Evaluation of Antioxidant, Cytotoxic and Antimicrobial activity of Ethanolic Extract of *Mikania cordata* (Burm. F.) Robinson

This Thesis Paper is submitted to the Department of Pharmacy, East West University in Conformity with the Requirements for the Degree of Bachelor of Pharmacy

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This research paper is dedicated to my all teachers who enlighten me by their light and my respective research supervisor Dr. Shamsun Nahar khan.

CERTIFICATE

This is to certify that, the research work on 'Evaluation of Antioxidant, Cytotoxic and Antimicrobial activity of Ethanolic Extract of *Mikania cordata* (Burm.F) Robinson' submitted to Department of Pharmacy, East West University, Jahurul Islam city, Aftabnagar, Dhaka-1212, in partial fulfilment of the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Taniza Tajrin (ID: 2009-3-70-013) under the guidance and supervision and that not part of thesis has been submitted for any other degree. We further certify that all the sources of information of this connection are duly acknowledged.

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LIST OF CONTENTS **Page number**

List of figures

List of Tables

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At the beginning, I would like to remember the mercy and kindness of Almighty Allah for completing my research work appropriately.

It is my pleasure and proud privilege to express my heartiest regards and gratitude to my respected teacher and supervisor **Dr. Shamsun Nahar Khan**, Associate Professor of Pharmacy Department, East West University.

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I remember here the inspiring words of my family members and to all my well wishers. I say many thanks to them for their wholehearted inspiration during my thesis work.

Abstract

The study was designed for pharmacological investigation of ethanolic crude extract of *Mikania cordata (*Family: Asteraceae, Bengali name – Asam Lata) and screening of their biological activities like, antimicrobial, cytotoxicity and free radical scavenging activities. In antioxidant activity the ethanolic extract show mild activity and in cytotoxic activity it showed moderate activity where it showed LD_{50} values at 29.49µg/ml and the antimicrobial activity of this ethanolic extract was not significant to mention.

Chapter-1
Introduction

1. Introduction

1.1. Definitions of medicinal plants

A considerable number of definitions have been proposed for medicinal plants. According to the WHO, "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis." When a plant is designated as 'medicinal', it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation. "Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes" (Ghani A,1998)

1.2 Why some of the plants are valued as medicinal plants

Many of the plants could be used as stimulants, poisons, hallucinogens or as medicine because of the presence of unique or rich biological-active plant chemicals (i.e. Chemical compounds that have a biological effect on another organism). Chemicals that make a plant valuable as medicinal plant are (1) Alkaloids (compounds has addictive or pain killing or poisonous effect and sometimes help in important cures, (2) Glycosides (use as heart stimulant or drastic purgative or better sexual health), (3) Tannins (used for gastrointestinal problems like diarrhoea, dysentery, ulcer and for wounds and skin diseases), (4) Volatile/essential oils (enhance appetite and facilitate digestion or use as antiseptic/insecticide and insect repellent properties), (5) Fixed oils (present in seeds and fruits could diminish gastric/acidity), (6) Gum-resins and mucilage (possess analgesic property that suppress inflammation and protect affected tissues against further injury and cause mild purgative), and (7) Vitamins and minerals (Fruits and vegetables are the sources of vitamins and minerals and these are used popularly in herbals) (Ghani A,1998)

1.3 The importance of medicinal plan in drug discovery

Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain. Several natural product drugs of plant origin have either recently been introduced to the United States market, including arteether, galantamine, nitisinone, and tiotropium, or are currently involved in late-phase clinical trials.(Marcy et al..,2005)

Globally, there have been concerted efforts to monitor quality and regulate the growing business of phytodrugs and traditional medicine. Data analysis confirms that the phytodrugs serve as crucial sources for the new drugs which can be further modified during drug development.

Studies reveal that the origin of the drugs developed in the last two decades showed that natural products or natural–derived drugs comprised of 28 per cent of all New Chemical Entities (NCEs). Not only were this 24 per cent of the NCEs synthetic or natural base compounds thus elevating the percentage of such products to nearly 52 per cent of all NCEs. This confirms the fact that the natural products including phytodrugs serve as crucial sources for the new drugs which can be further modified during drug development.

