In Vitro Biological Investigation of Leaves of *Eichhornia crassipes* (Pontederiaceae)

A project submitted by
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This project work is dedicated to my parents

Certification Statement

This is to certify that, this project titled 'In Vitro Biological Investigation of *Eichhornia crassipes* (Pontederiaceae)'submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Imon Rahman, Senior Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

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Abstract

*Eichhornia crassipes*is free floating aquatic plant and it belongs to the family Potenderiaecae and it is very native to South America. Though it is an aquatic plant but it also has therapeutic effect. Extracts of the various parts of the *E. crassipes* has therapeutic effect such as antimicrobial activity, anti-oxidant, wound healing, antitumor, cytotoxic, larvicidal activity. Leaf extract of this plat contain phenolic compounds, alkaloids, terpenoids, glycosides, sterols. The objective of this study was to investigate the bioactivity like cytotoxicity by lethal brine shrimp method, antimicrobial activity by disc diffusion methods from all the extractive of *Eichhornia crassipes*. The lethal brine shrimp study reveals the mild to moderate result by LC50. In antimicrobial test, this pant has negligible potentiality to act against bacterial and fungal strain.

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Abbreviations

WHO: World Health Organization

MESF: Methanolic Extract Soluble Fraction

PESF: Petroleum Ether Soluble Fraction

CSF: Chloroform Soluble Fraction

AQSF: Aqueous Soluble Fraction

VS: Vincristine Sulphate

DMSO: Dimethyl sulfoxide

NaCl: Sodium Chloride

HCl: Hydrochloric acid

FRAP: Ferric Reducing Ability of Plasma

MHA : Mueller Hinton Agar

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CHAPTER 1: INTRODUCTION

1.1 Rational of this Project Work

The use of medicinal plant was invented within early century. In early century some written evidence was found about utilizing the plant as purpose of cure verity of disease in the great civilization like Chinese, Indian, and North – African. It was the beginning of 19th century where the active particles of the medicinal plant were isolated to treat the diseases (Phillipson, 2000).

Today's clinical agents that are used frequently like Quinine from Cinchona bark, morphine and codeine from the latex of the opium poppy, digoxin from Digitalis leaves, atropine and hyoscine from species of the Solanaceae, those all were isolated before World War II (Phillipson, 2000).

Though the active particles were isolated during 19th century but the significance and importance of the natural product was experienced by the people as organic chemistry during the last century. (Macias, 2007)

To build the history of drug discovery the medicinal plants are used in treatment of various diseases from the ancient period. Plants part like roots, leaves, barks, flowers or fruits are being used to make the herbal medication. These parts of plants contain lots of chemical component and by isolating every desire component with the addition of bulk which leads to make the dosage form. To drug discovery, lots of new technologies are used such as combinatorial chemistry and computer-based molecular modeling design to create synthetic molecule day by day. Therefore the medicinal plants have a very significant history of clinical use which makes these more acceptable to the patient (Veeresham, 2012).

According to the ancient history, Dioscorides who wrote "De MateriaMedica" by studying medicinal plants was known as "the father of pharmacognosy'' according to the ancient history. He wrote this book it when he was travelling with roman army as he was a military physician by profession. His book is carrying lots of information about plant origin medications. In his book, total 944 drugs were describe and among 657 drugs of them were

from plant origins along with the description of appearance, locality, mode of collections and how to make the medicinal preparation and their therapeutic effects (Petrovska, 2012).

In 2008, World Health Organization (WHO) stated that among the most population of the world like 80% people depended on the medicinal plant that we called traditional medicine for their needs. WHO also state that there are approximately 600-700 plant based medications are available in Germany and 70% of the German physicians are more interested to prescribe those medications. Therefore, the history of use of traditional medicine has increased tremendously in United States over the last 20 years because of less tolerance of synthetic drug by the patient and cost of the drug (Derwich*et al.*, 2009).

In Bangladesh, the use of medicinal plant is also increased and acceptable to the patient. The reason behind to select an aquatic plant like *Eichhornia crassipes* for project work is that, *Eichhornia crassipes* is a widely distributed aquatic plant which belongs to the family of Pontederiaceae. In Asia, it is one of most known and available water hyacinth and mostly known as waste water weed. Bangladesh is the riverien country where aquatic plant such as *E. crassipes* has grown naturally with a vest source. If the lead compound have found in a significant amount, then this aquatic plant can be cost effective as an active ingredient and this will also be beneficial for the developing country.

1.1.1 Aim of the project

Aim of this project is to investigate and evaluate the bioactivity study of leaves extract of water hyacinth *Eichhornia crassipes*

1.1.2 Objective of the project

The objective of the project is to using methanolic leaf extract of *Eichhornia crassipes*to:

- Determine the Brine shrimp lethality test for the cytotoxic effects of all the fractions of water hyacinth.
- Determine the antimicrobial activity by disc diffusion method of all fraction of water hyacinth.

1.2 The plant family: Pontederiaceae

Pontederiaceae families' plants are a small flowering of aquatic plant which is belongs to the herbaceous monocotyledons. 6-9 genera and about 30-35 species are comprised of this family which is mostly native to the New World Tropics. Besides their native range many taxa had been spreading as weed or ornaments (Eckenwalder& Barrett. 1986).

It is recognized by The APG II system of 2003 where it spaces the family in the order Commelinales. This family is small where heterostylous aquatic plants are identified and these are found in tropical and subtropical level of waters. *Eichhornia crassipes* is known as water hyacinth in this family which is an invasive species in many waterways ((Angiosperm Phylogeny Group, 2009).

1.3 The plant genus: *Eichhornia*

Eichhornia is a water hyacinth which is belongs to the genus of aquatic flowering plants under the Pontederiaceae and very native to the South America.

1.4 Identification

The leaves are thick and dark and also flatten on top of the water like as mat. The purple flower is on the top of its mat along with its reproductive organ as the leaf is a floating macrophyte. The roots of water hyacinths are thick and dense by which many macro and micro use to invertebrates of small or juvenile fish. This plat lives in a colony that is created by them (Ellis, 2011).

Figure 1.1: Figure of *Eichhornia crassipes*

1.5 Distribution

In 1823, a German naturalist C. von Martius was discovered this species during his study of the flora of Brazil then he named this as *Pontederia crassipes*. After sixty years from the discovered this species, scientist Solms integrated this species in the *Eichhornia* genus followed by the description of Kuntz in 1829 (Tellez *et al*, 2008).Originally from the Amazon Basin, its entry into Africa, Asia, Australia, and North America was facilitated by human activities (Dagno et al., 2012).Wetland like lakes, streams, ponds, waterways, ditches, and backwater are widely found areas where waterhyacinth can grow over (Jafari, 2010).

