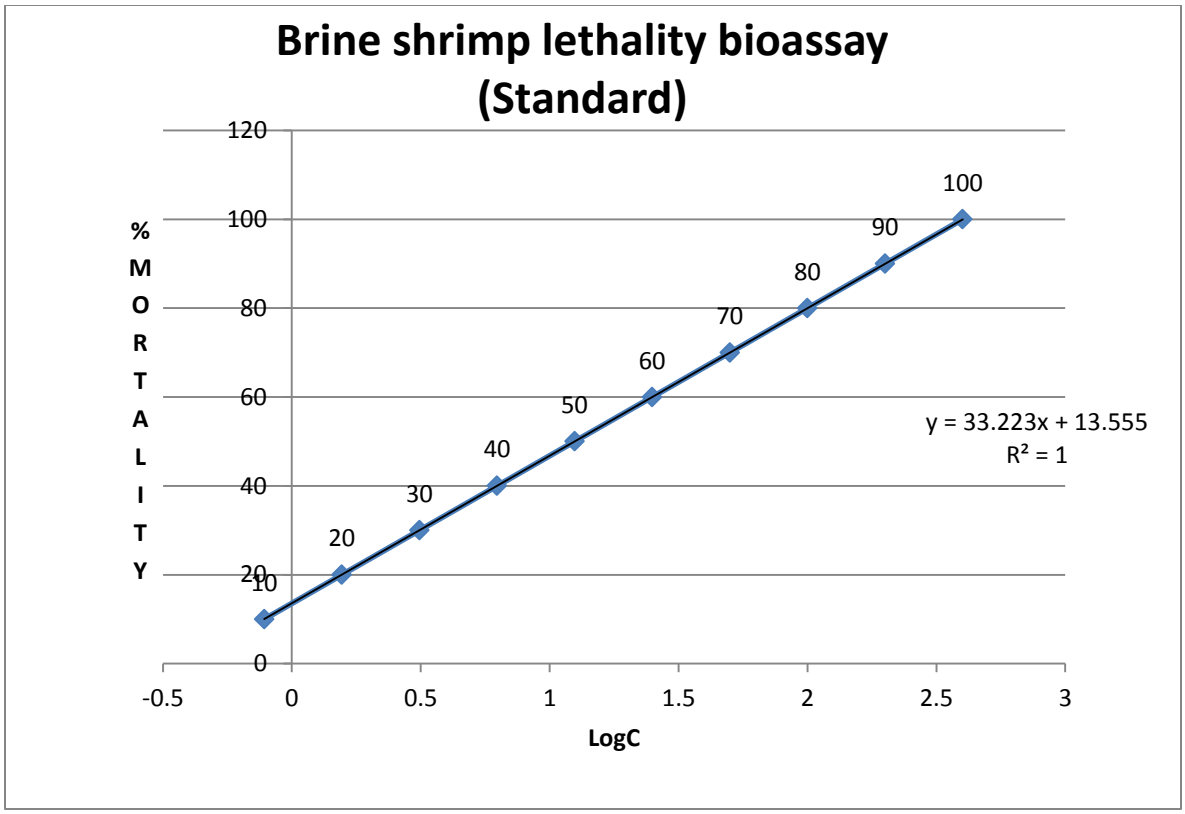

Figure 4.2: Plot of % mortality and predicted regression line of Tamoxifen (standard)

Table 4.4: Results of the bioassay of ethyl acetate (extract)

Test tube No.	Concentration (µg/ml)	LogC	No. of Nauplii alive	No. of Nauplii Dead	% mortality	LC_{50} (µg/ml)
1	400	2.602	2	8	80	
2	200	2.301	3	7	70	
3	100	2.000	4	6	60	
4	50	1.699	4	6	60	
5	25	1.398	5	5	50	27.17
6	12.5	1.097	5	5	50	
7	6.25	0.796	6	4	40	
8	3.125	0.495	8	2	20	
9	1.5625	0.194	8	2	20	
10	0.78125	-0.107	10	0	0	

