IN VITRO BIOLOGICAL INVESTIGATIONS OF CHLOROFORM EXTRACT OF *Wedelia trilobata* LEAVES

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

Submitted By

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DECLARATION BY THE CANDIDATE

I, Musarrat Sabnam, hereby declare that this dissertation, entitled **"*In vitro* biological investigations of chloroform 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 Nigar Sultana Tithi, Senior Lecturer Department of Pharmacy, East West University, Aftabnagar, 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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CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertation, entitled **"*In vitro* biological investigations of chloroform extract of *Wedelia trilobata* leaves"** is a bonafide research work done, under my guidance and supervision by Musarrat Sabnam (ID: 2011-1-70-011), in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled **"*In vitro* biological investigations of chloroform extract of *Wedelia trilobata* leaves"** is a bonafide research work done by Musarrat Sabnam (ID: 2011-1-70-011), in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.

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Thank you

Dedication

This Research Paper is dedicated to

My beloved parents and

My beloved teachers

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LIST OF ABBREVIATIONS

Abridged form	Full form
AAE	Ascorbic Acid Equivalent
AA	Ascorbic acid
DMSO	dimethyl sulfoxide
µg	microgram
µl	microgram
hr	hour
WTEt	*Wedelia trilobata* leaf extract
WT	*Wedelia trilobata*
IC50	The concentration of a drug which is required for 50% of inhibition of a specific test.
LC_{50}	The lethal concentration required to kill 50% of the sample population of a specific test.
nm	nanometer
mg	milligram
ml	milliliter
WHO	World Health Organization
STZ	Streptozotocin
UV	Ultraviolet
USDA	United States Department of Agriculture
ZOI	Zone of inhibition
E. coli	*Escherichia coli*
$CHCl_3$	Chloroform
E.g.	For example (exempli gratia)
i.v.	Intravenous
p.o	Per Oral
RGR	relative growth rate
g	gram
TBARS	thiobarbituric acid reactive substances
GSH	glutathione
FRAP	ferric reducing antioxidant power
DPPH	2,2-diphenyl-1-picrylhydrazyl
RBCs	Red blood cells
MMP	matrix metalloproteinase
CFA	complete Freund's adjuvant
BWE	biomass water extracts
MMC	minimum microbial concentration
MIC	minimum inhibitory concentration
ΦNPQ	xanthophyll-dependent thermal dissipation

ABSTRACT

The study was designed for pharmacological investigation of chloroform fraction of *Wedelia trilobata* leaves extract (Family: Asteraceae). The powdered leaves of *Wedelia trilobata* were extracted with methanol and then partitioned with petroleum ether, chloroform and ethyl acetate consecutively. The chloroform fraction was investigated for total flavonoid content, brine shrimp lethality bio-assay and antimicrobial test. The fraction contained 228.824mg AAE/g of total flavonoid content which shows that the fraction have prominent antioxidant property. Screening for cytotoxic properties using brine shrimp lethality bioassay with tamoxifen (LC_{50} value of 13.38µg/ml) as positive control showed that the fraction have considerable cytotoxic potency exhibiting LC_{50} value 27.17µg/ml. In antimicrobial activity investigation, the chloroform fraction showed low antibacterial and antifungal activity against the tested organisms compared to ciprofloxacin (30µg/disc) which was used as positive control. The chloroform fraction showed moderate cytotoxic activity, potent antioxidant activity and slight antimicrobial activity. Further investigations are needed for the proper identification and isolation of these bioactive compounds to produce safer drugs for treatment of harmful diseases.

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

Chapter One
Introduction

1.1 Medicinal Plants

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

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

1.1.1 Medicinal Plants as Drugs

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

According to the World Health Organization (WHO),

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

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

1.2 Medicinal Plants – History and Context

Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. Written evidence of herbal remedies dates 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 (Nunn, 2002; Robson *et. al.*, 2009, Hong, 2004; Ackerknecht, 1982).

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

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

1.2.1 Traditional Medicine in Bangladesh

Traditional medicines have existed in Bangladesh as an important basis of health care since olden times. Because of their potentialities and close association with the culture and tradition of the people, traditional systems of medicine have assumed a unique position in the health care of the people living in even the remotest areas of the country. Although the use of traditional medicine is so deeply rooted in the cultural heritage of Bangladesh the concept, practice, type and method of application of traditional medicine vary widely among the different ethnic groups. Traditional medical practice among the tribal people is

guided by their culture and life style and is mainly based on the use of plant and animal parts (Samy, et al., 2008).

1.2.2 Significances of Medicinal Plants to Mankind

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

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

1. Synergic medicine- The plants ingredients all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.
2. Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.

3. Preventive medicine- It has been proven that the component of the plants are also characterized 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 (Ghani, 1998).

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

1) They are used as sources of direct therapeutic agents.

2) They serve as raw materials base for elaboration of more complex semi synthetic chemical compounds.
3) The chemical structures derived from plant sources can be used as models for new synthetic compounds.
4) Finally plants can be used as taxonomic markers for the discovery of new compounds (Reddy, *et al.*2010).

1.2.3 Global scenario of Medicinal plants

According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Herbal medicine is a common element in Ayurvedic, homeopathic, naturopathic, traditional, and oriental, Native American & Indian medicine. Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. The present global herbal market is worth about US$ 62 billion per annum. The annual growth of herbal market is about 15 percent and the global herbal market by 2050 is expected to be about US$ 5 trillion (WHO, 2002).

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

1.3 Medicinal Plants in Bangladesh

Medicinal plants mainly used in the preparations of Unani and Ayurvedic medicine, also prescribed by practitioners of traditional medicine in different parts of the country and others are used as household remedies by the common people. South Asian countries have a large number of valuable medicinal plants naturally growing mostly in fragile ecosystems

that are predominantly inhabited by rural poor and indigenous community .In Bangladesh 5,000 species of angiosperm are reported to occur (IUCN, 2003). The number of medicinal plants included in the 'materia medica' of traditional medicine in this subcontinent at present stands at about 2,000.More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh Dhaka, Rajshahi , Shylet and Chittagong division is rich in medicinal plants.(Sadi,2012)

Table 1.1: Name and medicinal uses of some common plants in Bangladesh (Uddin, 2014)

Scientific Name: *Areca catechu* L. **Family:** Palmae **Name:** Supari (local name), Betel nut, Areca Nut (English name). **Plant part used:** Seeds. **Medicinal use:** abdominal pain, dyspepsia, Glaucoma, edema, antimicrobial, astringent.	
Scientific Name: *Dolichos lablab* L. **Family :** Leguminosae **Name**: Shim(local name) bonavista bean ,white hayacint bean ,baibiandou **Plant part used:** Seeds. **Medicinal use:** Malabsorption and diarrhea, nausea, vomiting, poor appetite leucorrhoea	
Scientific Name: *Eclipta prostate* L **Family :** compositae **Name:** Keshraj,bhimraj,kalokeshi(local)yerba de tajo. **Plant part used:** Whole plant **Medicinal use:** Uterine bleeding, premature graying of hair, tinea pedis	

Scientific Name: *Piper nigrum* L. **Family :** Piperaceae **Name**: Golmorich (local name), Black pepper(English name) **Plant part used:** Dried unripe fruit **Medicinal use:** Epilepsy, chronic bronchitis s, asthma, poor appetite.	
Scientific Name: *Thuju orientalis* L. **Family: Cupressaceae**. **Name:** Chinese arborvitae, oriental arborvitae, jhau (local name). **Plant part used:** Leaves, seeds. **Medicinal use:** GIT bleeding, alopecia, constipation, insomnia, chronic bronchitis.	
Scientific Name:.*Punica granatum* L **Family :**Punicaceae **Name:** Anar(local name), pomegranate(English name) **Plant part used:** Fruit rind **Medicinal use:** Massive uterine bleeding, dysentery, ascariasis, melena.	
Scientific Name: *Dillenia indica* L. **Family** :Dilleniaceae **Name**: Chalta (local name)elephant apple(English name) **Plant part used:** Fruit, leaves bark **Medicinal uses:** Astringent, expectorant, antifungal, antibacterial.	

Scientific Name:*Glinus oppositifollius* L. **Family :**Molluginaceae **Name:**Gima(local name),Jima(English Name) **Plant part used:** Whole plant **Medicinaluse:**CNS deppressent, diuretic,antiseptic.	
Scientific Name: *Aegle marmelos* **Family :**Rutaceae **Name:** Bel(local name),wood apple(English name) **Plant part used:** Fruits **Medicinal uses:** Dysentery, diarrhea	
Scientific Name: *Centella asiatica* **Family :** Apiaceae **Plant part used:** Whole plant **Name**: Thankuni(local name) Indian pennywort(English name) **Medicinal uses:** Antiprotozoal, diuretic, leprosy, Ulceration of womb, eczema, urinary and ovarian irritation.	