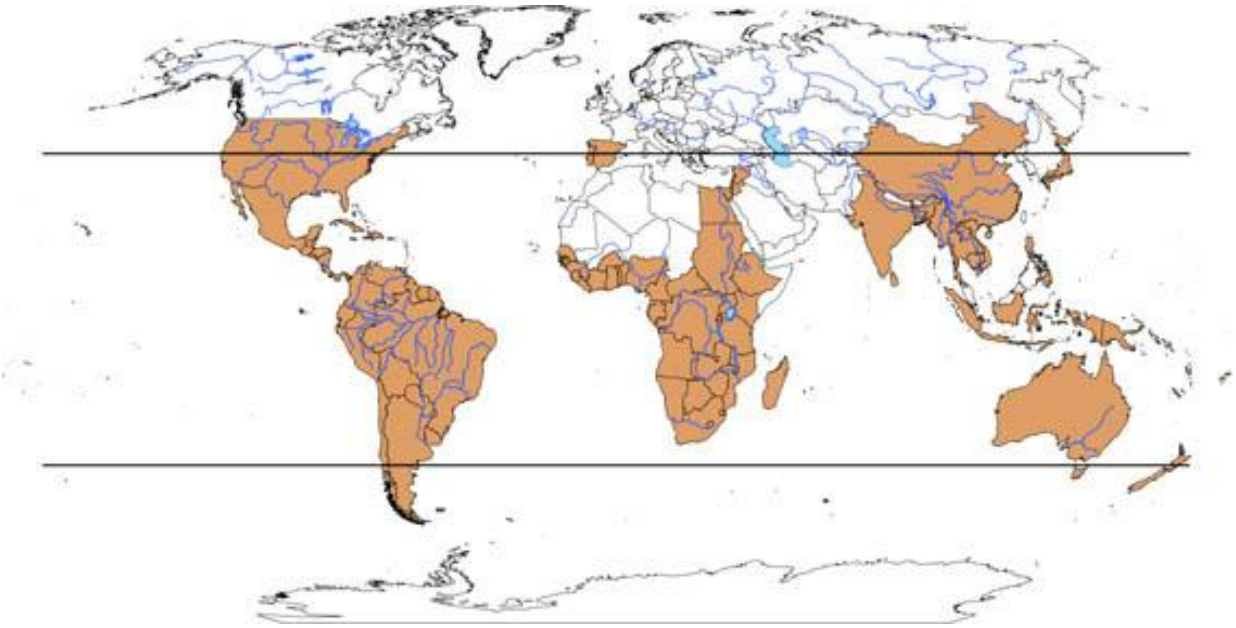

Figure 1.2: Distribution of water hyacinth throughout the world

1.6 Ecological Factors

Warm water which is rich in macronutrients is the best for growing heliopath plant like waterhyacinth. This plant is grown neutral pH but it can also tolerate pH values from 4 to 10.

This pH tolerance points out that *Eichhornia crassipes* can be used for treatment of different types of wastewater. *Eichhornia crassipes* can also tolerate drought well because it cansurvive in moist sediments within several months (Center *et al.*, 2002). Air humidity that is low from 15% to 40% can be limiting factor for uninterrupted growth of water hyacinth (Jafari, 2010).

1.7 Uses of *Eichhornia crassipes* (Jafari, 2010)

There are possible uses of *Eichhornia*c rassipes were found in the study of Jafari (2010). These are listed below

1.7.1 Paper

The production of paper of *Eichhornia crassipes* has been successful in a number of countries such as Philippines, Indonesia, and India though it was a small-scale cottage industry.

1.7.2 Fibre Board

Another application of water hyacinth is the production of fibre boards for general-purpose use is also another application of waterhyacinth and it is cost low.

1.7.3 Yarn and Rope

To make the rope, the waterhyacinth plant can be used as fiber from the stems. The process of rope making is similar to the jute rope. A local furniture manufacturer from Bangladesh, who is wins the rope around a cane frame to produce an elegant finished product by the use of rope.

1.7.4 Basket Work

Dried water hyacinth is used to make baskets and matting for domestic uses in Philippines. The traditional basket making and weaving skills are used to produce similar goods for the tourist industry in India.

1.7.5 Charcoal Briquetting

To deal with the rapidly expanding carpets of water hyacinth in Kenya charcoal briquetting has been a proposed idea.

1.7.6 Biogas Production

The conversion of water hyacinth to biogas is the biggest possibility. Many experiments were carried out to convert biogas from waterhyacinth along with animal waste.

1.7.6 Animal Fodder

In a study, it showed that the amounts of nutrients that are needed for animal are present in water hyacinth to ruminants. The use of water hyacinth as animal fodder in developing countries can help to solve some of the nutritional problems that exist in these countries.

1.7.7 Fertilizers

Water hyacinth can also be used on the land both as a green manure and as compost. The compost of waterhyacinth can increases soil fertility and crop yield and also improves the quality of the soil. In developing countries, water hyacinth is the best option to use as fertilizer on the land and also in poor soil quality to avoid expensive mineral fertilizer.

1.7.8 Fish Feed

Water hyacinth can be used indirectly to feed fish.

1.8 Phytochemicals Composition

Eichhornia crassipes a waterhyacinth is a fast growing plant which can be used for rapid removal of different kinds of pollution in water ensuing in positive outcomes. Waterhyacinth was estimated for its possible potential of heavy metal accumulations which further lead in the discovery of high cellulose content and its functional groups such as amino (-NH2), carboxyl (COO-), hydroxyl (-OH-), sulfahydryl (-SH) and towards the heavy metals adsorption was showed with high tolerance and affinity (Patel, 2012). Phytochemicals such as amino acids including glutamic acid theonine, leucine, lysine, methionine, tryptophan, tyrosine, and valine, flavonoids including apigenin, azaeleatin, chrysoeriol, gossypetin, kaempferol, luteolin, oientin and tricin are contained by *Eichhornia crassipes*. In this study, the dry mass of the plants is consisted of 5.2% nitrogen, 0.22% of phosphorous, 2.3% of potassium, 0.36% of calcium, 280 ppm of Iron, 45 ppm of Zn, 2 ppm of Cu and 332 of Mn (Patel, 2012)

CHAPTER 2: LITERATURE REVIEW

2.1 Previously studied pharmacological properties of *E. crassipes*

Literature reviews of *E. crassipes* have been carried out and the results indicated the different pharmacological activities like anti-microbial, anti-oxidant, wound healing, antitumor, cytotoxic, larvicidal activity.

2.1.1 Anti- microbial activity

The antimicrobial activity of different kind of plant extract has been evaluated by researches. The methanol extract and its fractions have been gone through under the investigation of antimicrobial (bacterial and fungal) and anti-algal activities (green microalgae and cyanobacteria) by using of the paper disc diffusion bioassay. The extract of water hyacinth showed the activity by using of technique against the bacterial strains like *Staphylococcus aureus, Escherichia coli, Penicillium* and *Aspergillus niger* under the condition of pH, concentration and action time. Water hyacinths metanolic extract showed the activity against *Alternaria alternata*, *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizoctonia solani,* and *Xanthomonas compestries* (Tyagi&Agrawal, 2014).

Another study has also been carried out for investigating of antimicrobial activity by using of disc diffusion method. Firstly, in a nutrient broth the bacterial isolates of *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, and Salmonella typhimureium* were grown for one day.

In a sterile petri plates the sub-cultured broth (500 µl) was transferred. Mueller Hinton Agar (MHA) around 1 mL was poured on it and rested for settle down (30 minutes). 150 µl of crude extracts were placed in plates with soaked filter paper and were kept in incubator for one day.

Antibiotic discs of ampicillin and tetracycline were used as control and the antibacterial activity was determined by the measuring of zone of inhibition around the disc wall. In this study, the extracts, though very low show a mild inhibitory effect on Bacillus species (Aravind*et al,* 2013).

2.1.2 Anti-oxidant activity

Hydroponically *Eichhornia crassipes* was exposed to various concentrations of metals like Ag, Cd, Cr, Cu, Hg, Ni, Pb and Zn for 21 days and the activity of catalase, peroxidase and superoxide dismutase were increased and differential inducement also increased among those metals. In this study, Zn had the least and Hg had the highest inducement of the antioxidant enzyme in *Eichhornia crassipes* and *Pistiastratiotes*. Increasing absorbance was showed by the reducing power of the aqueous extract and fractions such as ethanol, aqueous, methanol and aqueous. Waterhyacinth also evaluated for their reducing power capability at five different concentrations which was related to their high antioxidant capacity. The hydrolyzed extract has good DPPH scavenging activity which was showed by the DPPH scavenging assay of the light petroleum, acetone, ethyl acetate, aqueous, and hydrolyzed extracts, and fractions (Tyagi&Agrawal, 2014).