4.2.2 Preparation of ethyl acetate extract fraction curve

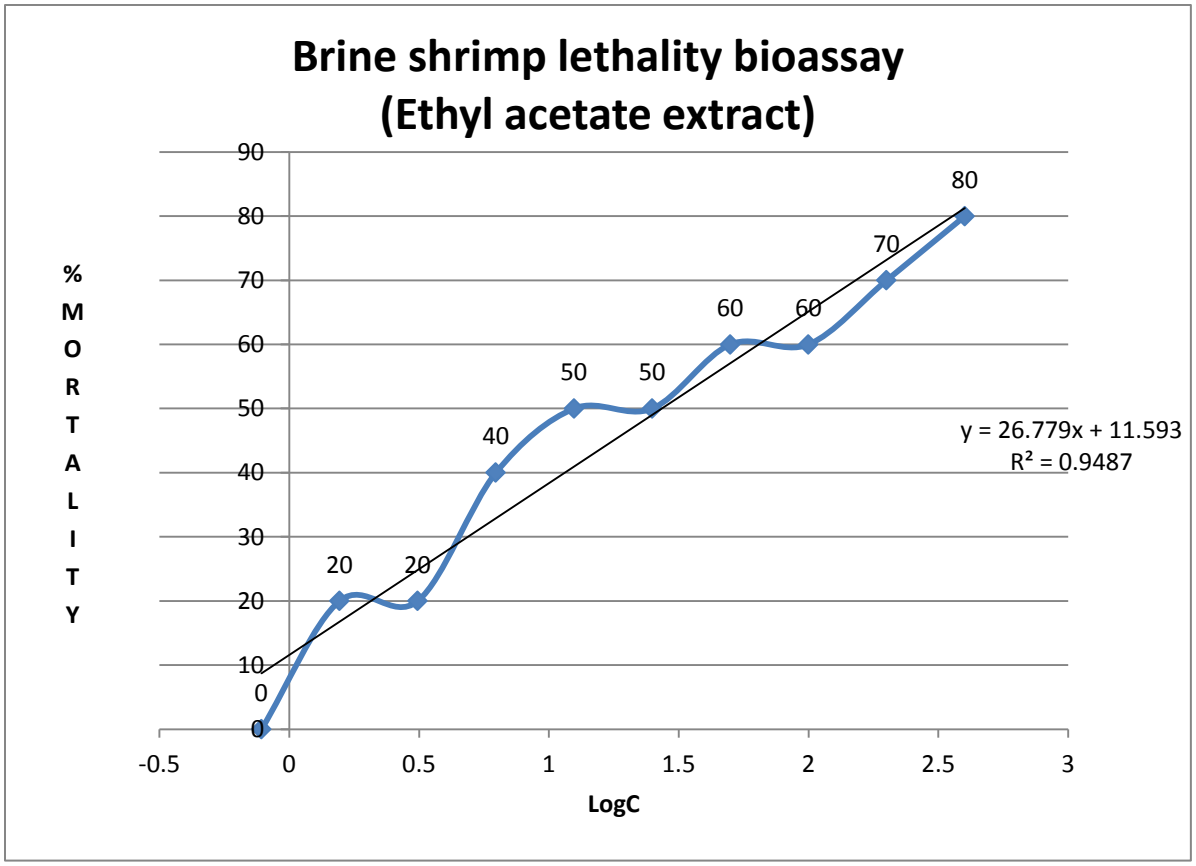

Figure 4.3: Plot of % mortality and predicted regression line of ethyl acetate extract

4.2.3 Discussion

In Brine Shrimp Lethality bioassay, varying degree of lethality was observed with exposure to different concentrations of the test samples. The degree of lethality was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and ethyl acetate fraction samples. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400µg/ml, whereas the least mortalities at lowest concentration 0.78125µg/ml as shown in Table 4.1and Table 4.2