1.4 Vernacular Names of *Wedelia trilobata*

Table 1.2: Showing the vernacular names of *Wedelia trilobata* (Invasive Species Compendium, 2015)

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
Malaysia	Wedelia kuning
Japan	America hama-guruma
Sweden	ampelkrage

1.5 Taxonomy of *Wedelia trilobata* (Invasive Species Compendium, 2015)

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Asterales

Family: Asteraceae

Genus: *Sphagneticola*

Species: *Sphagneticola trilobata*

- **Botanical Name** (Invasive Species Compendium, 2015)

Sphagneticola trilobata (L.) Pruski

1.6 Common Names

Bay Biscayne creeping oxeye, Bay Biscayne creeping-oxeye, creeping daisy, creeping ox eye, creeping ox-eye, creeping oxeye, creeping wedelia , rabbit's paw, Singapore daisy, trailing daisy, wedelia , yellow dots. (Invasive Species Compendium, 2015)

1.7 Asteraceae Family:

The name "Asteraceae" comes from *Aster*, the most prominent genus in the family,that derives from the Greek ἀστήρ, meaning star.This family has more than 23,000 currently accepted species, 1,620 genera and 12 subfamilies. Asteraceae, is an exceedingly large and widespread family of flowering plants (Angiospermae). The Asteraceae represent as much as 10% of autochthonous flora in many regions of the world. Most members of Asteraceae

are herbaceous. However a significant numbers are also shrubs, vines, or trees. The family is distributed world widely and is most common in the arid and semiarid regions of subtropical and lower temperate latitudes. This family has a remarkable ecological and economical importance. Some of the family members like lettuce, sunflower seeds, artichokes, provide cooking oils, sweetening agents, coffee substitutes, and herbal teas. Several genera are popular with the horticultural community, including marigold, pot marigold ,cone flowers, various daisies, fleabane, chrysanthemums, dahlias, zinnias, and heleniums. Asteraceae are important in herbal medicine, including *Grindelia, Echinacea*, yarrow, and many others. (Rahman, 2013 b)

The Asteraceae are characterized by having the flowers reduced and organized into an involucrate pseudanthium in the form of a head or capitulum. The leaves are alternate, opposite; stipules are absent. The flowers may be bisexual or unisexual. Where both types are found in a single head, the central flowers have tubular, usually 4-5- lobed corollas, and generally are bisexual, and the peripheral flowers have strap-shaped corollas generally with 3 distal teeth, and are usually female. The corolla is sympetalous with mostly 3-5 lobes. The androecium nearly always consists of 4 or 5 stamens that are united by their anthers and are adnate to the corolla tube or epigynous zone, alternate with the lobes. The gynoecium consists of a single compound pistil of 2 carpels, a single 2-cleft style, and an inferior ovary with one locule and one basal ovule. The fruit is an achene (cypsela) which may have a persistent pappus that commonly functions in fruit dispersal (Invasive Species Compendium, 2015).

1.7.1 Species of Asteraceae Family Available in Bangladesh

Plants of Asteraceae family are widely available and grow well in Bangladesh .they have good adaptability with the climatic conditions of Bangladesh. They are found in plain as well as hilly areas like Sylhet and Chittagong. In the present study 36 plant species 29 genera of the family Asteraceae have been recorded which are used by the local people in the ailment of human diseases and 18 species have been recorded which are used in the ailment of diseases of domestic animals. On the other hand 53 human diseases recorded by traditional medicine have been recorded. (Rahman, 2013 b)

Within the *Aracae*, genera such as *Alocasia*, *Arisaema*, *Caladium*, *Colocasia*, *Dieffenbachia*, and *Philodendron* contain calcium oxalate crystals in the form of raphides. When consumed, these may cause edema, vesicle formation and dysphagia accompanied by painful stinging and burning to the mouth and throat, with the symptoms occurring for up to two weeks (Rahman,2013 a).

Table1.3: Some plants of Asteraceae family available in Bangladesh (Rahman, 2013 a)

Scientific Name: *Tagetes patula* *Linn.* **Local name:** Genda. **Part used**: Flower, leaf. **Uses:** The leaves are good remedy for piles, Kidney troubles, candidiasis, muscular pain, Ear ache, opthalmia, flower is useful in fevers and epileptic fits.	
Scientific Name: *Zinnia peruviana* L. **Local name**: Zinnia **Part used:** Leaf, stem. **Uses:** The leaves and stems are also used in skin diseases, more particularly leprosy.	
Scientific Name: *Mikania cordata* (Burm. f.) Robinson **Local name:** Assamlata. **Part used:** Whole plant, leaf. **Uses**: Plant is used as a remedy for snakebite. Leaves are used for poulticing the wounds.	

Scientific Name: ***Lactuca sativa*** Linn. **Local name:** Lettuce. **Part used:** Whole plant, leaf, **Uses:** Leaves are hypnotic, stomachic, improve appetite; purify the blood,headache, bronchitis and cough due to heart disease. The fresh plant is a mild sedative, purgative, diuretic and antispasmodic.	
Scientific Name: ***Helianthus annuus*** Linn. **Local name:** Surjjamuki. **Part used:** Flower, seed. **Uses:** The oil of sunflower is used as heart disease. The flower is anthelmintic, cures skin diseases, itching, ulcers, leprosy.	
Scientific Name: ***Enhydra fluctuans*** Lour. **Local name:** Helencha. **Part used:** Whole plant, leaf. **Uses:** The leaves are laxative' cure inflammation, leucoderma, bronchitis, biliousness; good in smallpox. The leaves are antibilious. Juice of the leaves is used as demulcent in cases of gonorrhea.	
ScientificName: ***Chrysanthemum coronarium*** Linn. **Local name:** Chandramollica. **Part used:** Bark, flower, leaf, root. **Uses:** The bark is purgative and is used in syphilis. The leaves areapplied topically to lessen inflammation.	

Scientific Name: ***Blumea lacera*** (Burm. f.) DC. **Local name:** Kukurmuta **Part used:** Leaf, root. **Uses:** juice of the leaves is used as an anthelmintic, febrifuge, astringent, and diuretic; mixed with black pepper, it is given in bleeding piles.	
Scientific Name: ***Callistephus chinensis*** (Linn.) Nees. **Local name:** Aster **Part used:** Roots. **Uses:** Mainly used as ornamentals. The Chinese use the root for coughs and pulmonary affections, malaria and hemorrhages.	
Scientific Name: ***Launaea aspleniifolia*** (Willd.) Hook.f. **Local name:** Tikdana **Part used:** Whole plant, root. **Uses:** The root of this plant in combination with other drugs is given as a lactagogue .	
Scientific Name: ***Grangea maderaspatana*** (Linn.) Poir. **Local name:** Namuti **Part used:** Whole plant, leaf. **Uses:** Used in ovarian disorders. Ithe leaves are used as a stomachic and antispasmodic.	

1.8 *Wedelia trilobata*

Wedelia trilobata is one kind of species of the Asteraceae family commonly known as Bhringraj, Singapore Daisy, *Rabbits Paw, Trailing Daisy*, *Bay Biscayne creeping-oxeye*, Creeping oxeye, Trailing daisy, *Wedelia*, Yellow dots.it is native to north and central America and in the Indies. *Wedelia* includes 104 additional species and they are found mainly in the tropical areas in America. The genus is named in honor of German botanist and physician Georg Wolfgang Wedel (1645–1721). (Invasive Species Compendium, 2015).

Fig 1.1 *Wedelia trilobata* plants

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. (Weeds of Australia, 2011)

