Study on cyototoxicity and antimicrobial test of *Boerhavia diffusa* with ethyl acetate solvent

A project report submitted to the department of pharmacy, East West University in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

Submitted By:

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May, 2011

EAST WEST UNIVERSITY

Certificate

This is certify that, the thesis paper "Study on cyototoxicity and antimicrobial test of *Boerhavia diffusa* with ethyl acetate solvent" submitted to the Department of Pharmacy, East West University, 43, Mohakhali C/A, Dhaka; in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Farhana Ferdous Brishty (ID# 2007-1-70-006) under our guidance and supervision and no part of the thesis has been submitted for any other degree. We further certify that, all the sources of information and other facilities availed of in this concentration is duly acknowledged.

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30.6.2011

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Chairperson

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This Thesis Paper is Dedicated To

My Beloved Parents

& my Honourable Supervisor

Acknowledgement

At first I would like to thanks to my Almighty ALLAH for being kind giving strength to me to carry on and complete my research work. Then I would like to thanks to my parents for supporting me a lot by each and every means and I am really great full to them, as well as to my family too.

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Abstract:

Boerhaavia diffusa, commonly known as punarnava in Sanskrit, is a herbaceous plant of the family Nyctaginaceae. The whole plant or its specific parts (leaves, stem, and roots) are known to have medicinal properties and have a long history of use by indigenous and tribal people in India. The medicinal value of this plant in the treatment of a large number of human ailments is mentioned in Ayurveda, Charaka Samhita, and Sushrita Samhita. It has many ethnobotanical uses (the leaves are used as vegetable; the root juice is used to cure asthma, urinary disorders, leukorrhea, rheumatism, and encephalitis), and is medicinally used in the traditional, Ayurvedic system. Besides, the B. diffusa plant is reported to posses many pharmacological, clinical, and antimicrobial properties. Recently, the authors observed potent antiviral efficacy of this plant against phytopathogenic viruses. The antiviral agent isolated from this plant was found to be a glycoprotein with a molecular weight of 16– 20 kDa. Administered by foliar spraying in the field, this antiviral agent could protect some economically important crops against natural infection by plant viruses.The purpose of this research is to find out the activity of our research plant *Boerhavia diffusa* against cytotoxicity and microorganisms.During our research work we did Brine shrimp lethality bioassay to measure the activity of *Boerhavia diffusa* by using solvent Ethyl acetate and Potassium dichromate as Positive control.In terms of Antimicrobial test, we used 3 types of microorganisms – *Staphylococcus aureus*, *Shigella dysenteriae* and *Candida albicans*.In terms of first 2 micrroganisms The Ethyl acetate extract of *Boerhavia diffusa* showed resistant activity and for the last 1 it showed intermediate activity .

Keywords:

Chapter-1:

Introduction

Introduction:

Boerhavia diffusa is a species of flowering plant in the four o'clock family which is commonly known as tar vine, punarnava meaning that which rejuvenates or renews the body, or red spiderling. It is taken in herbal medicine for pain relief and other uses. The leaves of *Boerhavia diffusa* are often used as a green vegetable in many parts of India. It is believed to improve and protect eyesight. *Boerhavia diffusa* has diuretic properties and is used by diabetics to lower blood sugar.

Scientific classification :

Family: Nyctaginaceae

Genus: Boerhavia

Kingdom: Plantae

Order: Caryophyllales

Species: B. diffusa, hirsuta

Synonyms: *Boerhavia adscendens, B. caribaea, B. coccinea, B. erecta, B. paniculata, B. repens, B.viscosa*

Common Names: Erva tostão, erva toustao, pega-pinto, hog weed, pig weed, atikamaamidi, biskhapra, djambo, etiponia, fowl's lice, ganda'dar, ghetuli, katkatud, mahenshi, mamauri, ndandalida, oulouni niabo, paanbalibis, patal-jarh, pitasudu-pala, punar-nava, punerva, punarnava, purnoi, samdelma, san sant, santh, santi, satadi thikedi, satodi, spreading hog weed, tellaaku,thazhuthama,thikri,touri-touri,tshrana.

Part Used: whole herb, roots

Botanical Name : Boerhavia Diffusa

Habitat : Grows as common weed

Product offered: Roots

Description:

Annual to perennial herb up to 1 m tall, sometimes with thick taproot; stem branching mainly from the base, prostrate when young, ascending to erect when flowering, fleshy, green, often flushed with red, glabrescent to short or long hairy with multicellular hairs, often glandular, especially around the swollen nodes. Leaves opposite, simple, unequal; stipules absent; petiole 1–2.5(–3.5) cm long; blade broadly ovate to elliptical, 1.5–6 cm × 0.5–5 cm, base obtuse, cordate or truncate, apex acute to obtuse, margins sinuate, pale green to whitish beneath, sometimes with red marginal glands. Inflorescence an axillary, small, often congested irregular umbel, (1–)3–5(–7)-flowered, aggregated in a large diffuse panicle up to 40(–60) cm long, by reduction of leaves appearing terminal, elongating greatly after start of flowering; bracts and bracteoles small, fimbriate, caducous. Flowers bisexual, regular; pedicel up to 1 mm long; perianth tubular-campanulate, distinctly constricted halfway, lower part obconical, surrounding the ovary, 5-ribbed, green, upper part 5-lobed, 0.5–1.5 mm × 2 mm, red or purple, soon falling; stamens 1(–3), slightly exserted; ovary superior, seemingly inferior, 1-celled, style slightly exserted, stigma head-shaped. Fruit an achene enclosed by the thickened lower part of perianth (collectively called anthocarp); anthocarp obconical or club-shaped, (2.5–)3–3.5 mm × 1–1.5 mm, apex rounded, 5-ribbed, with rounded ribs, with glandular hairs, 1-seeded. Seed obovoid, pale brown. Seedling with epigeal germination; hypocotyl well developed; cotyledons rounded, with distinct midvein; first leaves alternate, shortly hairy, purplish beneath.

Figure 1.1: Boerhavia diffusa

Distribution of *B. diffusa*

Boerhavia diffusa is found in the tropical, subtropical and temperate regions of the world. It is distributed in China, India, Australia, Pakistan, Egypt, Sudan, Srilanka, U.S.A. and South Africa.

It is also found in a number of countries of the Middle East. This plant is indigenous to India and U.S.A. In India it is found in the warmer parts and up to an altitude of 2000m. It is found growing in waste lands, road sides, road dividers, near railway tracks, on ruins of old buildings, on rubles, and near old earthen ponds.

Boerhaavia diffusa Toxicity:

The acute and subchronic toxicity studies of *Boerhavia diffusa* (*B. diffusa*) leaves in albino mice and rats were investigated. Phytochemical analysis was also carried out. 500, 1000 and 2000 mg/kg of the aqueous leaf extract were administered orally to the test groups while distilled water was given to the control group. The parameters measured include food and fluid intake, body weight, absolute and relative weight of various organs, haematological parameters [total white blood cell (WBC) and packed cell volume (PCV)], and tests for liver function: glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase and total bilirubin. The lethal dose (LD_{50}) was found to be greater than 2000 mg/kg (*p.o.*) in both mice and rats. Rats treated with the extract had progressive increase in body weight, which was significantly ($p < 0.05$) different from control. The aqueous extract of *B. diffusa* leaves increased both food and fluid intake. There were no significant changes in both the absolute and relative organ weights between the control and the test groups. The liver enzymes and haematological parameters were statistically equal in all the groups. *B. diffusa* aqueous leaf extract is non toxic in albino rats.

Bloom Information:

Bloom Color: Red , Purple

Bloom Time: Apr , May , Jun , Jul

Cultivation:

Boerhavia diffusa is a weed of cultivated land and wasteland, often in lawns in drier areas. Although common, it is not a weed of importance. After mechanical cultivation the plant resprouts from its roots but relatively few cultivations are needed to exhaust it.

Medicinal properties:

Plant pacifies vitiated vata, pitta, fever, constipation, leucorrhoea, lumbar pain, myalgia, skin diseases, cardiac disorders, urinary infection, vesical stone, anemia, dyspepsia, constipation, and general debility.

Chemical Constituents:

Punarnava contains b-Sitosterol, a-2-sitosterol, palmitic acid, ester of b-sitosterol, tetracosanoic acid, hexacosonoic acid, stearic acid, arachidic acid, urosilic acid, Hentriacontane, b-Ecdysone, triacontanol etc.