Table 4.5: Cytotoxic activity of Tamoxifen and ethyl acetate fraction of *Wedelia trilobata* leaves

Sample	Linear regression	R2 value	LC50 (µg/ml, 24hr)
Standard (Tamoxifen)	y = 33.021x + 12.806	0.9891	13.38
Extract (Ethyl acetate)	y = 26.77x + 11.59	0.948	27.17

In this investigation, standard and ethyl acetate fraction exhibited cytotoxic activities with the LC_{50} values **13.38** and **27.17** micro gram / ml respectively as shown in table 4.5. for both standard and ethyl acetate fraction the R value is closer to one which indicates that the extract has potent activity against brine shrimp nauplii comparable to the standard.

4.3 Antimicrobial test results

The antimicrobial activities of ethyl acetate extract of *Wedelia trilobata* leaves were examined in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The ethyl acetate 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 ethyl acetate extract

Table 4.6: Antimicrobial activity of standard sample (Ciprofloxacin) and ethyl acetate extract.

Type of microorganism		**Zone of inhibition (mm)**	
		Standard sample	**Ethyl acetate fraction**
Gram positive bacteria	*Bacillus cereus*	38	10
	Bacillus magaterium	38	9
	Bacillus subtilis	40	9
	Staphylococcus aureus	40	8
	Sarcina lutca	37	6
	Salmonella paratyphi	38	8

Gram negative bacteria control	*Salmonella typhi*	36	8
	Vibrio parahemolyticus	40	8
	Vibrio mimicus	35	8
	Pseudomonas aureginosa	38	0
	Shegella Dysenteriae	38	7
	Escherisia coli	36	6
Fungi	*Saccharomyces cerevisiae*	35	6
	Candida bicans	26	9
	Aspergillus niger	30	10

4.3.2 Discussion

Ethyl acetate extract of *Wedilia trilobata* showed moderate to low antimicrobial activity when compared to ciprofloxacin. None of the zone of inhibition of ethyl acetate fraction is equal to ciprofloxacin against any bacteria or fungi. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against *Candida bicans* comparable to the standard (30mm).

Chapter Five

CONCLUSION

Conclusion

The results obtained in this study indicate that the ethyl acetate 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 ethyl acetate extract proved that the leaves contain flavonoid compounds. To determine the total flavonoid content of the test samples the standard curve was used. In1mg/ml concentration of ethyl acetate fraction of *Wedelia trilobata* (leaves) 212mg of AAE/gm of dried extract of flavonoid content was found. So this extract contains anti oxidative compounds. The ethyl acetate 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 (LC50) value. This represents the concentration of the standard or ethyl acetate extract that produces death in half of the test subjects after a certain period. The 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. In Brine Shrimp Lethality bioassay, varying degree of lethality was observed with exposure to different concentrations of the test samples. The degree of lethality was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration in both standard and ethyl acetate fraction samples. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400µg/ml, whereas the least mortalities at lowest concentration 0.78125µg/ml. In this investigation, standard and ethyl acetate fraction exhibited cytotoxic activities with the LC_{50} values 13.38 and 27.17 micro gram / ml respectively as shown in table 4.5. For both standard and ethyl acetate fraction the R value is closer to one which indicates that the extract has potent activity against brine shrimp nauplii comparable to the standard. The antimicrobial activities of ethyl acetate extract of

Wedelia trilobata leaves were examined in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The ethyl acetate fraction was subjected to the various bacterial and fungal cultures and from that zones of inhibition were measured. Ethyl acetate extract of *Wedilia trilobata* showed moderate to low antimicrobial activity when compared to ciprofloxacin. None of the zone of inhibition of ethyl acetate fraction is equal to ciprofloxacin against any bacteria or fungi. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against *Candida bicans* comparable to the standard (30mm).Since *Wedelia 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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