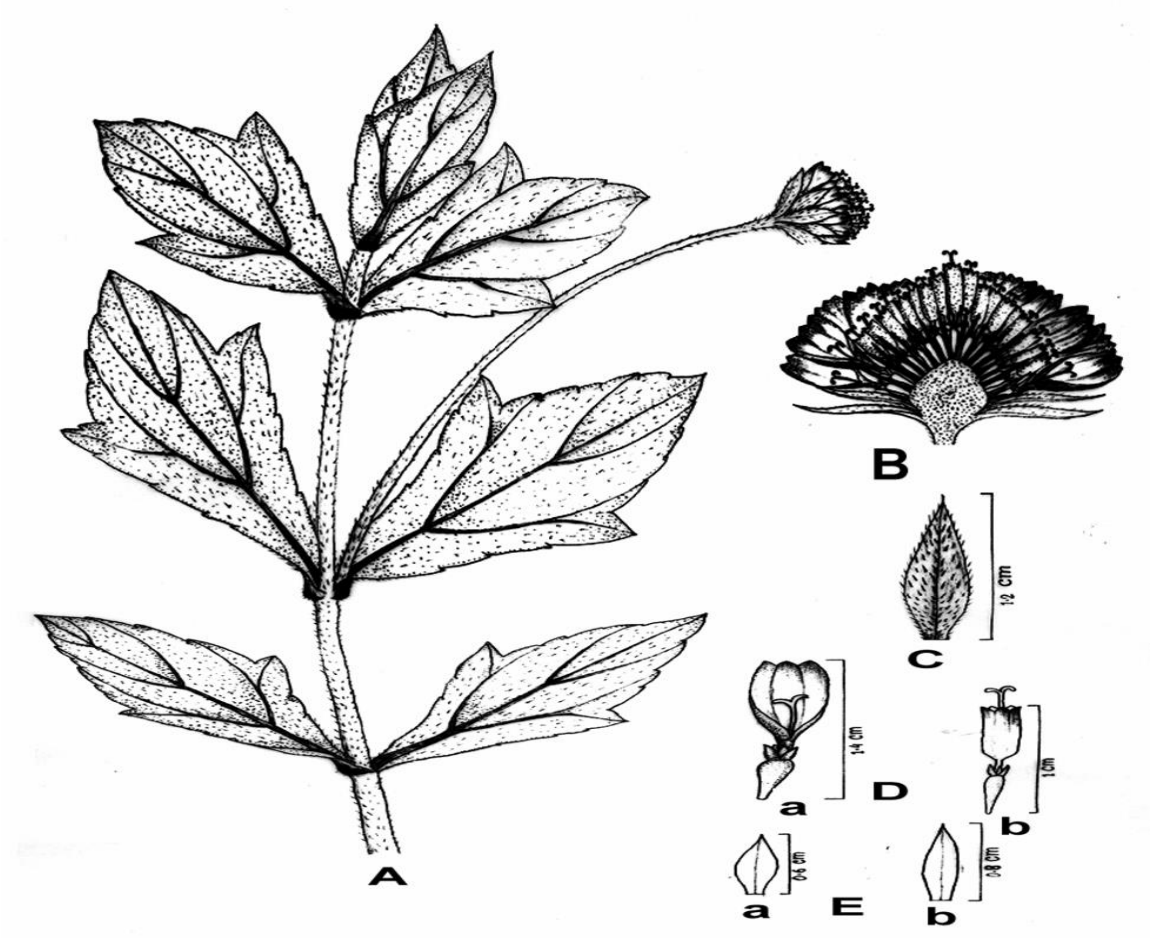

Fig 1.2 *Wedelia trilobata* (L.) A.S. Hitchc. A. habit sketch (2/3 nat. size); B. L.S. of a capitulum; C. involucral bract; D. a. female floret, b. a hermaphrodite disc floret; E. a. outer palea, b. inner palea.

1.9 Synonyms of *Wedelia trilobata*

Complaya trilobata (L.),*Silphium trilobatum(*L),*Thelechitonia trilobata* (L.) ,*Wedelia carnosa Wedelia paludosa* DC. *Sphagneticola trilobata.* (Weeds of Australia, 2011)

1.10 Distribution of *Wedelia trilobata*

Wedelia trilobata is native to the northern part of South America, Central America, Mexico and the West Indies but is also widely found in Bangladesh, India, China, Hong-Kong, Malaysia, Indonesia Hawaii, Vietnam, Kampuchea, Burma and Philippine, south Florida

and West Africa. It grows well in warm and humid climate. *Wedelia trilobata* is commonly cultivated as an ornamental groundcover in the warmer regions of Australia.) It often grows luxuriantly in valleys, raceways, field edge, roadway and humid lands and grows mostly in agricultural areas, coastland, natural forests, planted forests, range/grasslands, riparian zones, scrub/shrub lands and in the urban areas. It is an important weed and garden herb in South China .In Bangladesh it is widely grows in Chittagong, Dhaka, Mymensingh, Patuakhali, Tangail and Nijum Deep. (Weeds of Australia, 2011)

1.11 Distinguishing Features of *Wedelia trilobata*

- ✓ A mat-forming groundcover, or occasionally a low-climbing plant, with hairy stems.
- ✓ Its paired leaves are often three-lobed and have toothed margins.
- ✓ These leaves are glossy in appearance and mostly hairless.
- ✓ Its bright yellow daisy-like 'flowers' (20-30 mm across) are borne singly on stalks 3-15 cm long.
- ✓ Each flower has 8-13 yellowish 'petals' (6-15 mm long) with finely toothed tips. (Weeds of Australia,2011)

1.12 Habitant of *Wedelia trilobata*

It is a tropical perennial, mat-forming herbaceous plant with wide ecological tolerance range it is equally suited to dry and moist sites. However it grows best in sunny and well-drained area. It grows well on almost all soil types, including bare limestone, poor sandy beaches, swampy or waterlogged soils. It is also tolerant to inundation and high levels of salinity. It is widely available almost throughout all the areas of Bangladesh and also grows in wild. It is mostly planted and cultivated as a ground covering and ornamented plant in the cities, roads, parks and houses. These plants when cultivated in gardens, will quickly spread along fences and roadsides, up electricity poles, over nearby trees and into suburban bush land. (Weeds of Australia, 2011)

Fig 1.3: *Wedelia trilobata* whole plant

1.13 Morphology of *Wedelia trilobata*

Wedelia trilobata is a perennial herbaceous plant, with broad leaves and vegetatively propagated seeds. (Weeds of Australia, 2011)

1.13.1 Stem of *W. trilobata*

The stems are rounded, green or reddish in color, 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. Stems those are rooting at the nodes are cylindrical, much-branched, and procumbent. (Weeds of Australia, 2011)

Fig 1.4:*W. trilobata stem*

1.13.2 Leaf of *W. trilobata*

The oppositely arranged leaves are stalk less or shortly petiolate, opposite-decussate, ovate-dentate or 3-lobed, irregularly toothed or serrate, usually with a pair of lateral lobes, fleshy, strigose on both surfaces, 4-7 cm long and 1.5-2.5 cm wide. Capitula heterogamous, rayed, solitary on 3-10 cm long peduncles. Involucre campanulate , hemispherical; bracts 2-seriate, outer 1.0-1.2 cm long and 0.4-0.5 cm broad, ovate-lanceolate, chuffy, rigid, often recurved and exceeding the disk; inner shorter, lanceolate; receptacle convex, paleaceous. Paleae embracing the cypselas , concave. Ray florets 1-seriate, female, ligulate, 5-12 mm long; disc-florets many-seriate, tubular, bisexual. Corolla of the ray-florets golden yellow with 2-3-fid limb; that of disc-florets with 5-fid limb. Anthers appendaged , bases sagittate with minute auricles. Stylar arms of outer florets elongated, tips acute, hairy; florets flattened, with acute appendages, hairy. Cypselas of outer florets 3-angled, those of disc-florets sub-terete or sub-truncate, tuberculate. Pappus a crown of short fimbriate scales. (Weeds of Australia, 2011)

Fig 1.5: *W. trilobata leaves*

1.13.3. Flowers of *W. trilobata*

The bright yellow to orange-yellow flower-heads are daisy-like in appearance and 3-15 cm long born singly on upright stalks. Each flower has 8-13 yellowish 'petals' that are 6-15 mm long with finely toothed tips. In the center of these flower-heads there are numerous

tiny yellow tubular flowers (i.e. tubular florets) which is 4-5 mm long. The base of each flower-head (i.e. capitulum) is enclosed in a row of narrow) green bracts of about 1 cm long. Flowering occurs throughout the year, but is most common from spring through to autumn. (Weeds of Australia, 2011)

Fig 1.6: Flowers of *W. trilobata*

1.13.4 Seeds of *W. trilobata*

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

Fig 1.7: Unripe seeds of *W. trilobata*

Fig 1.8: Mature seeds of *W. trilobata*

1.13.5 Roots of *W. trilobata*

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).

Fig 1.9: *W. trilobata roots*

1.14 Reproduction and Dispersal

Wedelia trilobata usually reproduces by vegetative propagation by using fragments of their stem. Stems forms new plants where they come in close proximity with the ground and

those pieces readily brings out roots and ultimately develop into new plants. Plants usually develop few fertile seeds which are viable and rarely produced. It is commonly planted as an ornamental plant. (Weeds of Australia, 2011)

1.14.1 Vegetative Growth Stages

Flowering stage, Fruiting stage, Post-harvest, Pre-emergence, Seedling stage, Vegetative growing stage.

1.14.2 Biology and Ecology

1.14.2.1 Genetics

Chromosome number for *W. trilobata* is given as x=30, 2n=60 by the Missouri Botanical Garden (2008). Ren et al. (2012) give the karyotype formula 2n=56=24m+28sm+4st, and postulate that the basic chromosome number may be x=14 rather than x=15 and that *W. trilobata* may be tetraploid. (Invasive Species Compendium, 2015)Wu et al. (2013) report that *S. trilobata* in South China, where it is invasive, may be hybridizing with the native congener *Sphagneticola calendulacea* (Invasive Species Compendium, 2015).

1.14.2.2 Reproductive Biology

W. trilobata produces achenes which contain seeds that are often said to be sterile .However large numbers of seeds are present in the soil seed bank in *wedelia*-infested study sites in Fiji and that up to 17% of seeds were viable. The minimum generative time of these seeds is usually one year. However, *W. trilobata* usually propagates vegetatively, with stems forming new plants where they touch the ground. Stem fragments also readily form roots (Invasive Species Compendium, 2015).