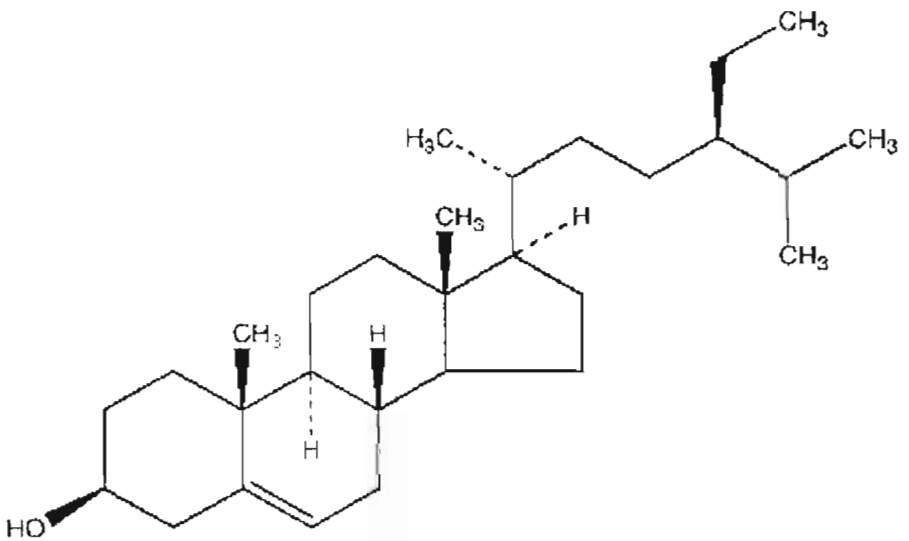


Figure: 1.2-Sitosterol

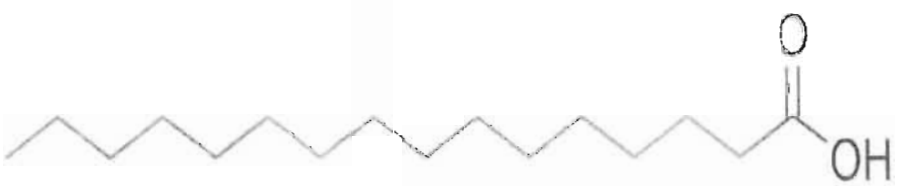


Figure: 1.3 Palmitic acid

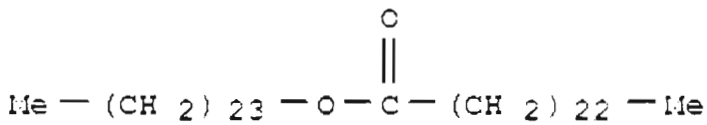


Figure 1.4: Tetracosanoic acid

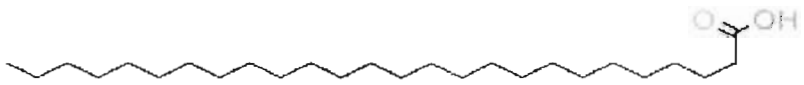


Figure 1.5: Hexacosonoic acid

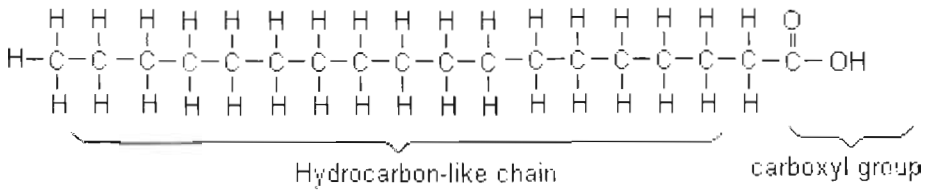


Figure 1.6: Stearic acid

Figure 1.7: Arachidic acid

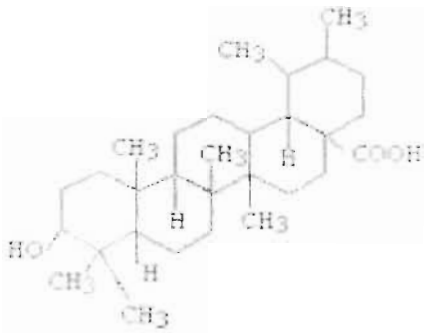


Figure 1.8: Ursolic acid

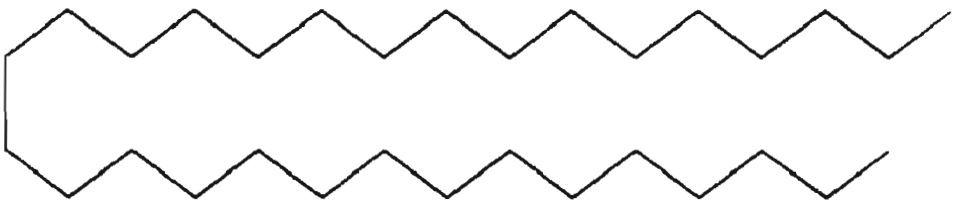


Figure 1.9: Hentriacontane

Figure 1.10: Triacontanol

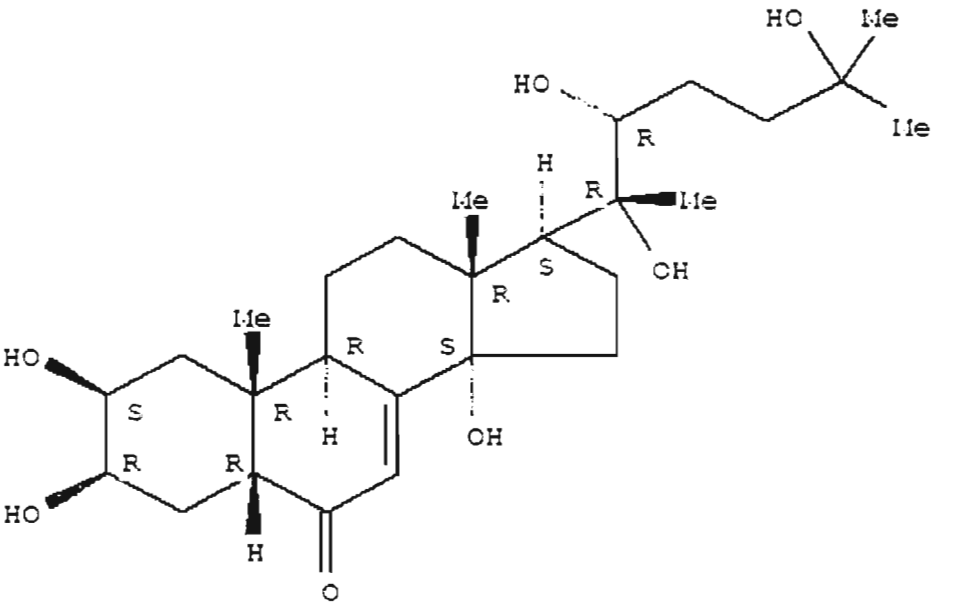


Figure 1.11: b-Ecdysone

Researches made on Pharmacological action of *Boerhavia diffusa*

Time	Name of the Researchers	Part of the plant	Assertions
2009	Chauhan C.K., Joshi M. J. and Vaidya A.D.B.	Full plant	Growth Inhibition of Struvite Crystals
2004	Nalamolu R.K., Boini K.M., Nammi S.,	Root	Reduction in blood glucose
2010	Goyal B. M., Bansal P., Gupta V., Kumar S., Singh R., Maithani M.,	Full plant	Cure asthma, urinary disorders, leukorrhea, rheumatism,encephalitis &vegetable
2009	Sreeja S.,	Full plant	Ant proliferative and ant estrogenic.
2010	Umamaheswari A., Nuni A., Shreevidya R.,	Leaves	Tested against the Gram-positive and Gram-negative bacterial strains by observing the zone of inhibition.
2010	Srivastava R., Saluja D., Dwarakanath B. S. and Chopra M.,	Root	Anti proliferative and immunomodulatory properties.

Table-2: Research findings

Time	Name of the Researchers	Part of the time	Findings
1979	LP Awasthi HN Verma	Root extract	Diuretics, anti-inflammatoy, antifibronolytic, anticonvulsant
2008	C.U.L gwe V.H.A Enemor	Aqeous leaf extract	Give anti nutnitive and nutnitive, antioxidant effect
2010	AK Meena MM Rao AK Yaday Uttam S Niranjan	Whole Plant	Used in the treatment of cancer enlargement of spleen, jaundice inflammation, dyspsia, abdominal pain, antistree agent

Growth and development:

Boerhavia diffusa can be found flowering and fruiting throughout the year, when sufficient water is available. The first flowers may appear 4 weeks after germination of the seeds.

Ecology:

Boerhavia diffusa occurs in ruderal localities and along roadsides, preferring sunny sites and a slightly seasonal climate, from sea-level up to 1200 m altitude. It is often a weed in cultivated land, usually on sandy soils, and is also found in lawns and grazing pasture.

Propagation and planting:

Boerhavia diffusa is propagated by seed, which germinates with the start of the first rains and continues to germinate throughout the rainy season. When the soil of arable fields is turned, pieces of root can sprout as well. Well-drained soils and sunny conditions are required. The mucous coat of the anthocarp shows a distinct sticky swelling when ripe, with which it clings to mammals and birds. *Boerhavia diffusa* has been successfully propagated by in-vitro induction of adventitious roots on stem explants, leaf or shoot tip cultures.

Diseases and pests:

In India several host-specific diseases have been identified on *Boerhavia diffusa*, i.e. *Cercospora diffusa* causing chlorotic leaf spots, and *Colletotrichum boerhaviae* causing brown necrotic spots. Also in India *Boerhavia diffusa* is recorded as a host for the virus causing aubergine mosaic disease (EMV), and in Costa Rica as a host of zucchini yellow mosaic potyvirus

(ZYMV). In Cameroon *Boerhavia diffusa* is an alternative host for the cotton aphid (*Aphis gossypii*), and in Nigeria caterpillars of *Aegocera rectilinea* and *Hippotion celerio* were found feeding almost solely on *Boerhavia diffusa*.

Medicinal use:

1) *Milk decoction:*

Punarnava is an important *rasayana dravyas* in Ayurvedic medicine, indicated by the translation of its Sanskrit name, 'once again new.' For this purpose *Punaranava* can be taken as a milk decoction. 10-24 grams of the root taken twice daily.

2) *Rejuvenating properties*:

The potent rejuvenating properties of *Punaranava* root are also made use of in a variety of rejuvenating formulae, including the famous *lehya Chyavanaprash. Punarnava* however also has a number of more mudane uses, especially for its ability to correct diseases of the urinary tract and treat edema. As a simple remedy for cystitis the *svarasa* or *churna* of *Punarnava* can be taken, 10-15 mL of the juice, or 3-5 grams of the powder, thrice daily until symptoms are gone.

3) *Edema treatment:*

In the treatment of edema 10-15 mL of the fresh juice of the leaves can be mixed with a small amount of *Maricha (Piper nigrum)* or *Shunthi (Zingiber officinalis),* taken twice daily for several weeks. The fresh juice is also taken in jaundice and in menstrual disorders. Lt. Col. Chopra found that *Punarnava* was efficacious in the treatment of edema and ascites due to early cirrhosis and peritonitis, using a liquid extract prepared from either the dry or fresh plant material of *Svetapunarnava*. Nadkarni adds that *Punarnava* is equally effective in edema secondary to heart disease from stenosis of the valves, in pleurisy and in other edematous conditions.

4) *Polyherbal formulations:*

In most cases *Punarnava* is used in polyherbal formulations to treat edema and other conditions. In the treatment of edema as well as colic, bloating, flatulence, constipation, hemorrhoids, intestinal parasites, and anemia, the *Chakradatta* recommends *Punarnavamandura*, comprised of equal parts *Punarnava, Trivit, Shunthi (Zingiber officinalis), Pippali, Maricha (Piper nigrum), Vidanga, Devadaru, Chitraka, Pushkaramula (Inula helenium* root), *Haridra, Danti (Baliospermum montanum), Chavya (Piper chaba), Indrayava, Katuka, Pippalimula (Pippali* root) and *Musta*, decocted in cow's urine.

5) *Urinary calculi and muscle pains:*

Another formula called *Punarnavadi taila* is mentioned by the *Bhavaprakasha* in the treatment of urinary calculi, muscle pains and hernia associated with the aggravation of *Kapha* and *Vata*, used in *vasti* (enemata) and internally.

6) *Abdominal enlargement:*

A decoction of *Punarnava, Devadaru, Haritaki* and *Guduchi* combined with *Guggulu* is stated to be effective in abdominal enlargement (*udararoga*), as well as intestinal parasites, obesity, anemia, edema and skin diseases.