In another study, FRAP (Ferric Reducing Ability of Plasma) was estimated. 40 μL plasma and 2mL of working FRAP solution were allowed to react which containing acetate buffer (pH 3.6),10mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40mM HCl, and 20mM FeCl3·6H2O in the ratio of 10:1:1 at 37 ^{0}C. In a UV-Vis double beam spectrophotometer, Fe+2-TPTZ complex was measured at 593 nm with time scanningat 30-second and intervals for 4 minutes. In FRAP content (6.25 μmol Fe (II)/L plasma), isoniazid treated rats were showed decrease compared with control group (12.38 μmol Fe (II)/L plasma). *E. crassipes* leaf aqueous extract (at 400mg/kg body weight) treated group showed increase in total antioxidant capacity of plasma (8.96 μmol Fe (II)/L plasma) content in Wistar rats Liv-52 treatment showed the appreciable mitigation of isoniazid induced decrease in FRAP content (Kumar *et al*,2014).

2.1.3 Wound Healing Activity

The methanolic extract of water hyacinth leaves were investigated for their wound healing activity in an excision experiment model of wounds rats in the form of an ointment which was prepared by two different concentrations (10% and 15%, w/w of leaf extract in a simple

ointment) and this study showed a significant wound contraction ability compared with controlled one (Tyagi&Agrawal, 2014).

2.1.4. Antitumor Activity

Methanolic extract of waterhyacinth (50%) leaves were investigated for antitumor activity at different doses and this showed a good response where this experiment was carried out against B16F10 *in vivo* melanoma tumor bearing hybrid mice models. In this study some fractions were exhibited selective anticancer activity against a liver cancer cell line, on the other hand rest of fractions were exhibited high anticancer activity against hormone dependent tumor types (cervix and breast cancers) (Tyagi&Agrawal, 2014).

2.1.5 Cytotoxic Activity

A study carried out by Kumar *et al* (2014) on anticancer activity of *S. podophyllum* and *E. crassipes* leaf extracts against T47D, PC3, NCI-H322, and A549 cell lines, where aqueous fraction of both *S. podophyllum* and *E. crassipes* leaf extracts showed 51% and 44% cytotoxic potential against NCI-H322 cell line.

2.1.6 Larvicidal Activity

From the different concentration of crude root extracts of E. crassipes, larvicidal activity was investigated with *Chironomus ramosus* Chaudhuri eggs and larva and this investigation showed 100% efficiency. With the light petroleum, ethyl acetate, and aqueous extracts, and methanol and ethanol fractions, larvicidal, pupicidal and repellent activity carried out against *Culexquinque fasciatus*in the laboratory showed good activity (Tyagi&Agrawal, 2014).

2.1.7 *Eichhornia crassipes* as Adsorbate

From this study, it showed that Waterhyacinths were efficiently removes a vast range of Pollutants, from suspended materials, nutrients and organic matter to heavy metals and pathogens (Tyagi&Agrawal, 2

CHAPTER 3: METHODOLOGY

3.1 Chemical Work

Eichhornia crassipes belonging to the family Pontederiaceae, was investigated for its chemical constituents.

3.2 Chemical investigation of *Eichhornia crassipes*

3.2.1 Collection and preparation of the plant material

The leaves of *Eichhornia crassipes* were collected from near Dhaka in March, 2016. The plant was identified at the Bangladesh National Herbarium though it is most known and available aquatic plant in this region, when a voucher specimen has been deposited for this collection. After collecting the leaves, this was properly washed and then the leaves were sun dried for several days. The dried leaves were then ground to a coarse powder using high capacity grinding machine.

3.2.2 Extraction of the plant material

280 gm of the powdered material was taken in a clean, round bottomed flask (3 liters) and soaked in 2.5 liter of methanol. Then the container was properly sealed with aluminum foil paper and kept for 12 days accompanying infrequent shaking and stirring. After then the whole mixture was filtered through a fresh cotton plug. Then the volume of the filtrate was reduced by using a Buchii Rota vapour and the weight was measured 80 gm.

3.2.3 Solvent-Solvent partition of crude extract by Modified Kupchan Partition (VanWagenen*et al*., 1993)

A protocol that designed by Kupchan and modified by Van Wagenen et al. (1993) was used to solvent-solvent partitioning. The crude extract (5 gm) was taken in a beaker and dissolved in 10% aqueous methanol. It was extracted with petroleum ether, then with chloroform and finally with ethyl acetate.

3.2.3.1 Partitioning with petroleum ether

The main solution was taken in a separating funnel. 100 ml of the petroleum ether was added to it and the funnel was gentle shaken and then kept undisturbed. Then the organic portion was collected. The whole process was repeated three times and the fractions collected were evaporated together.

3.2.3.2 Partitioning with chloroform

To the main solution that left after washing with petroleum ether, 16 ml of distilled water was added and mixed uniformly. The main solution was then taken in a separating funnel and extracted with dichloromethane ($CHCl_3$) (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction.

3.2.3.3 Partitioning with ethyl acetate

To the main solution that left after washing with petroleum ether, and dichloromethane, 20ml of distilled water was added and mixed uniformly. The main solution was taken in a separating funnel and extracted with ethyl acetate ($CH_3COOC_2H_5$) (100 ml x 3). The $CH_3COOC_2H_5$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction.

3.3 BRINE SHRIMP LETHALITY BIOASSAY

3.3.1 Introduction

Brine shrimp lethality bioassay is a kind of bioassay that is proficient of distinguishing a board spectrum bioactivity presence in a crude extract. Brine shrimp lethality bioassay is prognostic of cytotoxicity and pesticidal activity. In 1982, this BSLT was introduced and successfully implied for bioassay of active cytotoxic and antitumor agents with vivo lethality test (Pisutthanan *et al*, 2004). In 1998 McLaughlin's study claimed that brine shrimp lethality bioassay is one of the rapid and extensive bioassay for the bioactive compound. To test the bioactivity of the natural extracts, fractions and also the pure

compound brine shrimp lethality bioassay is used. Brine shrimp nauplii are used as an approving monitor of new bioactive natural products for selection and fractionation in the detection.

3.3.2 Principle (Olowa & Nuneza, 2013)

For determining cytotoxic effect, brines shrimp bioassay is one of the most renowned and conventional method by that cytotoxic effect of extract of any plant constitute easily determined. In simulated sea water brine shrimp eggs are hatched to get nauplii. To preparation of desire concentrated test samples required amount of dimethyl sulphoxide (DMSO) are added. After hatching the nauplii are counted by inspecting visually. The nauplii are taken into vials that containing simulated sea water (5ml) after that the different concentrated samples are added to the pre-labeled vials by micropipette. Then vials are reserved in well facilitated room for a day and nauplii were counted after 24 hours.

3.3.3 Materials

- *Artemiasalina* leach (brine shrimp egg)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes
- Micropipette
- Glass vials
- Magnifying glass
- Test tubes
- Test samples of experimental plant

Table 3.1. Test samples of experimental plants

Plant part	Sample code	Test Sample	Calculated amount (mg)
	ME	Methanolic extract	4.0

Leaves of *Eichhorniacrassipes*	PESF	Petroleum ether soluble fraction	4.0
	CSF	Chloroform soluble fraction	4.0
	Ethyl acetate soluble fraction	Ethyl Acetate soluble fraction	4.0
	AQSF	Aqueous soluble fraction	4.0

3.3.4 Experimental Procedure

3.3.4.1 Preparation of seawater

Firstly, 38g of sea salt (pure NaCl) was weighed and then dissolved in one liter of distilled water. After that the solution was filtered to get clear solution.