1.14.2.3 Physiology and Phenology

W. trilobata is a vigorous perennial herb capable of forming a continuous herbaceous ground cover. Flowering takes place year round. It produces the most flowers in open, frost-free locations. However, *W. trilobata* can grow in shade and still flower, although only sparingly. Where it has escaped from garden boundaries it can be found in lawns and

disturbed areas, where it will respond to mowing by flowering at ground level (Invasive Species Compendium, 2015).

1.15 Traditional Uses

- *W. trilobata* has traditionally been used to treat infections, indigestion and to treat hepatitis. It shows considerable anti-hepatotoxic and protective effects against carbon tetrachloride induced liver destruction.
- The leaves or aerial parts of this plant are used in traditional medicine in Caribbean and Central America for backache,muscle cramp, rheumatism, stubborn wounds, sores,swelling, and arthritic painful joints
- In Hong Kong it is utilized as substitute for *W.cinensis*, a traditional Chinese medicine used for treatment of the common cold, hepatitis, indigestion and infections (Invasive Species Compendium, 2015).

1.16 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.(Ghosh,2010)

1.17 Ethnopharmacological Uses:

- ✓ The aerial parts of this plant are used in traditional medicine in the Caribbean and Central America against bronchitis, colds, and 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 dysfunctioning, 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 (Ghosh, 2010).

Table 1.4: Biological and pharmacological activities (*in vitro* and *in vivo*) of *W. trilobata* (Balekar *et al*, 2014).

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*, *Shigella sonnei*
Ethanol extract of leaves	Strong inhibitory effect on *P. aeruginosa, K. pneumoniae, P. fluorescens*, *X. oryzae pv. oryzae*, *X. axanopodis* pv. malvacearum, moderately inhibited the *E.coli, C.michiganensis* sub sp. *michiganensis butless* activity was observed on *S. aureus.*
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 stem	**ANTIBACTERIAL ACTIVITY** Inhibitory effect on *Bacillus subtilis, Pseudomonas fluorescens, Clavibacter michiganensis* sub sp. *michiganensis*, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas axanopodis* pv. *malvacearum* and strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia.*

Ethanol extract of flower	Strong inhibitory effect on *Staphylococcusaureus, X.oryzae* pv. *oryzae* moderately inhibited the *K. pneumoniae, P. fluorescens, X. axanopodis* pv. *malvacearum* but less activity was observed on *E.coli, P. aeruginosa,Clavibacter michiganensis* sub sp. *Michiganensis.*
Methanol extract of flower	Moderate inhibitory activity against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and *Shigella flexneri.*
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, Fusarium solani, 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	**WOUND HEALING ACTIVITY** The WEA displayed antibacterial and fibroblast stimulatory activities while WCM exhibited antioxidant activity

ethanolic extract of *W. trilobata* leaves	
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 Two
Literature Review

2.1 Phytochemical Constituents

Phytochemical screening and antimicrobial activity of *Wedelia trilobata* L. plant

The medicinal 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 (Li *et al.*, 2012).

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 petroleum ether,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 . The highest yield was obtained from plants collected in winter, when it was registered low temperature and precipitation. The essential oil was characterized by high percentage of hydrocarbon sesquiterpenes, hydrocarbon monoterpenes and low levels of oxygenated sesquiterpenes. The major components were germacrene D (11.9-35.8%), α-phellandrene (1.4-28.5%), α-pinene (7.3-23.8%), *E*-caryophyllene (4.6-19.0%), bicyclogermacrene (6.0-17.0%), limonene (1.8-15.1%) and α-humulene (4.0-11.6%)(Silva *et al.*,2012).

2.2 Pharmacological Properties

S. trilobata is employed to treat backache, muscle cramps, rheumatism, stubborn wounds, sores and swellings, and arthritic painful joints .Anticonceptive activity was described for some extracts and the isolated compounds, kaurenoic acid and luteolin, from S. trilobata (Block *et al.*, 1998). Trypanosomicidal activity was observed for the ethanol extract (Chiari *et al.*, 1996) and the bioassay-directed fractionation leads to isolation of the diterpenes kaurenoic and grandiflorenic acids (Batista *et al.*, 1999).

2.2.1 Antidiabetic Activity:

Aqueous extracts of *Sphagneticola trilobata* attenuates streptozotocin-induced hyperglycaemia in rat models by modulating oxidative stress parameters

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 nitroprusside 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® (Kade *et al.*, 2010)

2.2.2 Central Nervous System (CNS) Depressant Activity

Central Nervous System Depressant Activity of *Wedelia trilobata* Leaves

The petroleum ether, chloroform, ethyl acetate and methanol extract of leaves of *W. trilobata* (30mg/kg) were evaluated for CNS depressant activity using pentobarbitone induced sleeping time, and locomotors activity in mice. The petroleum ether extract potentiated pentobarbitone sodium induced sleeping time in mice than other extracts. The

animal 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

Efficacy of a kaurenic acid extracted from the Venezuelan plant *Wedelia trilobata* (Asteracea) against *Leishmania* (Viannia) *braziliensis*

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 0.78g/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*. (Brito *et al.,* 2006)

2.2.4 Antioxidant Activities

Evaluation of antioxidant and antibacterial activities of methanolic flower extract of *Wedelia trilobata* (L.) Hitch

Wedelia trilobata (L.) Hitch is a member of the family Asteraceae, with attractive yellow flowers borne singly at the end of each stem. The work was conducted to investigate the antioxidant, antimicrobial and DNA protecting ability of the methanol extract of *W. trilobata* flower. Antioxidant properties were assessed by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis 3- thylbenzthiazoline-6-sulphonic acid (ABTS) assay. The methanol extract and standard ascorbic acid showed antioxidant activity with IC_{50} value of 90 µg/ml and 60 µg/ml respectively in DPPH radical scavenging assay. The *in vitro* antibacterial screening was evaluated by disc diffusion method against three Gram-positive and three Gram-negative human pathogenic bacteria. The extract showed zones of inhibition of 10-16 mm against different strains. The phenolic content of the extract was 250 µg/ml gallic acid equivalence (GAE in µg) which was measured by Folin-Ciocalteu

method. The extract was tested for pTZ57R/T plasmid DNA protection against hydroxyl radicals as evidenced by DNA fragmentation assay. (Chethan *et al.* 2012)

2.2.5 Antimicrobial Activity

Antimicrobial activity of *Sphagneticola trilobata* (L.) pruski, against some human pathogenic bacteria and fungi

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,* 2013)

Antimicrobial, antioxidant and in vitro anti-inflammatory activity of ethanol extract and active phytochemical screening of Wedelia trilobata (L.) Hitchc.

Ethanol extract of leaf, stem and flower of *Wedelia trilobata* was assessed for its antimicrobial, antioxidant and anti-inflammatory activity and phytochemical screening. Total phenolic content was assessed using Folin-Ciocalteu's method. The antioxidant activity was determined by measuring the scavenging activity of DPPH radical and FRAP assay. The antimicrobial efficacy was determined using paper disc method against different fungi and bacteria. Sensitivity in terms of zones of inhibition and phytochemical

composition of the all parts extracts were also determined. In vitro anti-inflammatory activity was evaluated using albumin denaturation, membrane stabilization assay and proteinase inhibitory assay. Aspirin was used as a standard drug for the study of anti-inflammatory activity. The results show that, all parts extracts effective against all the bacteria tested, whereas all the extracts were failure in inhibiting the growth of all *Alternaria sp.*, *Cercospora carthami* and *Nigrospora oryzae*, but other fungi also were showed moderate inhibition against all the three extracts. Phytochemical analysis revealed the presence of tannins, flavonoids, terpenoids, phenols and saponins. Leaf and stem ethanol fractions showed highest total Phenolic content. The leaf and stem ethanol extract possessed strong scavenging activity in both DPPH and FRAP methods. In DPPH and FRAP method, the leaf and stem had showed free radical inhibition of 86, 82 and 630.72, 508.81 respectively. The leaf and stem ethanol extract also showed in vitro anti-inflammatory activity by inhibiting the heat induced albumin denaturation and red blood cells membrane stabilization with 87.14 and 86.76 and 78.11, 74.17 g/ml respectively. Proteinase activity was also significantly inhibited by the leaf (84.19 g/ml) and stem (81.84 g/ml). From the result, it is concluded that phytochemicals (tannins, flavonoids, terpenoids, phenols and saponins) present in the *W. trilobata* extract may be responsible for the antimicrobial, antioxidant and anti-inflammatory activity. (Govindappa, *et al.*, 2011)

2.2.6 Wound Healing Activity

Wound healing activity of ent-kaura-9(11), 16-dien-19-oic acid isolated from Wedelia trilobata (L.) leaves

An ethanolic extract of *W. trilobata* leaves was subjected to column chromatography. Hexane, ethyl acetate (WEA) and chloroform: methanol (50:50) (WCM) fractions were obtained. The fractions were tested using relevant *in vitro* wound healing assays. WEA (3 g/mL) promoted fibroblast L929 viability up to more than 90% before and more than 85% after hydrogen peroxide induced oxidative stress. WEA (3 g/mL) induced a 70% migration rate in the *in vitro* scratch assay and the collagen content was increased to 261g/mL compared to the control (57.5g/mL) .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 of 15.62 g/mL against *S. aureus* and 7.81g/mL against *S. epidermidis*. The ent-kaura-9(11), 16-dien-19-oic acid (2.5-0.08g/mL) produced an increase in the percentage viability of mouse fibroblast L929 cells from 97-117% and protection of the fibroblast L929 cells against oxidative stress induced by hydrogen peroxide (94-80%). (Balekar, *et al.*, 2012)

Wound-healing potential of gradiflorenic 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 iflorenic acid (2.5µg/mL) produced percentage viability of B Jhuman 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 B J 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).