According to World Health Organisation (WHO), as much as 80 per cent of the world's population relies on traditional medicine. With increased concerns about rising healthcare costs, some governments are encouraging the use of indigenous systems of medicine rather than expensive imported drugs. This has been a strong driver for the resuscitation of phyto products. Today's healthcare systems rely largely on medicinal plant material. Much of the

world's population depends on traditional medicine to meet daily health requirements, especially within developing countries. Use of plant-based remedies is also widespread in many industrialized countries and numerous pharmaceuticals are based on plant compounds.

The pharmaceutical industry is both large and highly successful. At present, about 50 per cent of the total plant-derived drug sales come from single entities, while the remaining 50 per cent come from herbal remedies. Although the latter have greater volumes of consumption, the relatively low volumes of single entities, which are mostly prescription products, are more than compensated by their higher prices. Single entity plant drugs, which mostly treat serious medical ill, include atropine, digoxin, morphine, paclitaxel, pilocarpine, reserpine, scopolamine, topotecan and vincristine, among many others. Several of the compounds have outlived their usefulness in light of better alternatives, however, and are exhibiting decline in sales. On the other hand, as a consequence of new drug developments, single entities overall are projected to increase their market share of the combined total future dollar sales.

1.4 The medicinal plants of Bangladesh

South Asian countries have a large number of valuable medicinal plants naturally growing mostly in fragile ecosystems that are predominantly inhabited by rural poor and indigenous community.

Bangladesh has tremendous wealth of medicinal plants. But unfortunately, there is a lack of systematic efforts to explore and exploit this valuable potential except some sporadic attempts by a few institutions. One of the greatest difficulties confronting the research workers is the paucity of authentic informations on the identity, their distribution and availability, and the use of medicinal plants in this country. Medicinal plants mainly used in the preparations of Unani and Ayurvedic medicine, also prescribed by practitioners of

traditional medicine in different parts of the country and others are used as household remedies by the common people.

In Bangladesh 5,000 species of angiosperm are reported to occur. The number of medicinal plants included in the 'materia medica' of traditional medicine in this subcontinent at present stands at about 2,000. More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh. Dhaka, Rajshahi, Shylet and Chittagong division is rich in medicinal plants.

1.5 Objective of this study

The objective of this study is to determine the some pharmacological effects of *Mikania cordata*. In our study we mainly focus to determine the antioxidant activity, cytotoxic activity, antimicrobial activity of this plant.

1.6 Description of *Mikania cordata*

1.6.1 Scientific name: *Mikania cordata* (Burm. F.) Robinson

1.6.2 Common names: Assam lata, American rope,Chinese creeper, mile-a-minute weed.

1.6.3 Local names: American vally, silk vally, kaipu vally, Dhritharashtra pacha (Kerala,India), cheroma, ulam tikus(Malaysia), sembung rambat (Indonesia).

1.6.4 Taxonomic position

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida,

Order: Asterales

Family: Asteraceae

Genus: Mikania

Species: *Mikania cordata*

1.6.5 Plant Description

Mikania cordata is a very rapidly growing perennial vine which spreads over the ground and climbs any available support. The soft steams have internodes up to 6 cm long and carry many pairs of heart-shaped leaves. Nodes easily take root upon contact with moist soil. Dense heads of whitish flowers develop at the tips of braches and in the axils of leaves.

Figure 01: *Mikania cordata*

1.6.6 Geographical Distribution

Mikania is native to Central and South America, and has become a serious weed in West Africa through to India, South-East Asia, Indonesia and the Pacific Islands. Mikania was first found in Australia in 1998 at Ingham and Bingil Bay, and has since been detected at one location near Speewah, near Mareeba. (Francis, 1991)

1.6.7 Traditional use of *Mikania cordata*

The decoction of leaves is used in dyspepsia, dysentery, and gastric ulcer. The alkaloidal fraction of the leaf ethanolic extract showed anti-ulcer effects. The methanolic extract of the root showed anti-ulcer effects, stimulation of hepatic protein synthesis in carbontetrachloride-induced hepatotoxicity in mice, and significant anti-carcinogenic response. Scandenolide obtained from *M. cordata* exhibited significant anti-inflammatory (Ahmed et al., 2001).