7) *Combination therapy:*

Combination of *Punarnava*, *Devadaru, Guduchi, Patha (Cissampelos pariera), Bilva, Gokshura, Brhati (Solanum indicum), Kantakari, Haridra, Daruharidra, Pippali, Chitraka* and *Vasaka*, reduced to a fine powder and taken with cow's urine is used in abdominal enlargement secondary to intestinal parasites. In *Vataja* forms of edema a combination of *Punarnava,*

Shunthi, Eranda (Ricinus communis) and *Brhati (Solanum indicum)* is stated by the *Chakradatta* to be efficacious.

8) *Edema topical therapy:*

As a topical therapy for edema the *Sharangadhara samhita* recommends *Punarnavadi lepa*, prepared by combining equal parts powders of *Punarnava, Daruharidra, Shunthi, Siddhartha (Brassica compestris)* and *Shigru (Moringa pterygosperma)* with rice water. Given the ability of *Punarnava* to mobilize kidney function, and the importance this is given to promote the elimination of metabolic wastes in joints and muscles.

9) *Inflammatory joint disease:*

Punarnava is also used to treat inflammatory joint disease, including gout and rheumatoid arthritis. To this extent the *Chakradatta* recommends a formula called *Shatyadi kvatha*, comprised of a decoction of *Punarnava* with a paste of *Shati (Hedychium spicatum)* and *Shunthi (Zingiber officinalis)*, taken every day for at least one week. Similarly, the *Bhavaprakasha* advocates a complex formula called *Punarnava guggulu* in the treatment of gout, hernia, sciatica, muscular atrophy and inflammatory joint disease.

10) *Internal abscesses:*

In the treatment of internal abscesses the *Sharangadhara samhita* recommends a decoction of *Punarnava* and *Varuna (Crataeva religiosa)*. *Punarnava* is also valued in ophthalmic disorders, the *Sharangadhara samhita* recommending a collyrium *(anjana)* for itching, prepared by mixing the *churna* with milk; mixed with honey to treatment ophthalmic discharges; with *ghee* for corneal wounds; with *taila* for poor vision; and with rice water *(kanjika)* for night blindness.

III) Alcoholism:

In the treatment of alcoholism the *Chakradatta* recommends a decoction of *Punarnava* to restore ojas. In the treatment of diabetes *Punarnava* can be combined with *Shilajitu* and *Guduchi*. *Punarnava* is also consumed as a nourishing vegetable in India, rich in vitamins and minerals, and has undergone investigation for its potential in famine relief.

Chapter- 2

Literature Review

Boerhaavia diffusa is a plant of Ayurvedic, traditional, ethnoherbological and clinical- medicinal importance. Indigenous tribes of many countries have been reported to use different parts of the plant for food and medicine. Recent studies have found that the plant has anti-microbial including anti-viral properties. However the commercial use of the plant has not so far been encouraged except some companies selling its dried powder. The entire plant along with root is eaten as vegetable in curries and soups in some parts of the world. Sheep and goats like to graze the plant and it grows again and again each time it is grafted by the ruminants. *Boerhaavia diffusa* L. is a common herbaceous weed with ovate, fairly longstemmed leaves and crimson flowers in a small terminal cluster. It is a medicinal plant used in traditional medicinal practice and has been reportedly useful in the treatment of many diseases (Ayensu 1978). In the present paper medicinal use of *Boerhaavia diffusa* and it effect on animal is investigated. This literature review mainly focous on the importance of this medicinal plant, its effect on animal and investigate its efficacy. Indigenous tribes of many countries have been reported to use different parts of the plant for food and Medicine. The aim of the present study was to evaluate the phytochemicals and antimicrobial activity of various solvent extracts of *Boerhaavia diffusa.*

Boerhaavia diffusa, Linn (Fam: Nyctagenaceae) commonly known as "Punarnava" in the Indian system of medicine is a perennial creeping herb found throughout India. The plant serves as a non-conventional vegetable, specially in the North-Eastern region of India. A large number of tribes in India use the plant for the treatment of Jaundice and various other liver disorders. The plant is also reported to be diuretic and laxative and are given for the treatment of anasarca, ascites and jaundice. The roots of *Boerhaavia diffusa* have been found to have antiinflammatory, diuretic, fibrinolytic, nephrotic syndrome and anti-convulsant activities. Investigations on the

chemical constituents of the plant has indicated the occurrence of two novel alkaloids, Punarnavine-1 and Punarnavine-2, belonging to the group quinolizidine. From the roots, seeds and leaves of *Boerhaavia diffusa*, isolation of β-sitosterol, β-sitosterol-β-D-glucoside, tetracosanoic, hexacosanoic, stearic, palmitic, arachidic acids, hextriacantane, urosolic acid has been reported. (Kirtikar KR, Basu BD 1993).

Boerhaavia diffusa is a medicinal plant widely used in the Ayurvedic medicine (Lad, 1999).

Ayurveda is an ancient traditional medical system of health care of the Veda civilization, flourishing in India many thousand years ago (Figure 1). The term "AYURVEDA" means "Knowledge or Science of Life" (from "Ayus" meaning "life" and "Veda" meaning "acquaintance", "science") in Sanskrit. In fact, Ayurveda focuses on the physical, spiritual and mental aspects of an individual. It supports and nourishes all the beneficial values of life and, what is more important, this medical practice is not in discord with any other medicinal science.

Ayurveda uses the balancing power of Nature, the power that every plant contains, in order to correct the imbalances which are at the root of any disease. The fundamental nature of the Ayurvedic medical system is based on the harmony that exists between the microcosm and the macrocosm. This notion was applied to the body by the Ayrvedic physicians as they considered that the body was composed of the same elements as the universe was created (earth, fire, water, sky and air). According to the teachings of Ayurveda, most of the body activities are governed by the three basic body energy control principles known as "doshas" (humour) in Sanskrit. Each dosha is made up of one or two of the five basic elements and each has a particular relationship to the body functions. When the doshas are perfectly balanced, the result is the health with the

invaluable energy, but when this delicate equilibrium is disturbed (as it happens if the body, the mind and the spirit are not in coordination), either bodily or mental suffering develops.

Figure 2.1: Ayurveda system

Boerhaavia diffusa are wide species of vegetables that are relatively underutilized and in most cases neglected. Thus are seen as weeds in farms (Keary and Hepper, 1985). *B. diffusa*, commonly called hog weed, is known as *erimmirii* (which literally means water-food) by the Ibos of southeastern Nigeria. The leaves are cooked and eaten as vegetable. The plant is used in folkloric medicine to treat convulsions and as a mild laxative and febrifuge (Adesina, 1979). The roots and leaves are considered to have an expectorant action, to be emetic and diuretic in large doses and are used in the treatment of asthma. The thick roots, softened by boiling are applied as

poultice to draw abscesses and to encourage the extraction of guinea worm (Keary and Hepper, 1985). The neglection of these vegetables coupled to the growing reduction in consumption of vegetables prompted this study. It is aimed at evaluating the levels of some macro and micronutrients and as well screen for phytochemical compositions of *B.diffusa.* It is hoped that this study will increase interest in them.

Boerhaavia diffusa is a herbaceous member of the family Nyctaginaceae. It is widely distributed in the tropics and subtropics (CSIR, 1988). It has a long history of uses by indigenous and tribal people and in Ayurvedic or natural herbal medicines (Dhar *et al.*, 1968). The major active principle present in the roots is alkaloidal and is known as punarnavine. In the traditional system of medicine, *B. diffusa* roots have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, and abdominal pain (Kirtikar and Basu, 1956), and as an antistress agent. The worldwide use of *B. diffusa* roots to treat liver disorders was validated when researchers demonstrated, in 1980 and 1991, that its root extract had antihepatotoxic properties (Chandan *et al.*, 1991; Rawat *et al.*, 1997).

Pharmacological studies have demonstrated that *B. diffusa* possesses diuretic (Gaitonde *et al.*, 1974), anti-inflammatory (Bhalla *et al.*, 1968), antifibrinolytic (Jain and Khanna, 1989), anticonvulsant (Adesina, 1979), and antibacterial properties (Olukoya *et al.*, 1993), which makes it a very useful medicinal plant. The aqueous extracts of roots are also a rich source of a basic protein, known as systemic resistance inducing protein (BD-SRIP). The aqueous solution of this protein, when applied before virus infection/inoculation, induces strong systemic resistance in several susceptible plants against commonly occurring viruses (Verma and Awasthi, 1979). All these properties have made this plant very interesting, and the plant has played an important role in the treatment of human and plant diseases.

Confusion exists in the literature on the correct identity of punarnava (*Boerhaavia diffusa*). Several species of *Boerhaavia* are being used under the name *punarnava* in India. It is generally agreed that the red variety is *B. diffusa*, whereas the white variety has been identified as belonging to the *Trianthema* species. The roots of *Trianthema* are often used as an adulterant for *punarnava*. The two genera belong to different families, and *Trianthema* species neither contain the active constituent (punarnavine), nor have red flowers. The whole *Boerhaavia diffusa* plant (fresh as well as dried) is the genuine source of the drug *punarnava*, and is considered official in Indian Pharmacopoeia.

The plant was named in honor of Hermann Boerhaave, a famous Dutch physician of the 18th century (Chopra, 1969). *Boerhaavia*, a herbaceous plant, belongs to the Nyctaginaceae (four o'clock) family, order Thymilae, group Dicotyledons and phylum Angiosperms (Rendle, 1925). *Boerhaavia diffusa* is a perennial herb and it is ascribed the name *punarnava*, a drug known since long in the indigenous system of medicine in India. The name *punarnava* (*Punah punarnava bhawati iti*, in Sanskrit, translates as "that which becomes fresh again and again . . .") is probably derived from the perennial habit of the plant, which remains dry and dormant during summer and regenerates from the same old root stock in the rainy season, or from the Sanskrit phrase that denotes such therapeutic property (*Karoti shariram punarnavam*, in Sanskrit, translates as "that which rejuvenates the body") (Wahi *et al.*, 1997). Apart from *punarnava*, the plant has a host of other names in Indian languages: *biskhafra* in Hindi, *thazhuthama* in Malayalam, *mukaratte* in Tamil, *gadhapurna* in Bengali, *satodi* in Gujarati, *itsit* in Punjabi, etc. It is known as hogweed or pigweed in USA, and Erva Tostão in Brazil.

In Purulia (West Bengal), tribals eat this plant as vegetable. *Boerhaavia* leaves are cooked and eaten in Assam, where it is commonly found in the markets. Its roots are used in the treatment of

piles by the inhabitants of the Garhwal Himalaya (Uttaranchal). The root paste is used to cure bloody dysentery by the Bhils of the Jhabua district in Madhya Pradesh. The decoction of the plant is given in the treatment of nodules in the body. The root juice is used in treating asthma, scanty urine, and internal inflammation disorders. *Boerhaavia diffusa* is used for curing ailments such as leukorrhea, rheumatism, and stomach ache by the Sahariya tribe in the Lalitpur district of Uttar Pradesh. This plant is also used by the tribes of Ambikapur district (Madhya Pradesh) for the treatment of elephantiasis. In the Indo-Nepal Himalayan terai region, the tribals harvest this plant for medicinal purposes, mainly for flushing out the renal system, and to treat seminal weakness and blood pressure(Mitra and Gupta, 1997).