2.2.7 Antibacterial Activity

Studies on the antibacterial activity, antioxidation, anti-inflammation and anticancer activity of *Wedelia trilobata* (L.) Hitchc

Wedelia trilobata (L.) Hitchc. is used in folk medicine. It was reported that the plant shows antibacterial activity and contents antitumor chemical components. The purpose of this research was to evaluate antibacterial, antioxidation, anti-inflammation, and anticancer activity of *W. trilobata. 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 and antioxidative activities. We also evaluate its anti-inflammation and anticancer activity of the flower. 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. Most extracts from flowers, leaves and stems showed the good antioxidative activities especially the flower. The ethyl acetate extracts presented the best on DPPH scavenging activity and reducing power and had the highest total phenolic compounds content. We used the ethyl alcohol and water extracts of W. trilobata to treat RAW264.7macrophage, which induced inflammation by LPS. In the NO2 assay, the extracts of W. trilobata flower had better inhibitory ability .In transient transfection assay shows the n-hexane and ethyl alcohol extracts of W. trilobata flower can activate PPAR. In MTT assay of SK-Hep-1, we found that extract of W. trilobata flower had the best inhibitory ability. We also found the ethyl alcohol extract 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. (Wei L.C., 2009)

2.2.8 Anti-inflammatory Activity

Kaurenoic acid from Sphagneticola trilobata Inhibits Inflammatory Pain: effect on cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway.

Kaurenoic acid [ent-kaur-16-en-19-oic acid (1)] is a diterpene present in several plants including *Sphagneticola trilobata.* The only documented evidence for its antinociceptive effect is that it inhibits the writhing response induced by acetic acid in mice. Therefore, the analgesic effect of 1 in different models of pain and its mechanisms in mice were investigated further. Intraperitoneal and oral treatment with 1 dose-dependently inhibited inflammatory nociception induced by acetic acid. Oral treatment with 1 also inhibited overt nociception-like behavior induced by phenyl-p-benzoquinone, complete Freund's adjuvant (CFA), and both phases of the formalin test. Compound 1 also inhibited acute carrageenin- and PGE (2)-induced and chronic CFA-induced inflammatory mechanical hyperalgesia. Mechanistically, 1 inhibited the production of the hyperalgesic cytokines TNF-α and IL-1β. Furthermore, the analgesic effect of 1 was inhibited by l-NAME, ODQ, KT5823, and glybenclamide treatment, demonstrating that such activity also depends on activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway, respectively. These results demonstrate that 1 exhibits an analgesic effect in a consistent manner and that its mechanisms involve the inhibition of cytokine production and activation of the NO-cyclic GMP-protein kinase G-ATP-sensitive potassium channel signaling pathway. (Mizokami *et al*, 2012)

2.2.9 Hepatoprotective Activity

Hepatoprotective activity of *Wedelia calendulacea* L. against Acute Hepatotoxicity in Rats

The hepatoprotective activity of ethanolic extract of *Wedelia calendulacea* L. (Family: Asteraceae) was studied against CClinduced,acute hepatotoxicityin rats. Hepatoprotective activity of the ethanolic-leaf extract of *W. calendulacea* (EEWC) was studied by estimating serum enzyme activities of as part of aminotransferase (AST), alanine aminotransferase

(ALT), akaline phosphatase (ALP), protein and bilirubin. The treatment with EEWC showed a dose dependent reduction of CCl_4 induced elevated serum levels of enzyme activities with parallel increase in total protein and bilirubin, indicating the extract could preserve the normal functional status of the liver. The weight of the organs such as liver, heart, lung, spleen and kidney in CCl_4 induced experimental animals administered with EEWC showed an increase over CCl_4 control group. (Murugaian, 2008)

2.2.10 Stimulatory Activity

In vitro stimulatory effect of grandiflorenic acid isolated from *Wedelia trilobata* (L.) leaves on L929 fibroblast cells

Wedelia trilobata (L.) has been used in traditional medicine in the Caribbean and Central America for stubborn wounds, sores, swelling and arthritic painful joints. The aim of the study was to evaluate the activity of grandiflorenic acid obtained from leaves of *W. trilobata* on fibroblast cells which are responsible for wound healing. The grandiflorenic acid was assessed for its possible activity on fibroblasts cells by measuring collagen content, lactate dehydrogenase (LDH) activity after an oxidative stress induced by hydrogen peroxide, and an *in vitro* scratch assay. The grandiflorenic acid (2.5 μg/mL) increased collagen content of fibroblast L929 to 95.2 μg/mL as compared to the control (23.8 μg/mL). Cells treated with hydrogen peroxide exhibited 71% of LDH release whereas cells treated grandiflorenic acid (2.5 and 1.25 μg/mL) and then treated with hydrogen peroxide showed less percent release of LDH (25 and 28%) indicating protection of cell membrane integrity. The grandiflorenic acid (2.5 μg/mL) induced a 98.9% migration rate in the *in vitro* scratch assay on day 1, while control showed 57.9% migration rate on day 1. The present study provides scientific evidence that grandiflorenic acid has stimulatory effect on L929 fibroblast cells indicating its potential for wound healing activity. (Balekar, *et al.,* 2013)

2.2.11 Analgesic Activity

Analgesic activities of the medicinal plants of *Wedelia trilobata, Wedelia biflora* and *Eclipta alba* in standard experimental animal models

Comparative study on analgesic activity was carried out using ethanol extracts in animal models. *Wedelia trilobata* (*EEWT*), *Wedelia biflora* [*Melanthera biflora*] (*EEWB*) and *Eclipta alba* (*EEEA*) was evaluated by acetic acid induced writhing method and hot plate assay to assess analgesic activity in mice. It was found that the extract caused an inhibition on the writhing response induced by acetic acid in a dose dependent manner. Dose of 500 mg/kg EEWT, EEWB, EEEA and Aspirin could block the writhing response by 49.17, 49.45, 55.23 and 68.68% ($p<0.001$), respectively. It was also indicated that the EEEA showed significant antinociceptive action in hot plate reaction time method in mice. This effect was comparable to that of standard drug morphine treated controls. The results reflects that analgesic effects and therapeutic efficacy of the extract on animal models which are comparable with those of standard drugs such as aspirin and morphine (Kumar *et al.,* 2007).

2.3 Discussion

The present review emphasizes the phytochemical, traditional, pharmacological and**,** clinical reports on *W. trilobata*. Tannin, saponins, flavonoids, phenolic, terpenoids constitute major classes of phytoconstituents 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. Strong antioxidants, antimicrobial and anti-inflammatory activities specifically in the ethanolic leaf and stem extracts 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 phytoconstituents. One of the phytoconstituent isolated and evaluated for wound healing potential is grandiflorenic acid (ent- kaura-9(11)-dien-19-oic 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 phytomedicines 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 phytoconstituent 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 ultimatelyin clinical studies. (Balekar *et al*, 2014)

Rationale of the Study

Wedelia trilobata is an important medicinal plant in Bangladesh. In the rural areas the different parts of the plant are extensively used to treat varieties of ailments, such as diabetes, diarrhea, dysentery, hypertension, menstrual problems and fever (Rahmatullah, 2010). The plant parts proved to be effective for the treatment of various diseases and so it is profoundly used for its medicinal value. This makes *W. trilobata* a potential study material for both phytochemical and pharmacological investigations. So experimental studies were carried out to evaluate different pharmacological activities of the methanol extract of *W. trilobata* leaves.

Aim of the Study

- ✓ Phytochemical fractionation of the methanolic extract of *Wedelia trilobata* (leaves) using petroleum ether, chloroform and ethyl acetate, consecutively.
- ✓ Evaluation of total flavonoid content *Wedelia trilobata* leaf extract.
- ✓ Evaluation of cytotoxic activity of *Wedelia trilobata* leaf extract by brine shrimp lethality bioassay.
- ✓ Evaluation of antimicrobial activity of *Wedelia trilobata* leaf extract against different Gram positive bacteria, Gram negative bacteria and fungi.

Chapter Three
Methods & Materials

3.1 Collection and Preparation of Plant Material

Plant sample (leaves) of *Wedelia trilobata* was collected from Hatirjheel and Aftabnagar, areas of Dhaka, Bangladesh. 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, amber glass container (5 liters) and soaked in 3.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 39°C with a rotary evaporation.

Figure 3.1: Drying of extract using rotary evaporator

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

3.3 Preparation of Mother Solution

5gm of methanol extract was triturated with 90ml of methanol containing 10ml of distilled water.