3.3.4.2 Hatching of brine shrimps

Brine shrimp eggs (*Artemiasalina* leach) that was used for the cytotoxicity test, was collected from the pet shops. In a small tank, sea waterwas taken and shrimp eggs were added to the sea water tank. Then the rest portion of the tank was covered. Tomature the shrimp eggs as nauplii, shrimp eggs were allowed to hatch for one day. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment.10 living shrimps were added to each of the test tubes containing 5 ml of seawater through a Pasteur pipette.

3.3.4.3 Preparation of test samples of the experimental plant

The test samples (Table 3.1) were taken into the vials and to prepare stock solutions 100 µl of pure dimethyl sulfoxide (DMSO) was dissolved. Then in the first test tube, 50 µl of solution was taken which were containing 5 ml of simulated seawater and 10 shrimp nauplii. 400µg/ml was the final concentration in first test tube. Then using of serial dilution method different concentrated series of solutions was prepared from the stock solution. In every test tube, 50 µl of samples and fresh 50 µl of DMSO were added to vial. Consequently different concentrations were found in the different test tubes (Table 3.2).

Table 3.2: Test samples with concentration values after serial dilution

Test Tube No.	Concentration (µg/ml)
1	400.0
2	200 .0
3	100 .0
4	50 .0
5	25 .0
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

3.3.4.4 Control group preparations

There are two kinds of control groups for the determination of cytotoxic activity. There are

i) Positive control
ii) Negative control

3.3.4.4.1 Preparation of the positive control group

In a cytotoxicty study the positive control group is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In this study vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions are made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. Then the positive control solutions were added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

3.3.4.4.2 Preparation of the negative control group

DMSO of 100 µl was added to each of ten premarked glass vials which were containing 5 ml of simulated sea water and 10 shrimp nauplii so that it can be used 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.

3.3.4.4.3 Counting of nauplii

The vials were inspected with the help of using a magnifying glass after 24 hours and the numbers of survivors were counted. The percentage (%) of mortality was calculated for each dilution. By the help of linear regression, concentration-mortality data were analyzed statistically with a simple IBM-PC program. The (LC_{50}) median lethal concentration values are represent the effectiveness or the concentration-mortality relationship of plant product. This median lethal concentration represents the concentration of the chemical that makes death in half of the test subjects' animal after a certain period.

3.4 ANTIMICROBIAL SCREENING

3.4.1 Introduction

Infectious diseases are known as the major problem in worldwide. Synthetic antibacterial drugs are expensive and inadequate and also most of the time this showed the side effects on human health. The multidrug resistances of microbial strains are continuously increasing. In a study, it is showed that plants secondary metabolites have beneficial medicinal effects on humans because of their interaction with potential target sites (Kumar, 2014).

Antibacterial agents are the natural or semi-synthetic or synthetic compound that is capable to kills or inhibits the growth of bacteria without doing any damage to the host. Antibiotics is a Greek word which has meaning such as, anti means against, and bios means life which refers to all agents that act against microbial organisms. Bacteria are one cell organism with having different characteristics. Many bacteria lives in human body and helps in metabolism because not all bacteria are harmful to human health. Microorganisms are easily susceptible to new drugs but there is less possibility to invent new drugs which can meet the increasing demand of antibacterial agent. Discovery of new drug is getting essential day by day to prevent the antibacterial diseases. Nowadays medicinal plants are referred as a good source of antimicrobial agent which is able to gives significant of therapeutic effects (Prasad, Sudha, Khadri, &Riazunnisa, 2015).

In 1981, death caused by infectious disease was ranked in 5^{th} position and in 1992 this disease became in the 3rd leading cause of death with an increasing number of 58% . In USA, 8% of death is caused by the infectious diseases (Pinner *et al*., 1996).To inhibit the growth of multidrug resistant *Pseudomonas aeruginosa*, plant extracts from various parts of the plant have been used and the plant products are clove, jambolan, pomegranate, thyme, and lantana. To develop new infection-fighting strategies to control microbial infections, it is mandatory to search for new antimicrobial agents from natural sources (Kumar, 2014).

The antimicrobial screening is the very initial phase for antimicrobial drug research which will perform to determine the susceptibility of various microorganisms to any agent. This test measures the ability of every test sample to stop the *in vitro* microorganisms' growth. This ability can be estimated by disc diffusion methods (Ayafor, 1972).

3.4.2 Principle of disc diffusion method

By disc diffusion methods, sensitivity tests were performed by following NCCLS (1993) protocol (Chattopodhay *et al*, 2002). Antibiotics diffuse from a confine source through the nutrient agar gel and creates a concentration gradient, it is known as classical method. The test samples of known amount which become dried and sterilized filter paper discs (6 mm diameter) are placed on nutrient agar medium uniformly seeded with the test microorganisms. For positive and negative control standard antibiotic (Ciprofloxacin) discs and blank discs are used. For the maximum diffusion of the test materials both plates are kept at minimum temperature (4°C) for 24 hours in the surrounding media (Barry, 1976). Then the plates are reversed and incubated at 37°C for 24 hours for most favorable growth of the organisms. A clear zone is seen around the disc is indicated the test materials having antimicrobial property that inhibit microbial growth in the media and this distinct area defined as zone of inhibition. Then the antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976)

3.4.3 Experimental Work

3.4.3.1 Apparatus and reagents

Filter paper discs	Autoclave
Nutrient Agar Medium	Laminar air flow hood
Petridishes	Spirit burner
Sterile cotton	Refrigerator
Micropipette	Incubator
Inoculating loop	Chloroform
Sterile forceps	Ethanol
Screw cap test tubes	Nosemask and Hand gloves

3.4.3.2 Test organisms

The microorganisms strains used for the experiment were collected as pure cultures. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 3.3

Table 3.3: Different strains used in antimicrobial screening

Gram positive Bacteria	Gram negative Bacteria	Fungi
Bacillus cereus *Bacillus megaterium* *Bacillus subtilis* *Sarcinalutea* *Staphylococcus aureus*	*Escherichia coli* *Salmonella paratyphi* *Salmonella typhi* *Shigellaboydii* *Shigelladysenteriae* *Pseudomonas aeruginosa* *Vibrio mimicus* *Vibrio parahemolyticus*	*Aspergillus niger* *Candida albicans* *Sacharomyce scerevacae*

3.4.3.3: Test materials

Table 3.4: List of Test materials

Plant part	Sample code	Test Sample
Leaves of *E. crassipes*	ME	Methanolic extract of Leaves of *E. crassipes*
	PESF	Petroleum ether partitionate
	CSF	Chlorofrom soluble partitionate
	EA SF	Ethyl acetate soluble partitionate
	AQSF	Aqueous soluble partitionate

3.4.3.4: Ingredients of culture medium

The subsequent media was used usually to exhibit the antimicrobial activity and to make subculture of the test organisms.

Nutrient agar medium	
Ingredients	Amount
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
pH	7.2 + 0.1 at 250C

Nutrient agar medium is the most frequently used and also used in the present study for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

3.4.3.5: Preparation of the medium

Every ingredient were calculated and seized in a conical flask. Then the distilled water was purred into that conical flask to prepare the necessary volume of this medium. In a water bath the contents were heated for a clear solution. With the help of sodium hydroxide and hydrochloric the amount of 10 ml, the pH was adjusted at 7.2-7.6 besides to prepare plates and slants respectively, the medium (5ml) was transferred in screw cap test tubes. For making fresh culture of microorganisms the slants were utilized.