Different parts of the *B. diffusa* plant have been widely used by indigenous tribes in the traditional system of medicine. The roots have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, abdominal pain, abdominal tumors, and cancers (Kirtikar and Basu. 1956). The root powder, when mixed with *mamira* (*Thalictrum foliolosum*), is used to treat eye diseases. It cures corneal ulcers and night blindness (Gupta *et al.*, 1962), and helps restore virility in men. People in tribal areas use it to hasten childbirth (Shah *et al.*, 1983). The juice of *Boerhaavia* leaves serves as a lotion in ophthalmia. It is also administered orally as a blood purifier and to relieve muscular pain.

In old Indian books of medicine such as the Charaka Samhita and Sushrita Samhita, it is mentioned that the Ayurvedic preparations made from *punarnava* – namely, *punarnavastaka kvath*, *punarnava kshar*, and *punarnava taila* – were used for the treatment of various ailments. The whole plant of *B. diffusa* is a very useful source of the drug *punarnava*, which is documented in Indian Pharmacopoeia as a diuretic (Chopra, 1969). The active principle contained in the herb is an alkaloid, known as punarnavine. The roots and leaves with flowers

have been found to be highly potent (CSIR, 1988). In Ayurvedic medicine, different parts of this plant were reported to have various medicinal properties. It was used in renal ailments as diuretic (Anand, 1995); and to treat seminal weakness and blood pressure (Gaitonde *et al.*, 1974). It is also used in the treatment of stomach ache, anemia, cough, and cold, and as a diaphoretic, laxative, expectorant, and a potent antidote for snake and rat bites (Chopra *et al.*, 1956), in the treatment of nephrotic syndrome (Singh and Udupa, 1972), hepatitis, gall bladder abnormalities, and urinary disorders (Mudgal, 1975; Cruz, 1995). The flowers and seeds are used as contraceptive (Chopra *et al.*, 1956).

Pharmacological studies have demonstrated that *punarnava* possesses punarnavoside, which exhibits a wide range of properties – diuretic (Gaitonde *et al.*, 1974); anti-inflammatory (Bhalla *et al.*, 1968); antifibrinolytic (Jain and Khanna, 1989); anticonvulsant (Adesina, 1979); antibacterial (Olukoya *et al.*, 1993); antistress agent; antihepatotoxic (Mishra, 1980; Chandan *et al.*, 1991; Rawat *et al.*, 1997); anthelmintic febrifuge, antileprosy, anti-asthmatic, antiscabies, and anti-urethritis (Nadkarni, 1976); and antinematodal activity (Vijayalakshmi *et al.*, 1979). An aqueous extract of thinner roots of *B. diffusa* at a dose of 2 ml kg-1 exhibited marked protection of various enzymes such as serum glutanicoxaloacetic transaminase, serum glutanic-pyruvic transaminase, and bilirubin in serum against hepatic injury in rats (Rawat *et al.*, 1997). *Punarnava* possesses diuretic and anti-inflammatory activities and the maximum activity was observed in samples collected in the rainy season. Due to the combination of these two activities, *punarnava* is regarded therapeutically as highly efficacious for the treatment of inflammatory renal diseases and common clinical problems such as nephrotic syndrome, oedema, and ascites resulting from early cirrhosis of the liver and chronic peritonitis.

The plant is reported to be efficacious in abdominal tumors and cancers. The drug proved useful as a hematinic and as a growth promoter in children fed with milk fortified with the drug. In the form of a powder or an aqueous decoction, the drug was found to be useful in the treatment of nephrotic syndrome and compared well with corticosteroids. The drug decreased the albumin urea, the serum protein was increased and serum cholesterol level was lowered.

Singh and Udupa (1972) reported that dried root powder showed curative efficiency when administered orally for one month to children or adults suffering from helminth infection. The subjects became worm-free within five days of treatment. The drug, singly or in combination with other drugs, was found to be effective in liver disorders, heart diseases (hypertension, angina, cardiac failure, etc.), respiratory tract infections, leukorrhea, spermatorrhea, etc. The purified glycoprotein from *B. diffusa* exhibited strong antimicrobial activity against RNA (ribonucleic acid) bacteriophages (Awasthi and Menzel, 1986). With much of the clinical research validating its long history of different uses in natural medicine, the commercial bulk of punarnava in India represents heterogeneous medicinal uses.

The entire plant including the roots is eaten as vegetable, in curries and soups. The roots and seeds are added to cereals, pancakes, and other foodstuffs. They are also served as bird feed or poultry feed. The plants are grazed by sheep, goats, and cows, and in West Bengal, it is believed that the plant enhances lactation period and also the amount of milk in cattle (CSIR, 1988).

In view of the pharmacological, clinical, and medicinal potential of this plant, the authors screened the root, leaf, stem, flower, and seed samples (collected at different stages of plant growth and from different locations, both fresh and dried) for their antiviral activity against a number of isometric as well as anisometric phytopathogenic viruses, in various host/virus

combinations both in vitro and in vivo (Verma and Awasthi, 1979). Maximum antiviral activity, in each case, was recorded with the aqueous extract of dried root powder applied before virus inoculation. The active principle was purified and isolated (Verma *et al.*, 1979). The roots of *B diffusa* are a rich source of a basic protein, which is used for inducing systemic resistance in many susceptible crops against commonly occurring viruses (Verma and Awasthi, 1979: 1980: Verma *et al.*, 1979; Awasthi *et al.* 1984; 1985; 1989). This protein or antiviral agent was active against tobacco mosaic virus in *Nicotiana glutinosa*, *Datura metel*, *Chenopodium amaranticolor* and *Nicotiana tabacum* (Ky58 White Burley and NP31); sunnhemp rosette virus in *Cyamopsis tetragonoloba*, *Vigna unguiculata,* and *Crotalaria juncea*; and gomphrena mosaic virus in *Chenopodium amaranticolor, Vigna unguiculata,* and *Gomphrena globosa* when applied a few hours (2–24 h) before inoculation by the respective inocula of viruses (Verma and Awasthi, 1979; Awasthi *et al.*, 1984). The antiviral agent was a basic glycoprotein (70– 80% protein and 8–13% carbohydrates) with a molecular weight of 16–20 kDa as determined by gel filtration chromatography (Verma *et al.*, 1979). The resistance-inducing protein was found to be extremely thermostable (Verma and Awasthi, 1979). Following treatment with the systemic resistance inducing protein, the susceptible healthy host produced a virus inhibitory agent (VIA). The VIA showed the characteristics of the protein, and upon incubation with the virus, reduced infectivity of the viruses both in vitro and in vivo (Verma and Awasthi, 1980). Upon gel filtration on Sephadex G- 75®, two active fractions, exhibiting protein characteristics, were recovered (Verma and Awasthi, 1980, Awasthi *et al.*, 1987). The VIA was present both in treated as well as untreated leaves. The biophysical characteristics of induced VIA were also studied and it was found to be a basic protein (Awasthi *et al.*, 1987). The glycoprotein occurring in *B. diffusa* roots

functions as a signal molecule, and is of great interest as it has a role in stimulating the defence systems of plants against viruses.

Owing to the high antiviral efficacy of *B. diffusa* under laboratory conditions, it was tested under field conditions as well against a few viral diseases of economically important crops. The purified glycoprotein from *B. diffusa* reduced infection and multiplication of tomato yellow leaf curl virus (Awasthi and Rizvi, 1999), papaya ring spot virus (Awasthi, 2000), and cucumber green mottle mosaic virus (Awasthi *et al.*, 2003). The aqueous crude extract from the dried roots was also found significantly active against a number of viruses – mung bean yellow mosaic virus (Awasthi, 2000); bean common mosaic virus (Singh and Awasthi, 2002); water melon mosaic virus (Awasthi, 2002); bottle gourd mosaic virus in muskmelon (*Cucumis melo*), ridged gourd (*Luffa acutangula*), and bottle gourd (*Lagenaria siceraria*) (Awasthi and Kumar, 2003); cucumber mosaic virus in cucumber (*Cucumis sativus*) and muskmelon and water melon mosaic virus in watermelon (*Citrullus lanatus*) (P Kumar, personal communication); and mung bean yellow mosaic virus in mung bean (*Vigna radiata*) (S Singh, personal communication).

Boerhaavia diffusa is an important medicinal plant. The plant is widely used for the treatment of oedema, dropsical condition, and urinary troubles. A large number of publications on the chemistry, pharmacology, and several other aspects have been made, but no homogenous, pure, active principle of the plant in the form of a modern standardized drug has been introduced. A basic protein showing high systemic resistance inducing activity against plant viruses has been isolated, but it has not yet been purified to homogeneity and commercially made available. However, the plant is abundantly available in wild form over large tracts of land.

Chapter- :3

Plant preparation

3.1) Collection and proper identification of plant sample:

The fresh whole plant was *Boerhaavia diffusa* was collected from Shere Bangla Krishi University of Dhaka district. It was identified by the Bangladesh National Herbarium, Mirpur, Dhaka as *Boerhaavia diffusa* the identification no. is- 35440

DACB

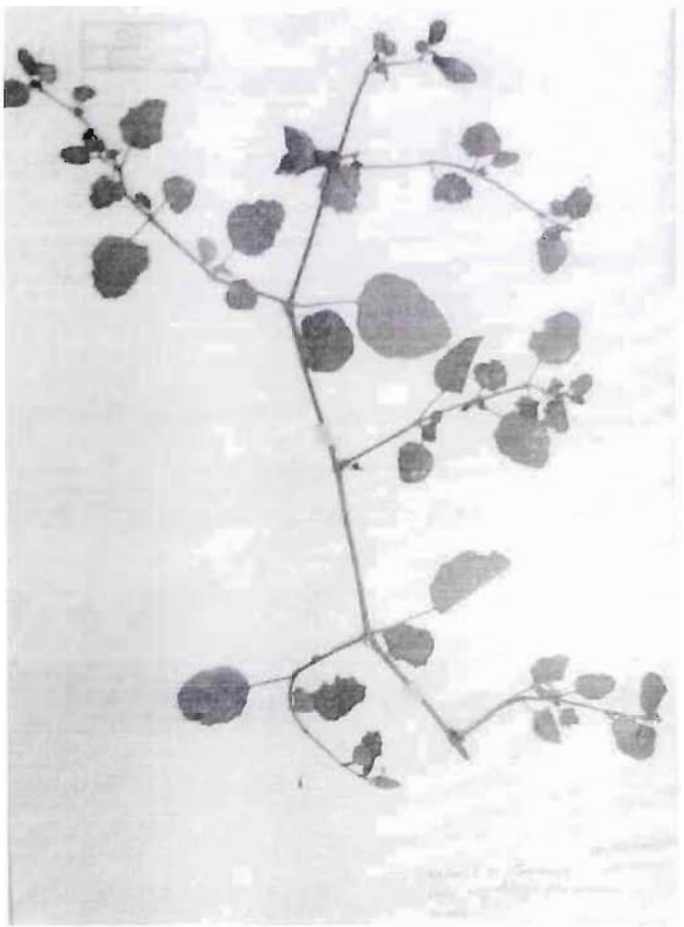


Figure 3.1: *Boerhaavia diffusa*

3.2) Preparation:

We collected 1.25 kg of *Boerhaavia diffusa* plant and then dried it for about 12 days under sunlight. After drying the plant we measured the amount in electrical balance and the weight is

gm.then these are grinding in the grinding machine. Again we measured the weight and is 480.4 gm.