The crude extract was dissolved completely. This is the mother solution.

3.4 Partition of Mother Solution

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

3.4.1 Partition with 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.

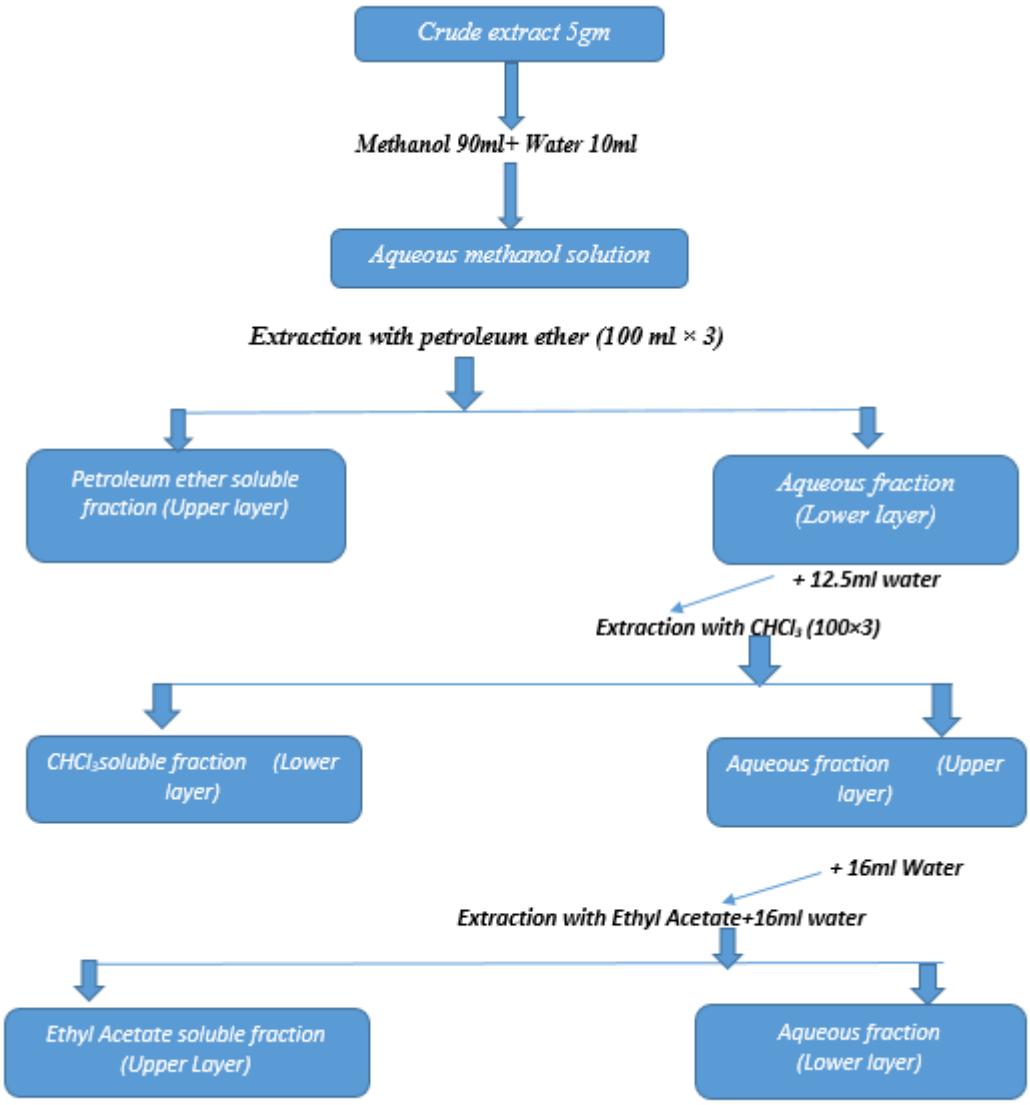

Fig 3.2: Schematic representation of partitioning of methanolic crude extract of *Wedelia trilobata* leaves.

3.4.4 Collection of Chloroform Fraction

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

3.5 Antioxidant Activity

3.5.1.1 Principle

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

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

3.5.1.2 Apparatus and Reagents

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

3.5.1.3 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.

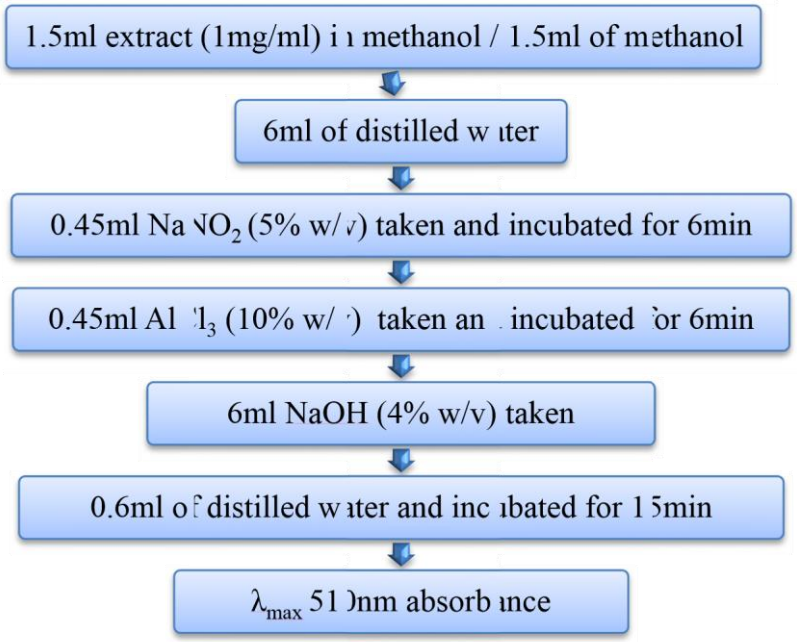

Figure 3.3: Schematic diagram of flavonoid content test

3.6 Brine Shrimp Lethality Bioassay

3.6.1 Principle

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as

a wide range of pharmacological activities e.g. anticancer, antiviral, and pharmacological activities of natural products etc. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus (*in-vivo*) lethality, a simple zoological organism, (Brine shrimp napulii- *Artemia salina*) can be used as a convenient monitoring for screening and fractionation in the discovery of new bioactive natural products(Meyer *et al,*1982).Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. Brine shrimp is the English name of the genus *Artemia* of aquatic crustaceans. *Artemia* is the only genus in the family Artemiidae (Olowa and Nuñeza, 2013; Rishikesh *et al.*, 2013).

3.6.2 Apparatus and Reagents

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

3.6.3 Procedure

3.6.3.1 Preparation of Sea Water

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000ml by distilled water in a 1000ml beaker for *Artemia salina* hatching. 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 *Artemia salina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. The eggs of *Artemia salina* were hatched at room temperature (25-30ºC) for 18-24hrs. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. 10 living shrimps were then collected by a pipette and then added to each of the test tubes containing 5ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay.

Fig 3.4: *Artemia salina* 24hours 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 tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

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

3.6.3.5 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 (Meyer *et al.*1982).

$$\text{Percentage (\%) Mortality} = \frac{\text{No of Shrimp Died}}{\text{Total No of Shrimp in Each Test}} \times 100$$

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, 1976:21).

3.7.2 Apparatus and Reagents

3.7.2.1 Materials

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

3.7.2.2 Test Sample of *Wedelia trilobata*

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

3.7.2.3 Test Organisms

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

Table 3.2: List of microorganisms

Type of microorganism	**Name of microorganism**
Gram +ve bacteria	*Bacillus cereus* *Bacillus magaterium* *Staphylococcus aureus* *Sarcina lutca* *Bacillus subtilis*
Gram –ve bacteria	*Escherichia coli* *Salmonella paratyphi* *Pseudomonas aeruginosa* *Shegella dysenteriae* *Vibrio mimicus* *Vibrio parahemolyticus* *Salmonella typhi*
Fungi	*Saccharomyces cerevisiae* *Candida bicans* *Aspergillus niger*

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 3.5: Autoclave machine

3.7.3.2 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in laminar hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the laminar hood. Petri dishes and other glassware were sterilized by autoclaving at a temperature of

121°C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Fig 3.6: 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, a 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 3.7: Incubator

3.7.3.8 Determination of Antimicrobial Activity by Measuring the Zone of Inhibition

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

Chapter Four
Results & Discussion

4.1 Antioxidant Test Result

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 Chloroform extract of *Wedilia trilobata* (leaves) was determined by following method:

- Determination of total flavonoids content

4.1.1Total Flavonoid Content Result

The chloroform 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 (C) (μg/ml)	Absorbance (nm)	Regression value	R^2 value
50	0.05	Y=0.0017x-0.042	**0.991**
100	0.13		
150	0.19		
200	0.29		
250	0.39		

After absorbance were taken of different solution of ascorbic acid of concentrations ranging from 50μg/ml to 250μg/ml, a linear relationship was observed when the absorbance 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.