3.4.3.6: Sterilization procedure

The antimicrobial screening was done in Laminar Hood so that no contamination, or cross contamination can be occurred by the test organisms. And precautions were taken and maintained. UV light in the Laminar Hood was switched on one hour before working and petri dishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Each and every equipments were also sterilized be start the work by UV light.

3.4.3.7: Preparation of subculture

The test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures under laminar air cabinet in an aseptic condition. For their optimum growth the inoculated strains were then incubated for 24 hours at 370C.

3.4.3.8: Preparation of the test plate

The test organisms were transferred to the test tubes containing about 10 ml of dissolved and sterilized agar medium in an aseptic area. To get a uniform suspension of the organisms the test tubes were shaken by rotation. The In the sterilized petri dishes bacterial and fungal suspension was immediately transferred and the petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

3.4.3.9: Preparation of discs

Measured amount of each test sample was measured and dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. In a blank petri dish

Sterilized metrical filter paper discs were taken under the laminar hood. After that discs were soaked with solutions of test samples and dried.

Table 3.5: Preparation of sample Discs

Plant part	Test Sample	Dose µg/disc	Required amount for 20 disc (mg)
Leaves of *E. crassipes*	Methanolic extract of leaves	400	8.0
	Petroleum ether partitionate	400	8.0
	Ethyl acetate soluble partitionate	400	8.0
	Chloroform soluble partitionate	400	8.0
	Aqueous soluble partitionate	400	8.0

Standard Ciprofloxacin (30 µg/disc) discs were used as positive control to determine the activity of standard antibiotic against the test organisms and also for comparison. And for negative controls blank disc were used that guarantee that the remaining solvents and the filter paper were not vigorous for microbial growth.

3.4.3.10: Diffusion and incubation

In a sample disc the bacteria and fungi were positioned, the standard antibiotic discs and the control discs on the previously marked zones in the agar plates pre-inoculated. Then the plates were kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

3.4.3.11: Determination of the zone of inhibition

The zone of inhibition was determined by observation of their activity. And this zone of inhibition indicated the antimicrobial potency of the test samples. The clear zone of inhibition surrounding disc was stopping the further expansion of the microorganisms. When incubation was done then by measuring the diameter of the zones of inhibition in millimeter with a transparent scale, the antimicrobial activities of the test materials were determined.

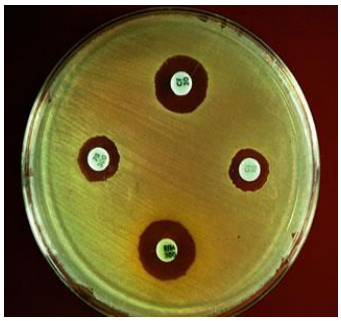

Figure 3.1A: Clear zone of inhibition inhibition

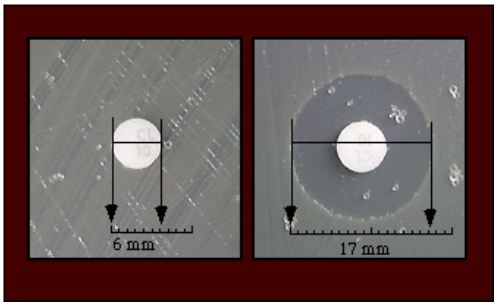

Figure 3.2B: Determination of clear zone of

CHAPTER 4: RESULT and DISCUSSION

4.1 Result and discussion of Brine Shrimp Lethality Test of *Eichhornia crassipes*

The methanolic extract (ME) of leaves of *Eichhornia crassipes* and its different partitionates i.e. petroleum ether (PESF), chloroform (CSF),ethyl acetate soluble fraction and aqueous soluble fractions (AQSF) were tested for brine shrimp lethality bioassay following the procedure of Olowa&Nuneza, 2013. The LC50 of the all test samples were determined by plotting the percentage of mortality rate of the brine shrimps against the logarithm of the sample concentration and the curve data of regression analysis helps in obtaining the best fit-line. In the present study, vincristine sulphate was used as positive control and the LC50 value was found to be 0.45µg/mL. The positive control VS gave significant mortality compared to the negative control. The LC50 values of different partitionate of extracts were compared to the positive control.

Among all the extractive MESF showed the highest lethality with value of 6.841 µg/ml. The LC_{50} values of PE, CSF, Ethyl acetate SF, and AQSF were found to be 10.186 µg/ml, 18.662µg/ml, 11.902 µg/ml and 79.054µg/ml, respectively

Table 4.1: LC_{50} values of the test samples of leaves of *Eichhornia crassipes*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
VS	y = 30.99x + 60.76	0.963	0.45
ME	y = 28.81x+25.94	0.969	6.841
PESF	y = 28.68x + 21.09	0.890	10.186
CSF	y = 32.39x+8.833	0.865	18.662
EASF	y = 27.10x+20.23	0.932	11.902
AQSF	y = 22.66x+6.993	0.836	79.054

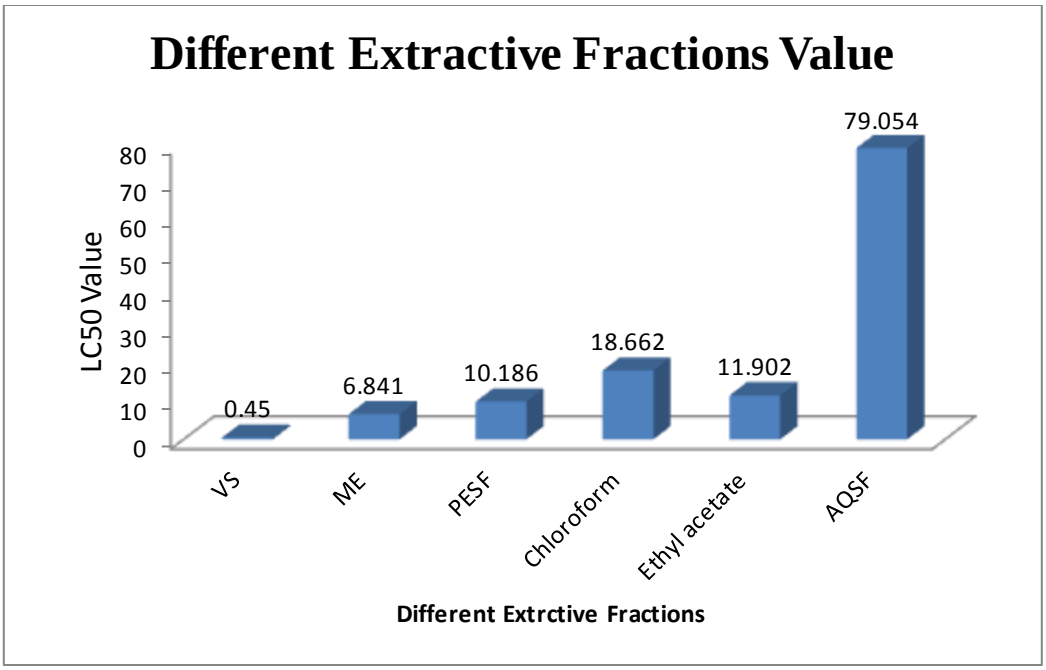

Figure 4.1: LC_{50} values of the different extract fraction of leaves of *Eichhornia crassipes*

Table 4.2: Effect of Vincristine sulphate (positive control) on shrimp nauplii

Conc. (µg/mL)	Log_{10} Conc.	% Mortality	LC_{50} (µg/mL)
0		0	0.45
0.039	-1.4089	20	
0.078125	-1.1072	30	
0.15625	-0.8061	30	
0.3125	-0.5051	40	
0.625	-0.2014	50	
1.25	0.09691	70	
2.5	0.39794	80	
5	0.6989	80	
10	1.00	90	
20	1.30102	100	