3.3) Solvent extraction:

gm powder of *Boerhaavia diffusa* powder were soaked into the ethyl acetate solvent 72 hour. Then these powder solution are given in the rotary evaporator to get crude extract. After this these crude extract were used in the cytotoxicity and antimicrobial test.

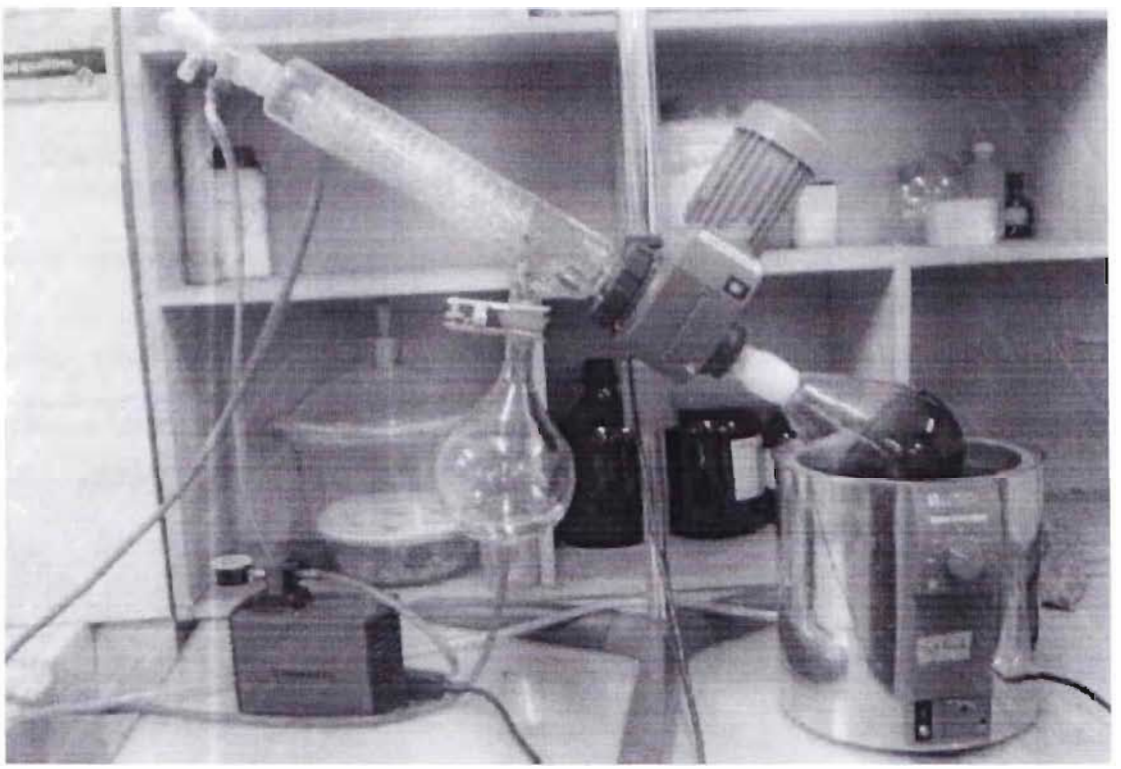


Figure 3.2 : Rotary Evaporator (IKA ®RV05 Basic, Biometra, Germany)

3.4) Preparation of plant materials

170 gm plant part was collected in fresh condition. It was shade dried effectively to make it suitable for grinding purpose. The coarse powder was then soaked in 38.79 gm ethyl acetate solvent in 1000ml beaker. After 72 hour these preparations are filtered in another beaker by

using Double rings.9 cm. Then again we took the weight of the filtered solution.Then this solution are giving to the 1000ml Round bottom flask (BOROSIL) then it placed in the rotary evaporator to evaporate the solvent by uand thus we get the crude extract. The evaporation was done at 58 degree Celsius temperature. The number of rotation per minute was selected as 150 RPM .The pressure of the vacuum pumper machine (Biometra) was 6bar. The evaporation was done at 58 degree Celsius temperature. The number of rotation per minute was selected as 150 RPM The pressure of the vacuum pumper machine (Biometra) was 6bar.This process we did agin by using remaining 100ml of Ethyl acetate . After this we collected the extract and wrapped the beaker with foal paper and kept it for 24 hour and kept in cool, dark, and dry place.

3.5) Cold extraction

In cold extraction, the powdered plant material is submerged in a methanol in an air tight flat bottom container 3 days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extractor as solution. Then this solutions are dividing into two portion for further use. 7.49 gm crude extract was obtained.

Figure 3.3: Crude extracts of *Boerhaavia diffusa*

The commercial bulk of *B. diffusa* represents a heterogeneous population. Consequently, it quite often results in poor quality roots and biomass. The cardinal feature of modern cultivation of any plant with impressive uniformity and high productivity for end product is thus grossly lacking in *B. diffusa*. The need, therefore, is to genetically improve the available commercial bulk of *B. diffusa* in order to meet the natural requirement of the valuable products. Improved varieties with enhanced drug yields hold great promise. Mutation breeding may have an important role in this direction and may improve the yield and quality. Recently, Shukla (2002) has reported a substantial amount of genetic variability in *B. diffusa*. Of the seventy-one genotypes tested, only a few were elite lines and were found to have desirable material for commercial use. Therefore, commercial manufacture of active constituents from these improved elite lines would be useful and profitable.

Chapter- 4

Cytotoxic Test

4.1) Brine shrimp lethality bioassay:

The Pharmacological evaluation of substances from plants is an established method for the identification of lead compounds, which can lead to the development of novel and safe medicinal agents (Huang et al,1998).The in-vivo lethality in a simple zoo logic organism can be used as a convenient monitor for screening and fractionation in discovery and monitoring of bioactive natural products. Meyer et al, 1982 focused on Artemia salina .Leach as a test organism and developed a protocol for Brine shrimp lethality bioassay to monitor cyotoxicity of a compound.

The method is attractive because it s very simple, inexpensive and low toxin amounts are sufficient to perform the test in the micro well scale. In the present study, Methanol,N-hexane and Ethyl acetate extract of *Boerhavia diffusa* were screened for their toxicity using brine shrimp lethality test.

4.2) Cytotoxicity Bioassay:

Principle:

Brine shrimp eggs are hatched in the stimulated seawater to get nauplii. Sample solutions are prepared by dissolving the test materials in pre calculated amount of dimethyl sulfoxide DMSO. Ten nauplii are taken in vials containing 10 ml of stimulated seawater .The samples of different concentrations are added to the pre marked vials with a micro pipette. The assay is performed using three replicates. Survivors are counted after 24 hours .These data are preceded in a simple program for profit analysis to estimate LC 50 values with 95% confidence intervals for statistically significant comparisons of potencies.

4.3) Materials :

- Brine shrimp eggs
- Sea salts (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipetts
- Micropipette
- Glass vials
- Magnifying glass
- Test samples of experimental plants.
- Aquarium air pump (SB2488, Sovo)
- Crude extracts
- Test tubes and racks
- Aluminum foil
- Electric Balance; SHIMADZU AY220 & SCALTEC SPB31
- Filter paper (Double Rings 102 – 11cm, HANGZHOU XINHUA PAPER Industry Co. Ltd., China)
- DMSO (Merck, Germany)
- TWEEN80 (Merck, Germany)
- K2Cr2O7(Merck, Germany)

4.4) Procedure:

38 gm sea salt was weighed,disloved in 1 litre of distilled water adjust ed to ph – 8.5 using 1N NaOH and was filtered off to get clear solution.

4.5) Htaching of brine shrimps:

Brine shrimp eggs were collected from pet shops used as the test organism/Articial seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then that side was covered.The tank was kept under constant aeration for 48 hours to hatch the shrimp and to be matured as nauplii.The hatched shrimps were attracted to the lamp through the perforated dam and with the help of a Pasteur pipette 10 living shrimps were added to each of the test tubes containing 10 ml of brine solution

4.6) Preparation of test solutions:

1.30 test tubes were cleaned with acetone and then every test tube was filled with 10ml of water and then the water level was tagged with masking tape.

2.Then the water removed from all the test tube and kept for the assay. A serial number was also written on the masking tape for maintaining the drugs concentration.

3.In each test tube 10 nauplii was taken into each test tube with a dropper from the freshly hatched free-swimming red nauplii. And the sea water level in the test tube was below 10 ml for the further adjustment with crude drugs.While taking the nauplii we have to be very careful to avoid any egg to enter into the test tube to get the ultimate result.

4.While giving the drug, 10 ml of DMSO and 2 drops of TWEEN 80 was given in the test tubes.

5.Then those test tubes were kept for 24 hours to measure the cyotoxicity result of crude extract of *Boerhavia diffusa.*

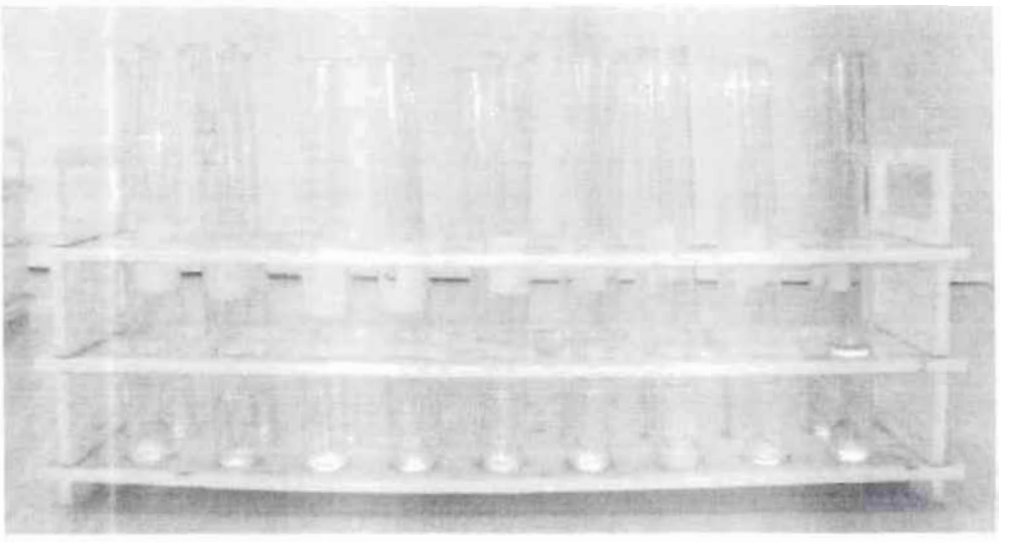


Figure 4.1: Labeled Test tubes.