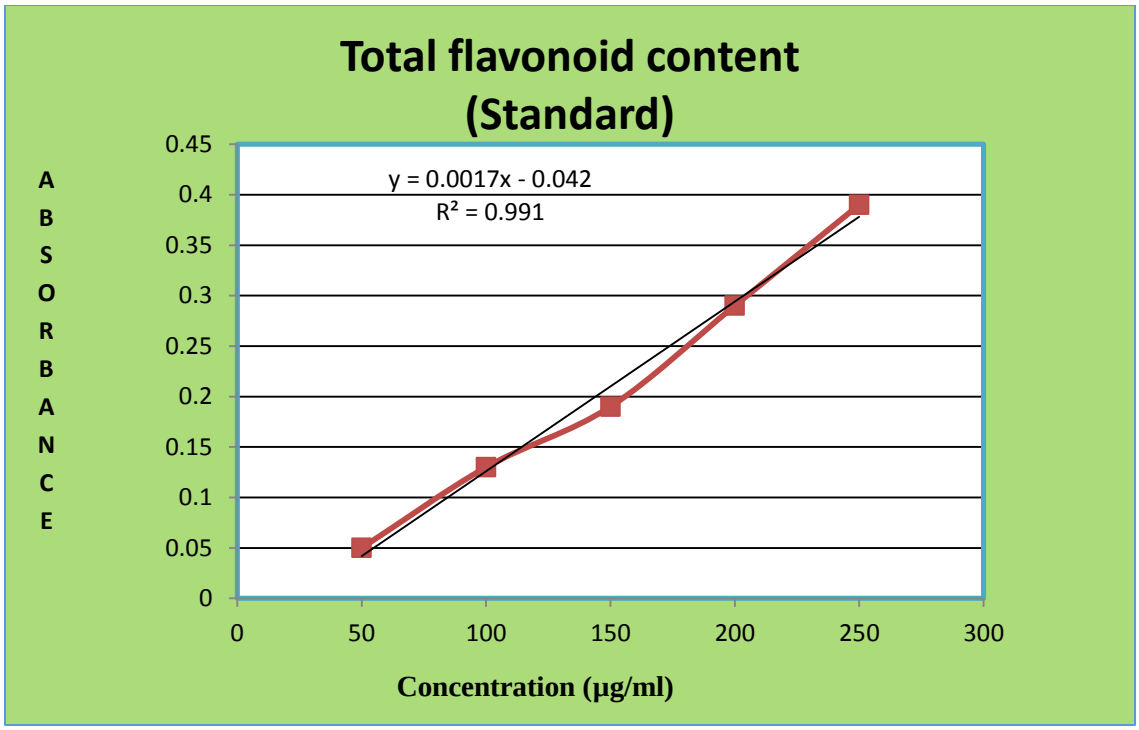

Figure 4.1: Graphical representation of assay of flavonoid content of ascorbic acid

4.1.1.2 Total Flavonoid Content Present in Chloroform 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 extracts calculated and is given in Table 4.2.

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

Sample	Concentration (C) (mg/ml)	Absorbance (nm)	Total flavonoid content(mg of AAE/g of dried extract)
Chloroform 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. In 1mg/ml concentration of chloroform fraction of *Wedelia trilobata* (leaves) 228.824mg 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 chloroform extract of *Wedelia trilobata* leaves were subjected to brine shrimp lethality bioassay following the procedure (Meyer *et al.*, 1982). After 24hrs, the test tubes were inspected using a magnifying glass and the number of survivors counted. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a median Lethal Concentration (LC_{50}) value. This represents the concentration of the standard or chloroform extract that produces death in half of the test subjects after a certain period. The percentage mortality at each concentration was determined using the following formula:

$$\text{Percentage (\%) Mortality} = \frac{\text{No of Shrimp Died}}{\text{Total No of Shrimp in Each Test}} \times 100$$

The LC_{50} of the test samples was obtained by a plot of percentage of the shrimps died(% Mortality) against the logarithm of the sample concentration (Log C) and the best-fit line was obtained from the curve data by means of regression analysis. The concentration-% mortality data were analyzed by using Microsoft Office Excel 2010.

4.2.1 Preparation of Standard Curve

Tamoxifen was used as positive control.

Table 4.3: **Re**sults of the bioassay of Tamoxifen (standard)

Test Tube no.	Concentration (C) (µg/ml)	Log C	Number of nauplii alive	Number of nauplii dead	% Mortality	LC_{50} (µg/ml)
1	400	2.602	0	10	100	**13.38**
2	200	2.301	1	9	90	
3	100	2.000	2	8	80	
4	50.0	1.699	3	7	70	
5	25.0	1.398	5	5	50	
6	12.5	1.097	5	5	50	
7	6.25	0.796	6	4	40	
8	3.125	0.495	7	3	30	
9	1.5625	0.194	8	2	20	
10	0.78125	-0.107	9	1	10	

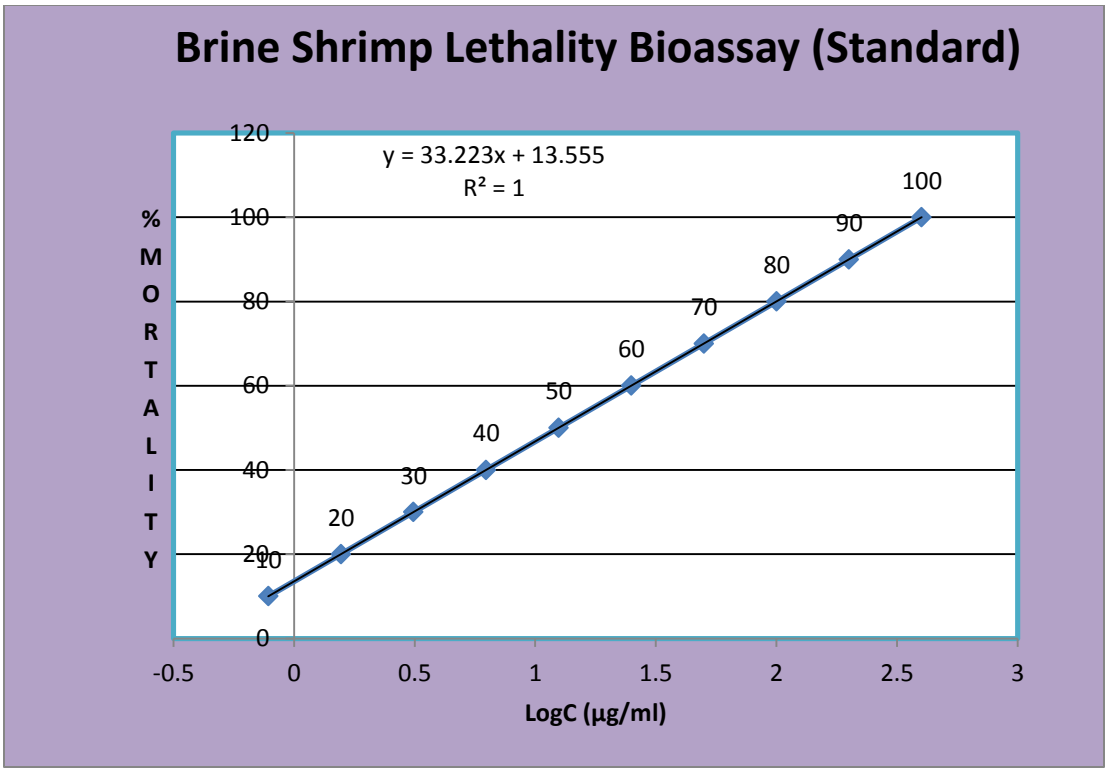

Figure 4.2: Plot of % mortality and predicted regression line of Tamoxifen (standard)

Table 4.4: Results of the bioassay of chloroform (extract)

Test Tube no.	Concentration (C) (µg/ml)	Log C	Number of nauplii alive	Number of nauplii dead	% Mortality	LC_{50} (µg /ml)
1	400	2.602	0	10	100	**27.17**
2	200	2.301	4	6	60	
3	100	2.000	3	7	70	
4	50.0	1.699	5	5	50	
5	25.0	1.398	5	5	50	
6	12.5	1.097	6	4	40	
7	6.25	0.796	6	4	40	
8	3.125	0.495	8	2	20	
9	1.5625	0.194	9	1	10	
10	0.78125	-0.107	10	0	0	