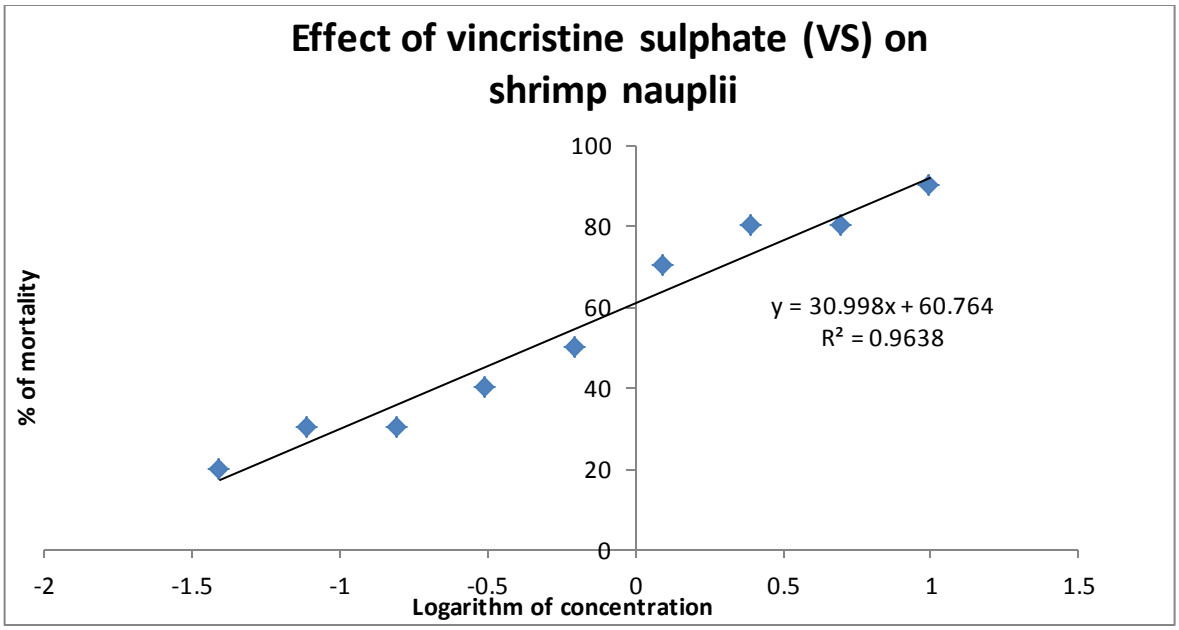

Figure 4.2: Plot of % of mortality and predicted regression line of VS

Table 4.3: Effect of the methanolic extract (ME) of leaves of *Eichhornia crassipes* on shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50} (µg/mL)
0	-	0	6.841
0.78125	-1.1072	0	
1.5625	0.19382	20	
3.125	0.49485	40	
6.25	0.79588	50	
12.5	1.09691	60	
25	1.39794	70	
50	1.69897	70	
100	2	80	
200	2.30103	100	
400	2.60206	100	

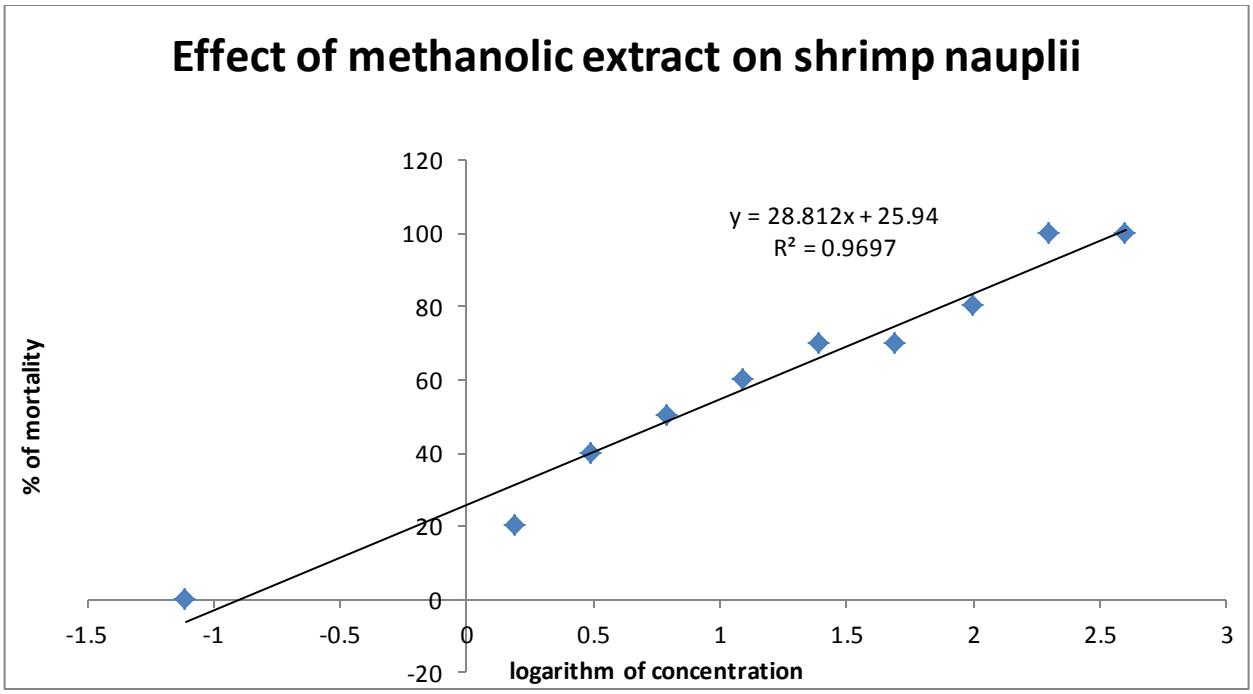

Figure 4.3: Plot of % mortality and predicted regression line of ME

Table 4.4: Effect of petroleum ether soluble fraction (PESF) of the methanolic extract of leaves of *Eichhornia crassipes* shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50}(µg/mL)
0	-	0	10.186
0.78125	-1.1072	10	
1.5625	0.19382	10	
3.125	0.49485	20	
6.25	0.79588	40	
12.5	1.09691	50	
25	1.39794	60	
50	1.69897	70	
100	2	90	
200	2.30103	90	
400	2.60206	100	