Thus the concentrations of the obtained solution in each test tube were as:

-50 microgram/ml

-75 microgram/ml

-100 microgram/ml

-125microgram/ml

-150 microgram/ml

-200 microgram/ml

-250 microgram/ml

-250 microgram/ml

4.7) Preparation of control groups:

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other factors are nullified. We have Used positive control.

-Positive control (Potassiun Dichromate)

Positive control in a cytotoxicity studies a widely used accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. 10 test tubes were taken in which 10 nauplii were taken previously. For positive control K2Cr2O7 were used as the standard drug.0.05 gm of K2Cr207 was taken then the test tube were adjusted with normal sea-water up to 10ml, so the final concentration of the first test tube was 1 µg/µl. This solution were given to the other test tubes for positive control test in the same manner but the final concentration varied as different amount of solution to each test tubes. Then these test tubes were kept for 24 hours to get the result of brine shrimp lethality test.

Table 1 :

Serial number	Amount of K2Cr207 solution	Final volume	Final concentration
1	2 µl	10 ml	1 µg/ml
2	4 µl	10 ml	2 µg/ml
3	6 µl	10 ml	3 µg/ml
4	8 µl	10 ml	4 µg/ml
5	10 µl	10 ml	5 µg/ml

6	12 µl	10 ml	6 µg/ml
7	14 µl	10 ml	7 µg/ml
8	16 µl	10 ml	8 µg/ml
9	18 µl	10 ml	9 µg/ml
10	20 µl	10 ml	10 µg/ml

Figure 4.2: Hatching of Nauplii

Cytotoxicity test

4.8Counting of Nauplii and analysis of data :

After 24 hours ,the test tubes were inspected using a magnifying glass and the number of survivors was counted.The percent (%) mortality was counted for each dilution.The concentration-mortality data were analyzed by using Microsoft exel 2007.The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC50) value,which discloses the concentrations responsible for death.

4.9) Result and Discussion:

Chart 4.1: Effect of Ethyl acetate extract of Boerhavia diffusa on brine shrimp nauplii

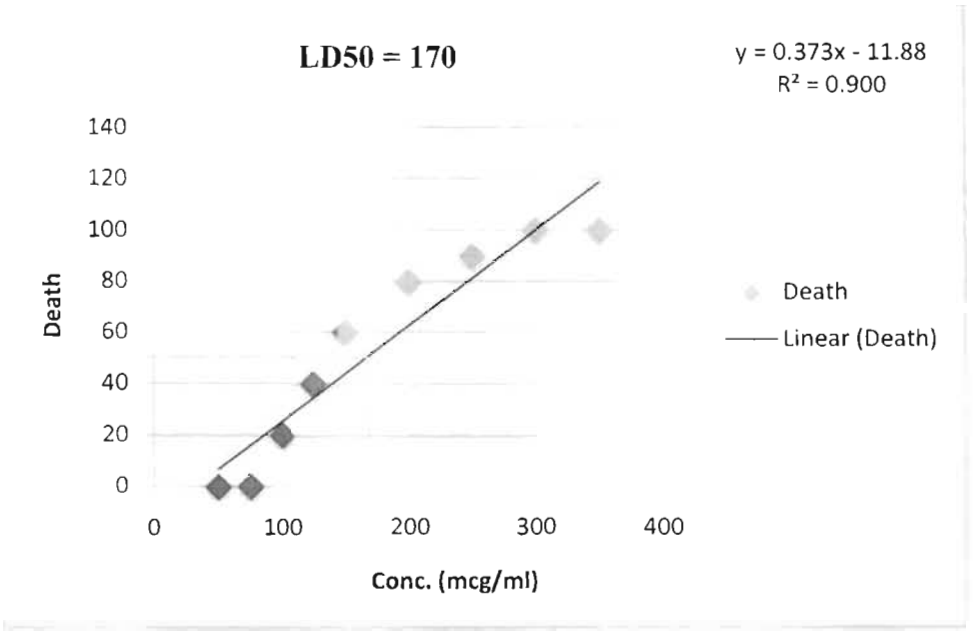
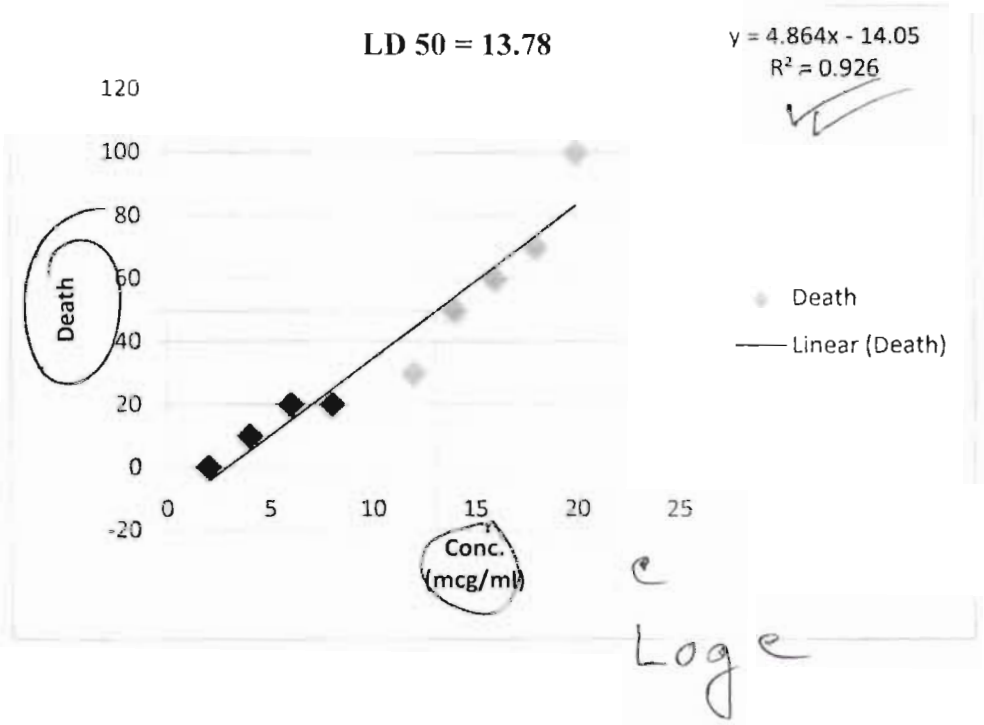


Chart 4.2: Effect of Potassium dichromate on brine shrimp nauplii.



Result and Discussion

It is known that when R^2 Value is 0.9 then it gives significant biological activity. Our *Boerhaviad diffusa* also showed significant biological activity compared to the positive control potassium dichromate. The LD 50 of *Boerhavia diffusa* 170 and the LD 50 of the positive control is 13.78 from the graph we can see the experimental value shows that the more the concentration increases the more number of death. The maximum number of death occurs at the concentration of 300 mcg/ml. *Boerhavia diffusa* shows cytotoxic activity successfully.

Chapter-5

Anti microbial Test

Anti Microbial test of *Boerhavia diffusa*

Microbiology is the study of living organisms of microscopic size, which include bacteria, fungi, algae, protozoa and the infectious agents at the borderline of life that are called virus. It is concerned with their form, structure, physiology, metabolism, and classification. It includes the study of their study in nature, their relationship to each other to other living organisms, their effects on human beings and on other animals and plants. Microorganisms can cause disease, spoil food and deteriorate materials like iron pipes, glass lenses, and wood pilings.

5.1) Methods:

Antibiotics diffuse from a confine source through the nutrient agar gel and create a concentration gradient. Solutions of known concentration of the test sample are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper disc containing the test samples of known amount are placed on nutrient agar medium with micropipette uniformly seeded with the test microorganisms. Standard antibiotic (Ciprofloxacin) disc and blank disc (impregnated with Ethyl acetate) are used as positive control. Theses plates are kept at low temperature (4C) for 24 hours to allow maximum diffusion. During this time, dried disc absorb water from the surrounding media and then the test material are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecule through agar gel. As a result, there is a gradual change of the test material's concentration in the media surrounding the disc.

The plates are then inverted and incubated at 37 C for 24 hours for optimum growth of the organisms. The test materials having antibacterial property will inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area, defined as `ZONE OF INHIBITION`. The antimicrobial activity of the test agent is then determined by measuring the diameter of Zone of Inhibition expressed in millimeter. The experiment is carried out more than once and of the reading required.

5.2) Apparatus and Reagent:

- Filter paper discs
- Sterile cotton
- Micropipette
- Laminar air flow hood (ESCOr Laminar Flow Cabinet)
- Refrigerator
- Petridishes
- Sterile forceps
- Screw cap test tubes
- Inoculating loop
- Nose mask
- Incubator (EHERT)
- Sterile tips
- Crude extracts of experimental plant
- Micropipette (Eppendrof, Germany)
- Micropipette tips (Eppendrof, Germany)
- Spirit burner
- Hand gloves
- Autoclave (HIRAYAMA, Japan)
- Hot air oven (FN-500, Niive)
- Nutrient Agar (TECHNO PHARMCHEM, India)
- Filter paper (Double Rings 102 – 11cm, HANGZHOU XINHUA PAPER Industry Co. Ltd., China)
- 0.9% Nacl solution

5.3) Preparation of agar solution:

A standard rule is, 28 gram Nutrient Agar should dissolve in 1000 ml of water. 8.4 gram Nutrient Agar was weighed and then 300 ml of distilled water was added to prepare 300 ml Agar solution. This preparation was kept in a 400 ml glass container. And then the glass container was kept in the autoclave machine for 15 minutes at 121°C temperatures. After that the agar solution were replaced in the laminar air flow cabinet.

5.4) Preparation of 0.9% Nacl solution :

0.9 gm of Nacl was dissolved in 100 ml of water.