4.2.2 Preparation of Chloroform Extract Fraction Curve

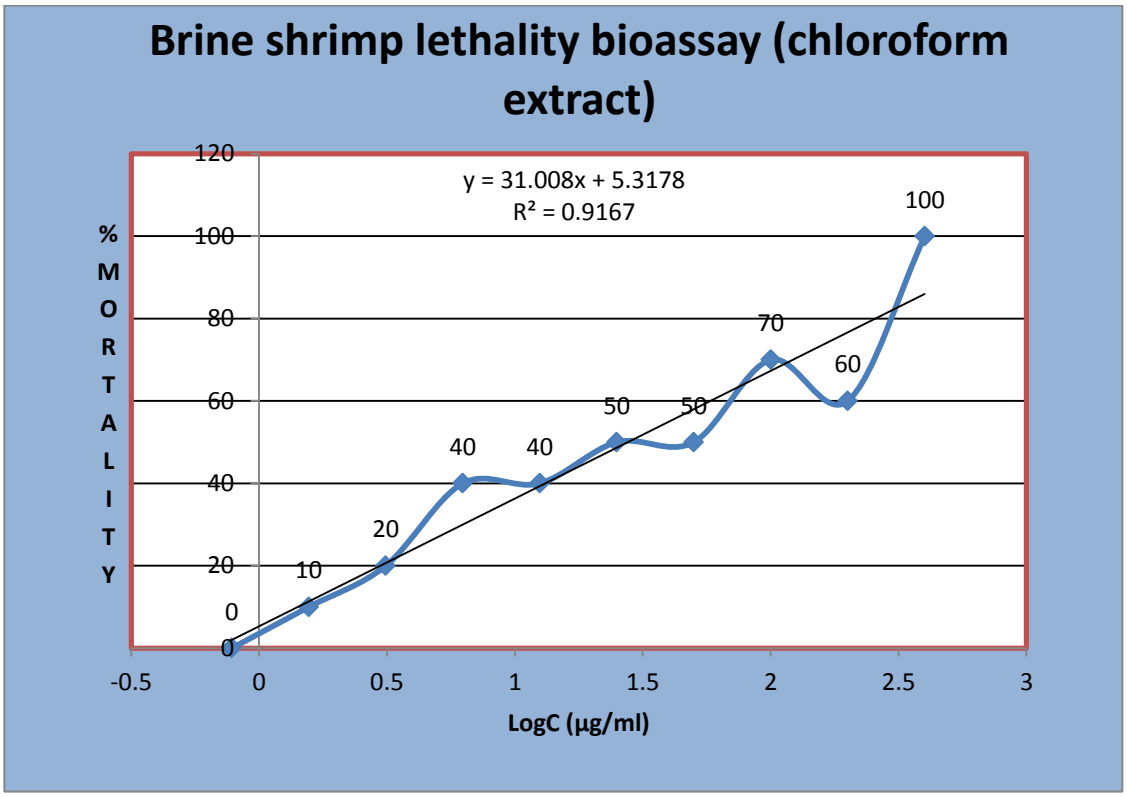

Figure 4.3: Plot of % mortality and predicted regression line of chloroform extract

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 chloroform 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 4.3and Table 4.4

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

Sample	Linear regression equation	R^2 value	LC_{50} (µg/ml, 24hr)
Standard(Tamoxifen)	y = 33.021x + 12.806	0.9891	13.38
Extract (Chloroform)	y = 31.00x + 5.317	0.916	27.61

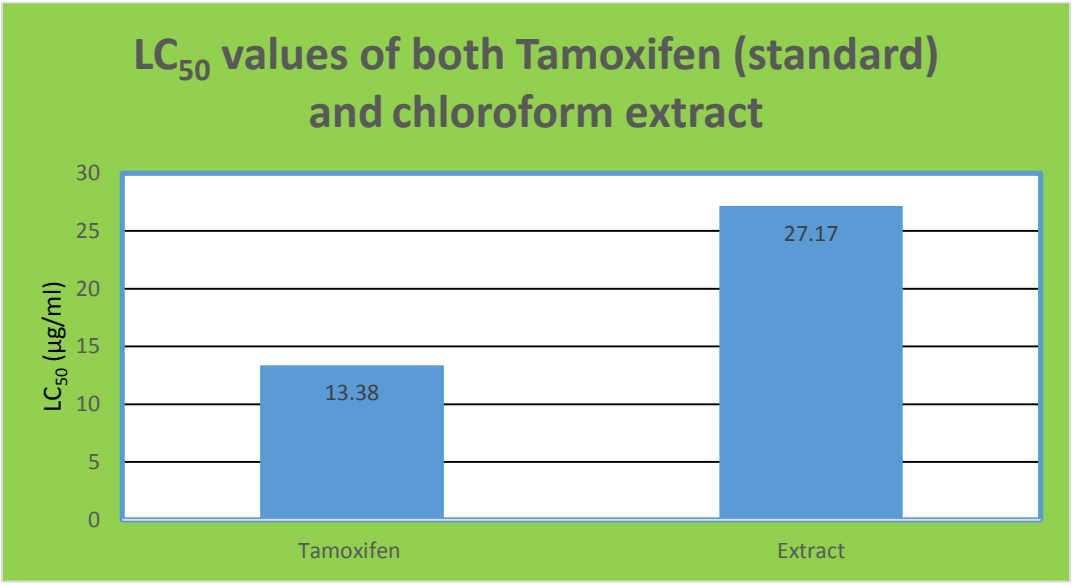

Figure 4.4 : Comparison of LC_{50} values of both Tamoxifen (standard) and chloroform extract

In this investigation, standard and chloroform 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 chloroform 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 chloroform extract of *Wedelia trilobata* leaves were examined in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The chloroform 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 (mm) of Standard and Chloroform Extract

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

Type of microorganism		**Zone of inhibition (mm)**	
		Standard	**Chloroform fraction**
Gram positive bacteria	*Bacillus cereus*	38	9
	Bacillus magaterium	38	0
	Bacillus subtilis	40	11
	Staphylococcus aureus	40	0
	Sarcina lutca	37	7
Gram negative bacteria	*Salmonella paratyphi*	38	8
	Salmonella typhi	36	6
	Vibrio parahemolyticus	40	0
	Vibrio mimicus	35	6
	Pseudomonas aureginosa	38	0
	Shegella dysenteriae	38	0
	Escherichia coli	36	8
	Saccharomyces cerevisiae	35	0

Fungi	*Candida bicans*	26	8
	Aspergillus niger	30	8

4.3.2 Discussion

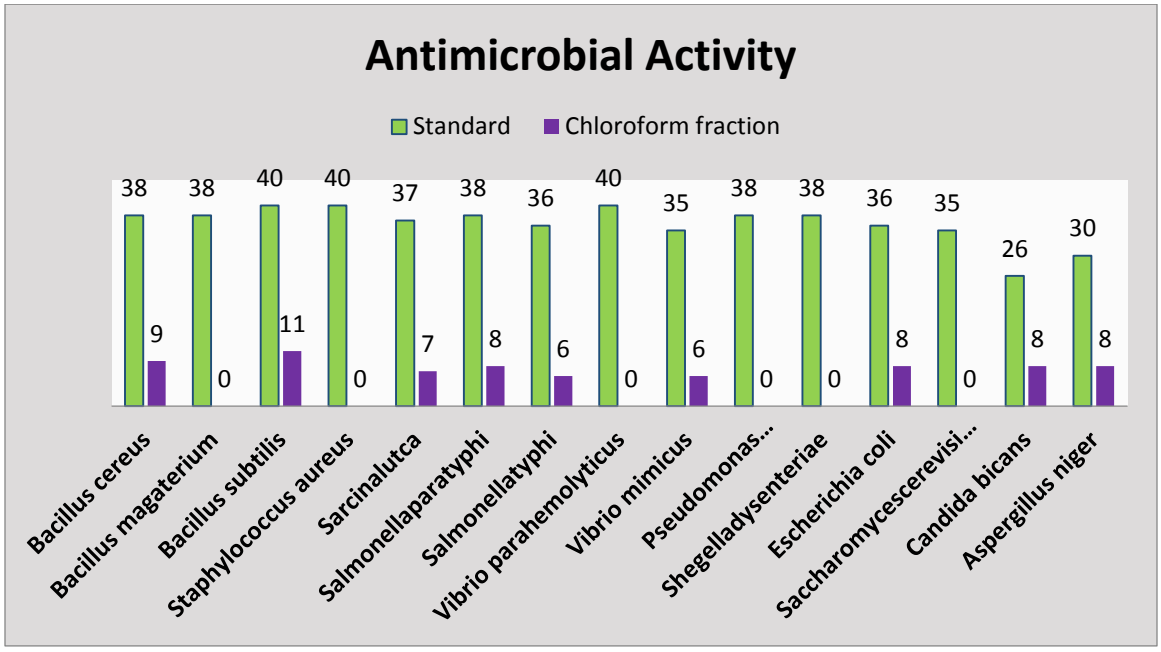

Fig 4.5: Comparison of activity between ciprofloxacin and chloroform fraction

Chloroform extract of *Wedilia trilobata* showed moderate to low antimicrobial activity when compared to Ciprofloxacin. None of the zone of inhibition of chloroform 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 (26mm).

Chapter Five
Conclusion

5.1 Conclusion

The results obtained in this study indicate that the chloroform extract of *Wedelia trilobata* leaves have significant cytotoxic activity. Investigations performed on the chloroform extract proved that the leaves contain higher amount of flavonoid compounds which represents that the extract have potent antioxidant property. Experimental evaluation showed that the leaves of this plant also possess slight antimicrobial properties. Since *W. trilobata* leaves exhibited potent cytotoxic activity, so the leaves can be further evaluated for anticancer, antiviral, pesticidal and antitumor properties (Meyer *et al*, 1982). 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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