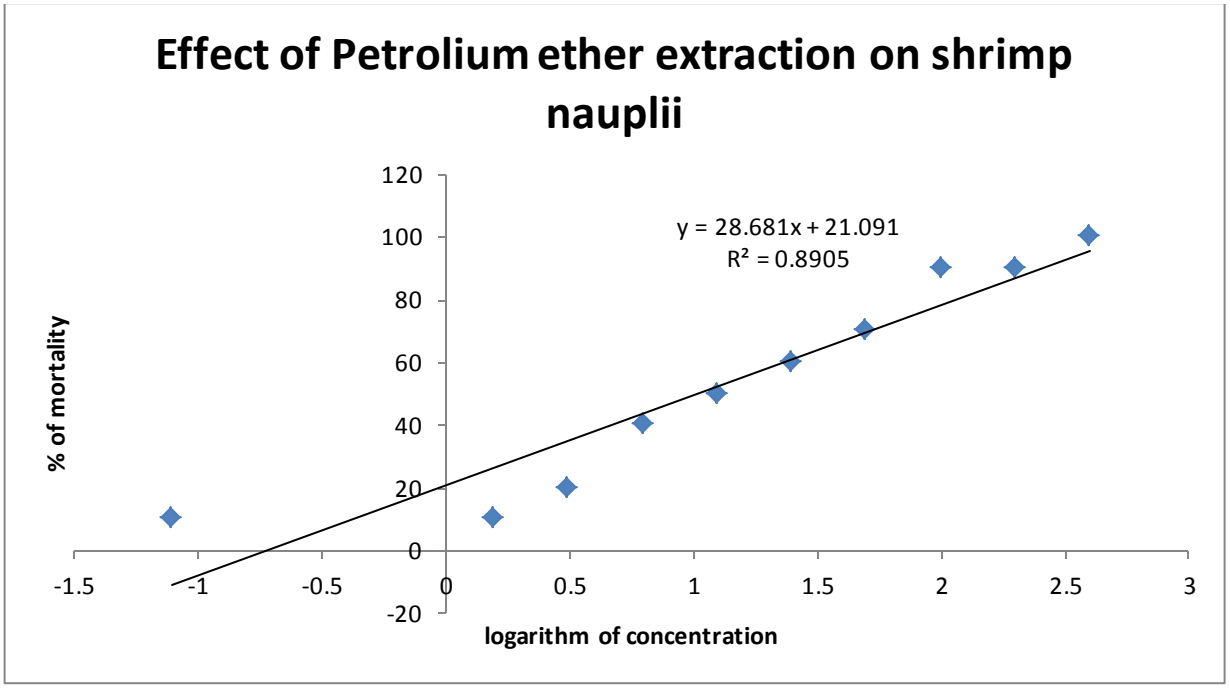

Figure 4.4: Plot of % mortality and predicted regression line of PESF

Table 4.5: Effect of Chloroform soluble fraction (CSF) of the methanolic extract of leaves of *Eichhornia crassipes* on shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	% of mortality	LC_{50}(µg/mL)
0	-	0	18.662
0.78125	-1.1072	0	
1.5625	0.19382	0	
3.125	0.49485	10	
6.25	0.79588	20	
12.5	1.09691	40	
25	1.39794	50	
50	1.69897	60	
100	2	80	
200	2.30103	100	
400	2.60206	100	

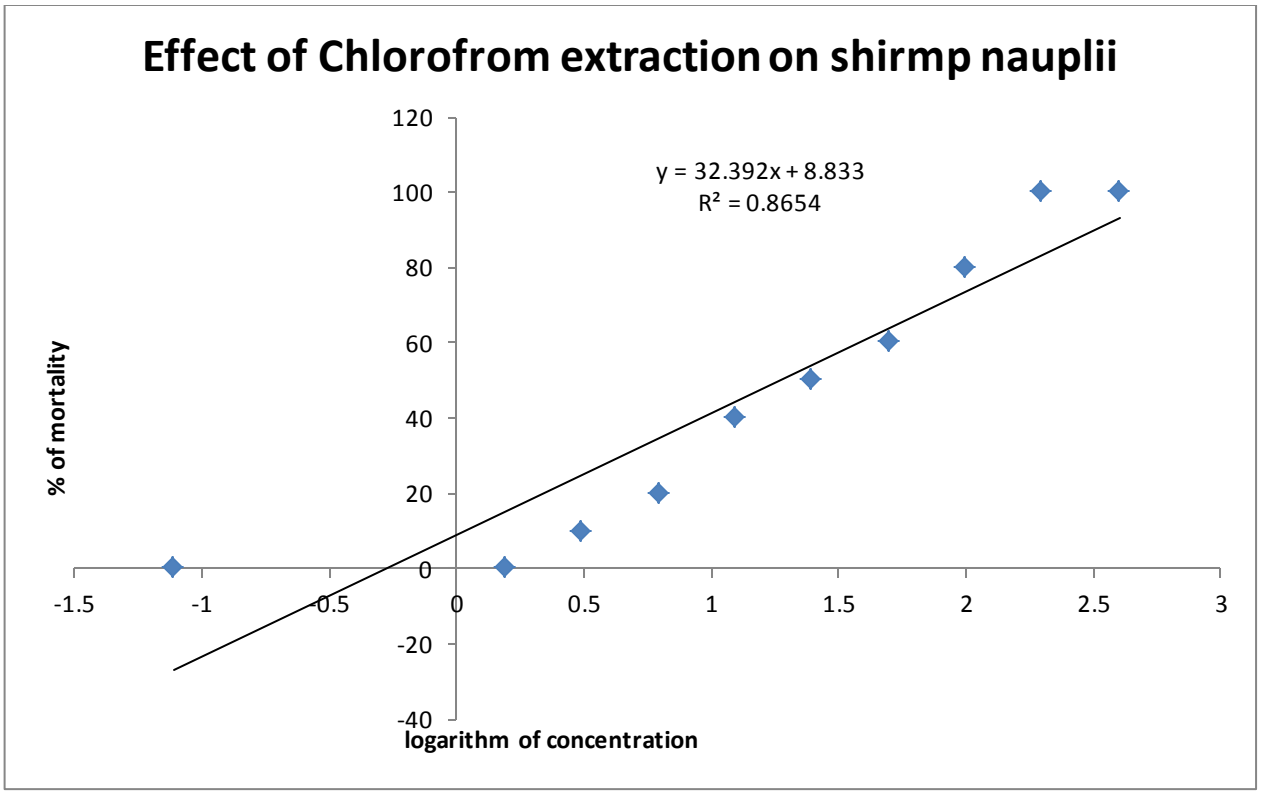

Figure 4.5: Plot of % mortality and predicted regression line of CSF

Table 4.6: Effect of Ethyl acetate soluble fraction (EASF) of the methanolic extract of leaves of *Eichhornia crassipes* shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	No. of surviving among 10	LC_{50}(µg/mL)
0	-	0	11.902
0.78125	-1.1072	0	
1.5625	0.19382	20	
3.125	0.49485	20	
6.25	0.79588	40	
12.5	1.09691	50	
25	1.39794	60	
50	1.69897	70	
100	2	80	
200	2.30103	90	
400	2.60206	90	