5.5) Sterilization of Petri dishes:

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 as switched on 1 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.

3 Petri dishes were wrapped with paper and placed inside an autoclave machine (HIRAYAMA, Japan) for sterilization at 121°C for 15 minutes. After that, Petri dishes were washed with detergent soap properly. After washing, allowed them to dry and then placed these Petri dishes into hot air oven (FN-500, Niive) for 20 minutes. Then these Petri dishes were place into the laminar flow cabinet for prevention of further contamination.

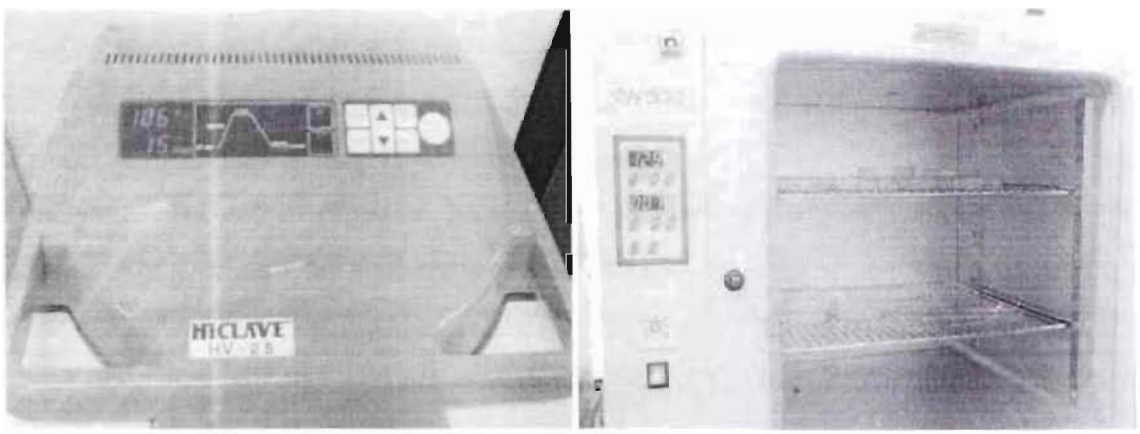


Figure 5.1: Autoclave (HIRAYAMA, Japan) and hot air oven (FN-500, Niive)

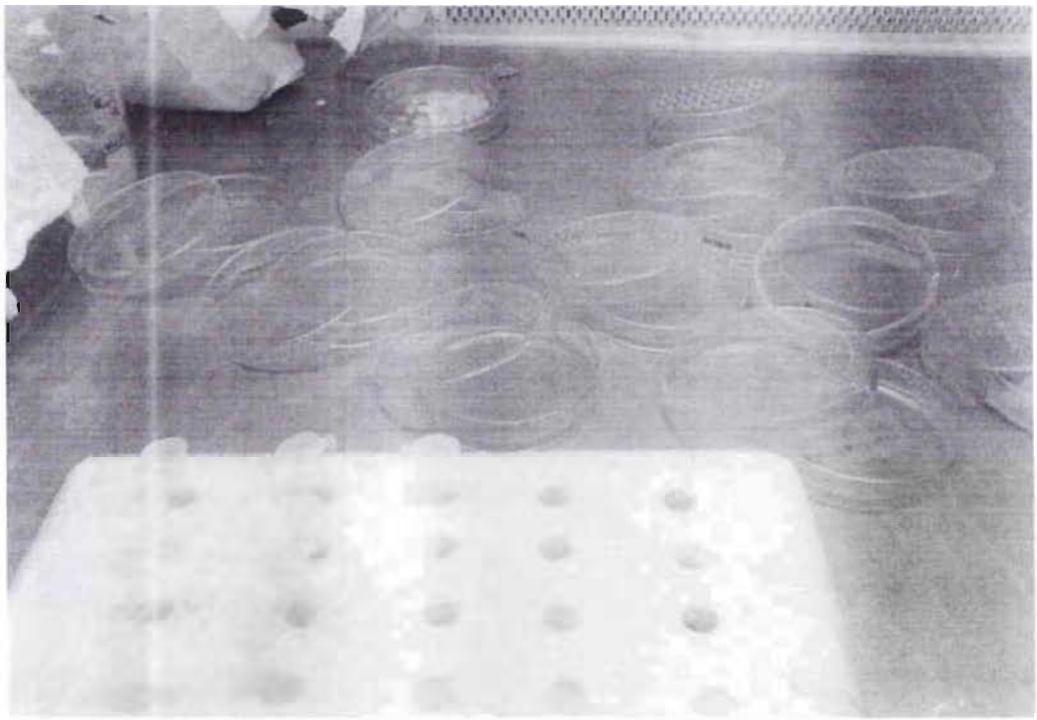


Figure 5.2: Prepared agar media for bacterial cultures

5.6) Inoculations:

The prepared Agar solution was poured into each of the nine Petri dishes in a way so that each Petri dish gets 12-15 ml agar medium. Agar medium was dispensed into each Petri dish to get 3-4 mm depth of agar media in each Petri dish. After pouring the agar medium, all Petri dishes were kept in room temperature so that agar medium can become properly solidified. Petri dishes were labelled with the name of the microorganisms.

0.5 ml of bacterial suspension was taken from the each vial with micropipette and place on the surface of the agar media on each Petri dish according to their labelled name. After that the suspension was spread with glass rod spreader gently on the agar media. Then the ciprofloxacin disc and other two discs (one discs having 500μg and another having 1000 μg of crude drugs) containing crude extracts were placed into the agar media. All these work were done in the laminar air flow cabinet.

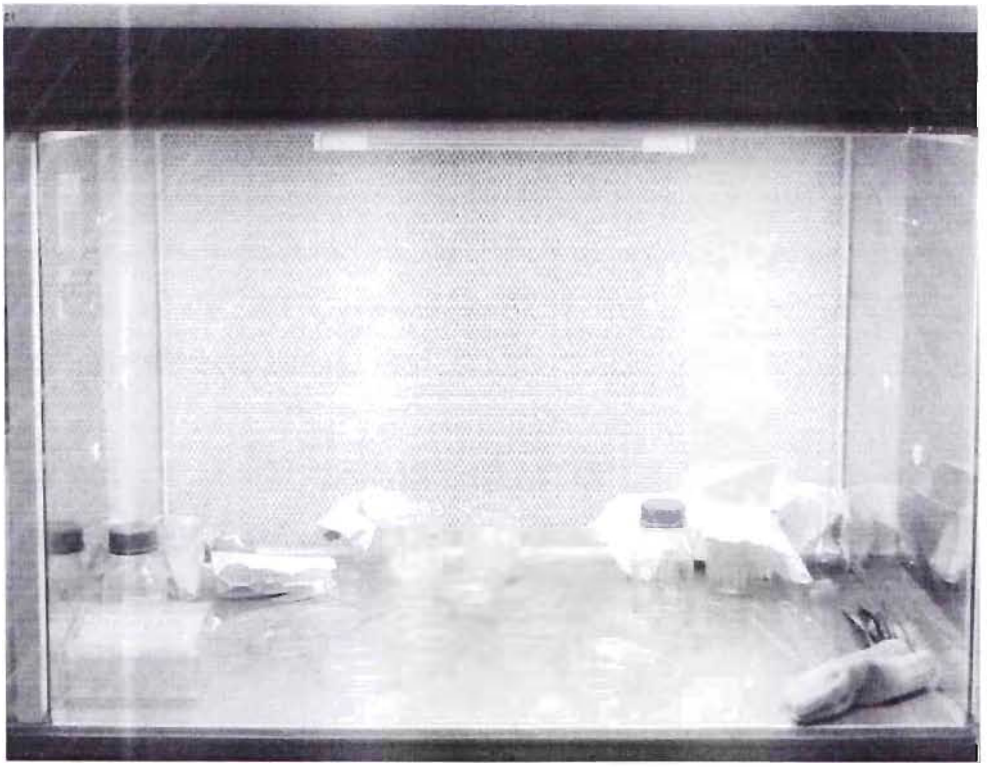


Figure 5.3: Laminar air flow cabinet (ESCO, Singapore)

5.7) Preparation of culture:

In an aseptic condition under Laminar air cabinet; the test organisms were transferred from the collected slants to the Petri dishes containing about 15 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 C for their optimum growth. These fresh cultures were used for the antimicrobial test.

5.8) Preparation of Test Plate:

The test organisms (*Staphylococcus aureus, Shigella dysenteriae, Candida albicans*) were transferred from the subculture to the test tubes containing isotonic saline with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by a rotation to get a uniform suspension of the organisms. The bacterial suspension was immediately transferred to the sterilized Petri dishes. The Petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

5.9) Preparation of Discs:

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 experiment we used Antibiotic Ciprofloxacin as the standard disc.

5.10) Preparation of sample discs with test samples:

0.2 gm of each Ethyl extract of *Boerhavia Diffusa* was dissolved in 2ml of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of and dried.

5.11) Diffusion and Incubation:

The Sample discs and the standard discs were placed gently on the previously marked zones in the agar plates pre-incubated with test bacteria. The plates were then kept in a refrigerator at 4C for 24 hours upside on 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 C for 24 hours.

Figure 5.4 : Incubation

Anti microbial test

5.12) Determination of Antimicrobial activity:

The antimicrobial potency of the Ethyl extract of *Boerhavia Diffusa* is measured by their activity to prevent the growth of the microorganisms surrounding the disc which gives clear Zone of Inhibition. After incubation, the antimicrobial activities of the test agent were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

5.13) Colony forming unit (CFU) :

In quantitative microbiology ,we are concerned with determining the concentration CFUs in our sample i.e. the number of CFUs per ml or per gram of our sample. If fewer than 30 colonies then we run into statistical inaccuracy, if greater than 300, the colonies would be tedious to count and also would tend to run together.

Table 5.1: Colony Forming Unit (CFU)

SA	4	Bacteria	Dilution	CFU
10		SA	3	16
11		SD	3	50
12		SD	4	35
13		CA	4	21
14		CA	3	74

5.15) Results:

The results of the experiments carried out on the antimicrobial effect on the plant *Boerhavia diffusa* leaves with solvent Ethyl Acetate again clinical pathogens like .*Streptococcus aureus, Shigell dysenteriae* and *Candida albicans.* Antibacterial activity of at different concentrations against different clinical pathogens with control is shown in the table below:

Table 5.2: *Staphylococcus aureus*

Staphylococcus aureus		
	500 mcg	1000 mcg
Solvent	Zone(mm)	
Ethyl acetate	0	0
Ciprofloxacin	Zone (mm)	
30 mcg	42	

Shigella dysenteriae		
	500 mcg	1000 mcg
Solvent	Zone(mm)	
Ethyl acetate	0	0
Ciprofloxacin	Zone (mm)	
30 mcg	38	

Candida albicans		
	500 mcg	1000 mcg
Solvent	Zone(mm)	
Ethyl acetate	0	7
Ciprofloxacin	Zone (mm)	
30 mcg	47	

New BSAC criteria for disc diffusion testing have since been developed including ciprofloxacin disc content, zone diameter breakpoints and corresponding MICs,

specifically for urinary pathogens.[5] These guidelines indicate MICs ≥ 8 mg/L and zones of inhibition ≤17/19 mm for resistant Gram-positive/negative isolates and MICs ≤ 4 mg/L and inhibition zones measuring ≥18/20 mm for susceptible Gram-positive/negative isolates. laboratory method for testing the effectiveness of antibiotics. It is usually done on organisms known to be potentially resistant to antibiotic therapy in vitro. A report of a "resistant" finding means the antibiotic is not effective in inhibiting the growth of a pathogen, whereas use of an effective antibiotic results in a "sensitive" report In ethyl acetate extract of *Boerhavia diffusa* plant was found to be resistant against *Staphylococcus aureus* and *Shigella dysenteriae.* And intermediate against *Candida albicans*.Each zone size is interpreted as follows:

- Sensitive: The zone size is equal to, longer than or not smaller than 11 mm than the control.
- Intermediate: The zone size of the test strain at least 4 mm, also at least smaller than that of the control strain.
- Resistant: The zone size of the test is smaller.

The ethyl acetate extract of *Boerhavia diffusa* is resistant to *Staphylocossus aureus* and *Shigella dysenteriae* as in both cases the zone diameter is 0 mm,that means the ethyl extract of Boerhavia diffusa is not effective against these 2 microorganisms.But this shows resistant to intermediate activity against the microorganism candida albicans as the zone diameter was 0 mm in terms of 500 mcg which indicates the resistant activity and zone diameter 7 mm indicates the intermediate activity in terms of 1000 mcg of Ethyl acetate extract of *Boerhavia diffusa.*

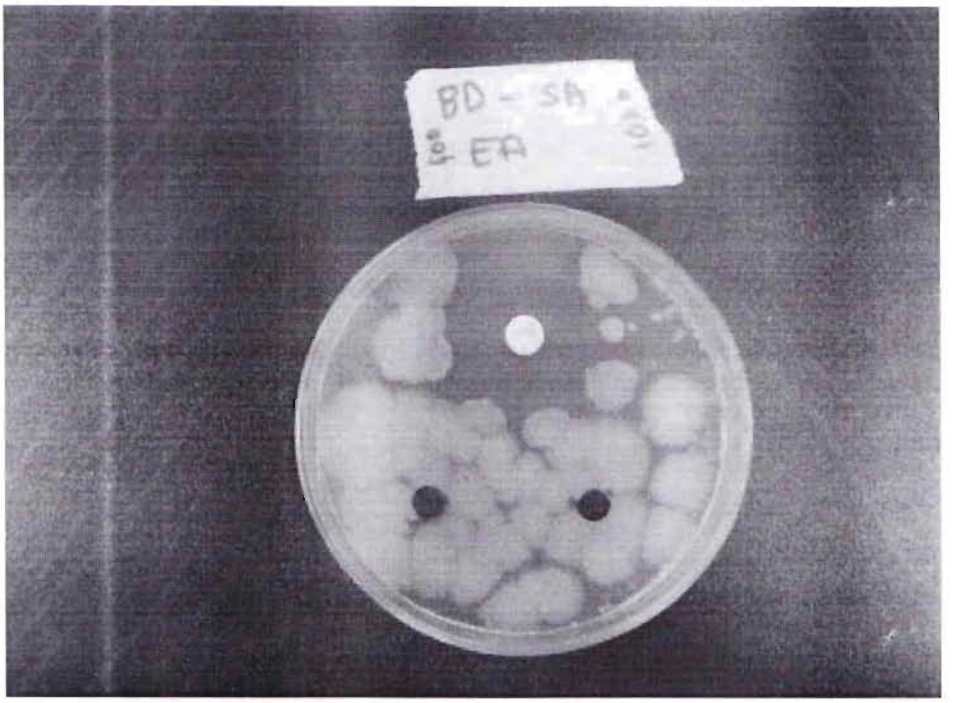


Figure: Antimicrobial effect of Ethyl acetate extract of *Boerhaavia diffusa* against *Staphyllococcus aureus*

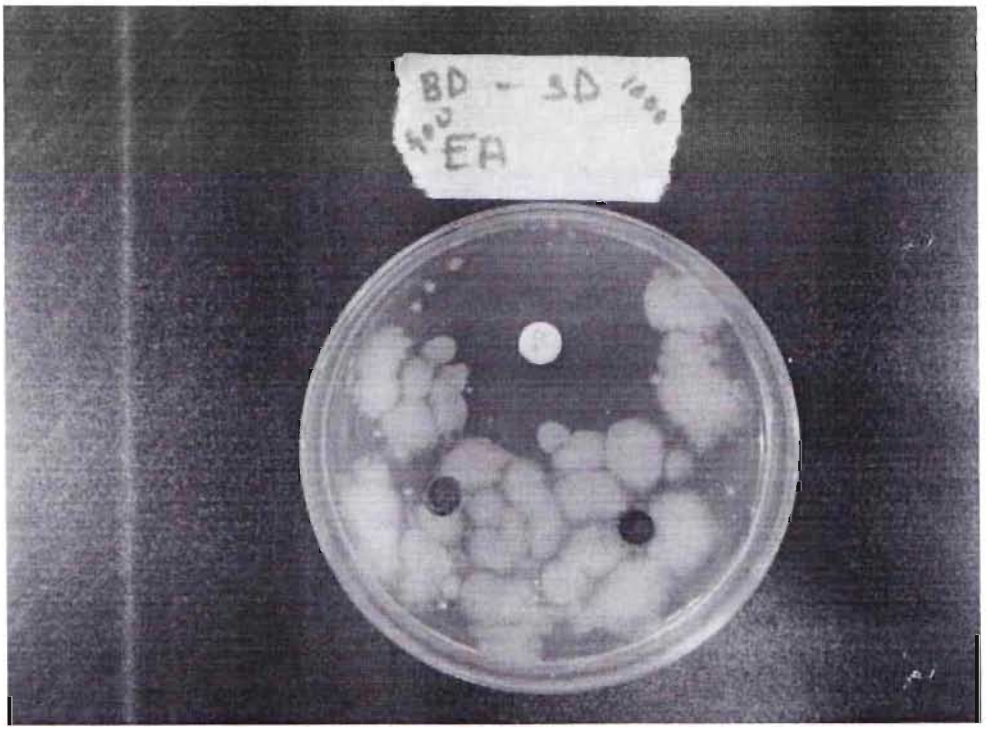


Figure: Antimicrobial effect of Ethyl acetate extract of *Boerhaavia diffusa* against *Shigella dysenteriae*

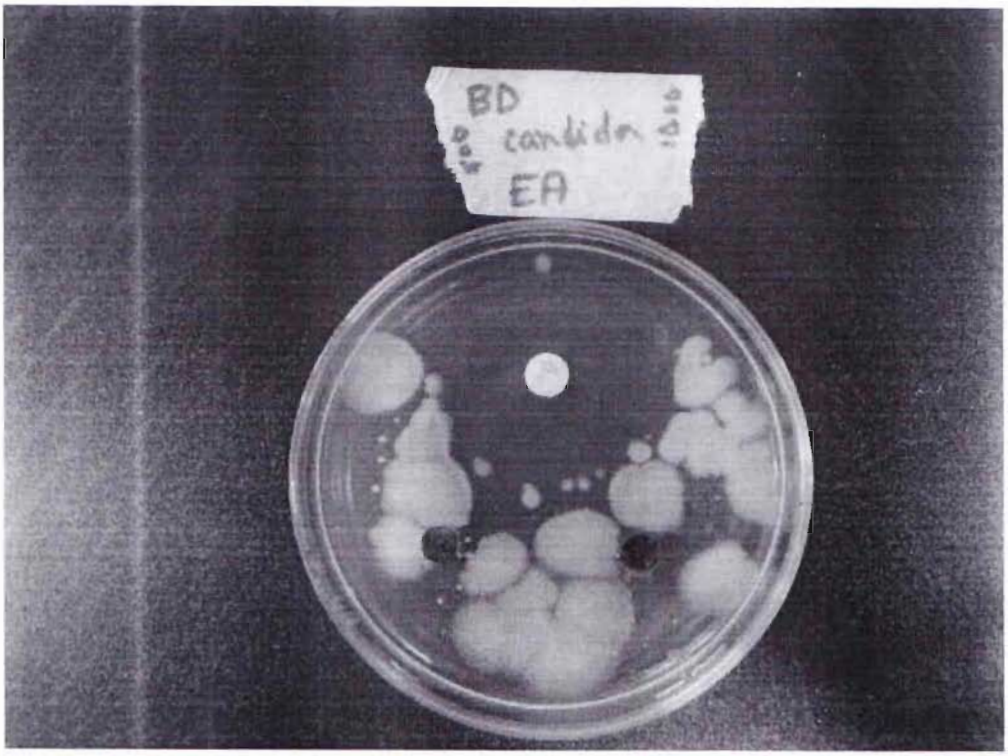


Figure: Antimicrobial effect of Ethyl acetate extract of *Boerhaavia diffusa* against *Candida albicans*

Chapter-6

Conclusion

Conclusion

Based on the results of the present study it is concluded that the *B. diffusa* leaves have potent antibacterial activity against various Gram-negative and Gram-positive bacteria which might be due to the phytochemicals present in the leaves. Also, there is further scope to study the identification and purification of active compound(s) involved in this antibacterial activity of *B. diffusa*.

The study was aimed at testing the efficacy of Ethyl acetate extract of medicinal plants for their antimicrobial activity and study the influence of plant extract on the virulent factors on microorganisms. The plants were selected based on the traditional knowledge that has accumulated in the past. The antimicrobial activity was tested against bacterial pathogens thatwere resistant to various antibiotics (Warrier et al., 1997). The Ethyl actetate extract of *Boerhavia diffusa* has shown poor antimicrobial activity against *Staphylococcus aureus*,*Shigella dysentariae* and is less effective against *Candida albicans* compared to the antibiotic Ciprofloxacin which was used as positive control. *Boerhavia diffusa* also showed anticancer activity by testing the brine shrimp lethality assay done with the Ethyl acetate extract as long as the concentration was increased the mortality rate also increased. Comparing to the positive control Potassium dichromate we found the anticancer activity of the *Boerhavia diffusa*

Chapter-7

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