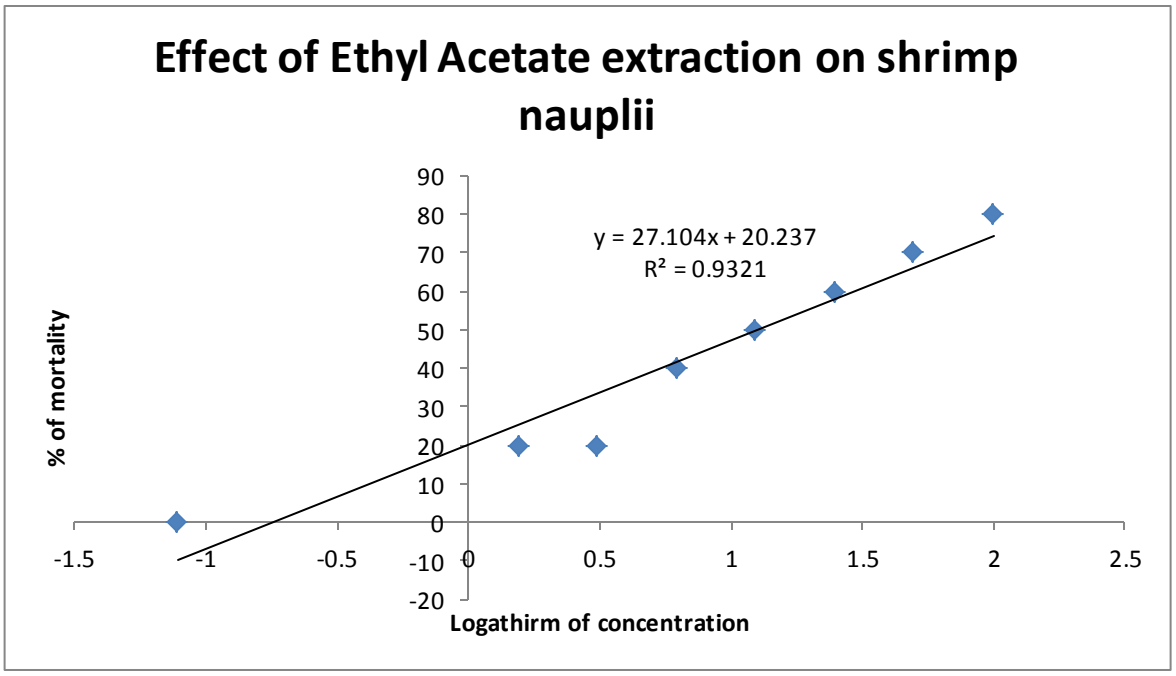

Figure 4.6: Plot of % mortality and predicted regression line of EthylacetateSF

Table 4.7: Effect of aqueous soluble fraction (AQSF) of the methanolic extract of leaves of *Eichhornia crassipes* on shrimp nauplii

Conc. (µg/mL)	Log_{10} conc.	No. of surviving among 10	LC_{50}(µg/mL)
0	-	0	79.054
0.78125	-1.1072	0	
1.5625	0.19382	10	
3.125	0.49485	0	
6.25	0.79588	20	
12.5	1.09691	30	
25	1.39794	30	
50	1.69897	40	
100	2	50	
200	2.30103	70	
400	2.60206	80	

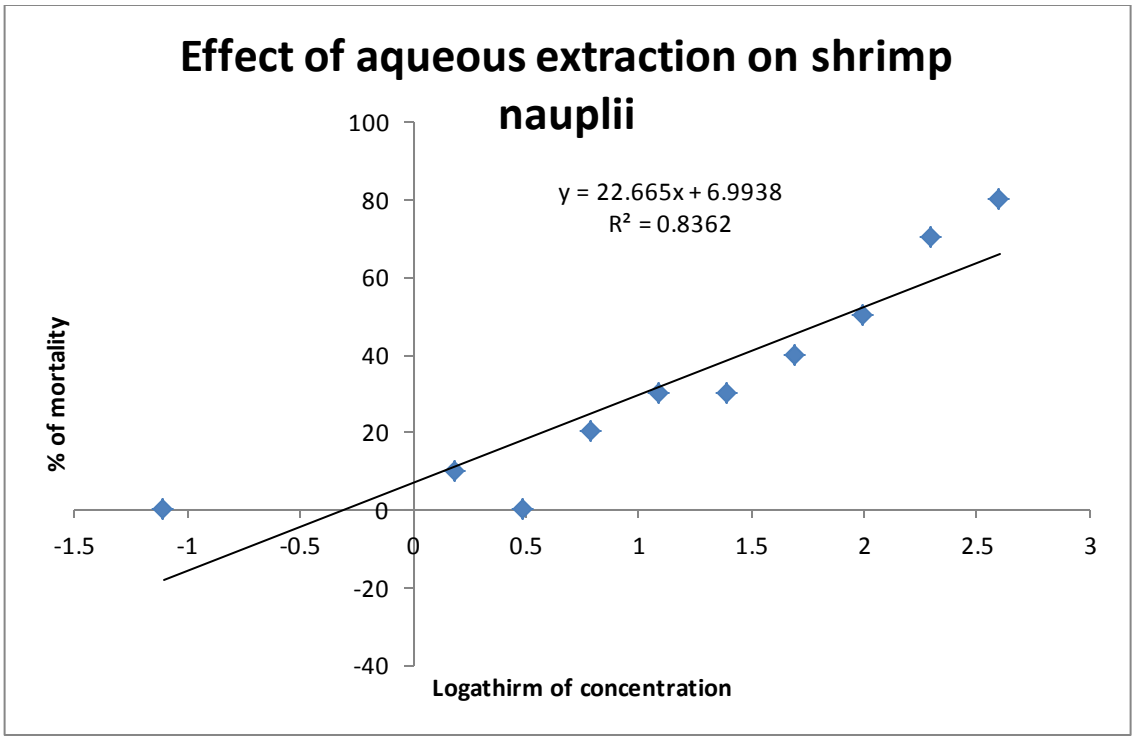

Figure 4.7: Plot of % mortality and predicted regression line of AQSF

4.2 Results and discussion of In vitro antimicrobial screening of *E. crassipes*

The crude extract of leaves of *Eichhornia crassipes* and its different extractive parts i.e. pet-ether (PESF), Chloroform (CSF) , ethyl acetate SF and aqueous (AQSF) soluble fractions of leaves of *E.crassipes* were investigated for antimicrobial screening with a concentration of 400 µg/disc .Among all extractives PESF and ethyl acetate showed negligible antimicrobial activity against different microorganisms. The results are given in the Table 4.8.

The pet-ether soluble fraction and ethyl acetate exhibited kind of same inhibition against microbial growth having zone of inhibition and the ranged from 7.0 mm to 10.0 mm. The maximum zone of inhibition produced by PESF was found to be 10.0 mm against *S. typhi*. This partitionates showed zone of inhibition against other microorganisms but these can be negligible.

Table 4.8: Antimicrobial activity of test samples of leaves of *Eichhornia crassipes*

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PESF	CSF	Ethyl Acetate SF	AQP	Ciprofloxacin
Gram positive bacteria						
Bacillus cereus	0	8	0	7	0	41
B. megaterium	0	7	0	7	0	42
B. subtilis	0	7	0	8	0	46
Staphylococcus aureus	0	9	0	8	0	45
Sarcinalutea	0	8	0	9	0	45
Gram negative bacteria						
Escherichia coli	0	7	0	7	0	43
Pseudomonas aeruginosa	0	8	0	0	0	40
Salmonella Paratyphi	0	8	0	7	0	40
S. typhi	0	10	0	7	0	47
Shigellaboydii	0	7	0	8	0	49
Sh. dysenteriae	0	7	0	0	0	46
Vibrio mimicus	0	8	0	8	0	40
V. parahemolyticus	0	7	0	7	0	41
Fungi						
Candida albicans	0	0	0	0	0	42
Aspergillusniger	0	7	0	8	0	48
Sacharomy cescerevacae	0	7	0	7	0	40

The results of *In vitro* microbial screening of *Eichhornia crassipes* indicate that, this plant extract has very low amount of antimicrobial activity against the microorganisms

CHAPTER 5: CONCLUSION

The methanolic extract (ME) of leaves of *Eichhornia crassipes* and its different partitionates i.e. pet-ether (PESF), chloroform (CSF) , ethyl acetate SF and aqueous (AQSF) soluble fractions of leaves of *E. crassipes* were subjected to chemical investigation biological investigations such as anti microbal activity, brine shrimp lethality assay.

Solvent-solvent partition of crude extract from leaves of *Eichhornia crassipes* was done by Modified Kupchan Partition method.

The investigations confirmed us that leave of *Eichhornia crassipes* has negligible antimicrobial effect against different strains of bacteria and fungi while the methanolic and petroleum ether soluble fractions of leaves exhibited moderate to strong cytotoxic activity against brine shrimp.

Hence, after taking into account all the potential bioactivity that was found during study, further extensive study can be done to discover the unknown usefulness of this plant for the uses as medicine.

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