- ✓ In southern Nigeria, decoction used for coughs; leaf juice used as remedy for sore eyes
- ✓ In East Africa, the Tongas used the plant as remedy for snake and scorpion bites. Infusion of plant given for affections of the stomach and intestines.
- ✓ Leaves used by the Malays by rubbing it onto the body for itches.

- ✓ In Java, leaves used for poulticing circumcision wounds and other wounds.
- ✓ In India, leaves used for itchiness and as wound plaster.
- ✓ In Bangladesh, decoction used for treatment of gastric ulcer.
- ✓ In southern Africa, leaves applied to wounds.

Chapter-2
Literature Review

2. Review of literature

2.1 Phytochemical studies

Out of 430 species identified from genus *Mikania*, 55 of them provide over 300 different chemical compounds, among terpenes and derivatives, some alkaloids, saponins, sterols and flavonoids.

2.1.1 Previously isolated classes of constituents

Different classes of compounds were previously isolated from various *Mikania* parts, which can be associated to this plant's pharmacological activities. The main groups are: coumarins and derivatives, sesquiterpenes, sesquiterpenes lactones, diterpenes, phytosterols or terpenoids and flavonoids. Caffeoylquinic acid derivatives beyond others chemical compounds are found in smaller amount. Diterpenes such as kaurenoic acid and benzoylgrandifloric acid (class of kauranes), have also attracted interest for their pharmacological action (Gasparetto et al., 2010).

2.1.1.1. Coumarins and derivatives

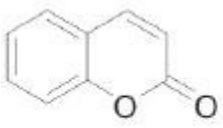
The most characteristic class of compounds in *Mikania* genus are the coumarins and derivatives, frequently responsible for pharmacological activity. A wide variety of biological activities is assigned for these compounds, such as antimicrobial, antiviral, anti-inflammatory, antispasmodic, antitumoral, anticoagulant, bronchodilator and antioxidant (Pereira et al., 1992; Hoult & Payá, 1996). The coumarin (1,2-benzopyran) (1), dihydrocoumarin and*O*-coumaric acid were identified in extracts of *M. glomerata* (Vidal et al., 2006) and *M. laevigata* (Oliveira et al., 1984). Herz and Kulanthaivel (1985) discovered in *M. congesta* aerial parts, growing in the state of Pará-Brazil, some similar compounds such as scopoletin (2), *O*-geranylscopoletin. In *M. shushunensis*, collected in Peru, herniarin (7-

methoxycoumarin) (3) and 2,6-dimethoxyquinone (Gutierrez & Herz, 1988) has been described.

2.1.1.2. Sesquiterpenes and terpenes, diterpenes and sesquiterpenes lactones

Sesquiterpenes are abundant in *Mikania* genus, related to that the most commom are germacrene D, isocomene and γ -humulene. These compounds were reported in around 15% of *Mikania* species that already had their chemical composition determined, among them *M. arrojadoi* (Bohlmann et al., 1982b), *M. officinalis, M. sessilifolia, M. luetzelburgii* and *M. belemii* (Bohlmann et al., 1981).

Likewise, terpenes, diterpenes and sesquiterpene lactones are often found, mainly the dilactones type mikanolide and miscandenin derivatives, which have analgesic activity (Ahmed et al., 2001), antibacterial (Facey et al., 2010).



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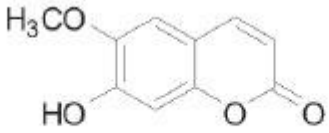

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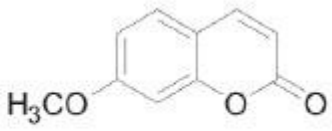

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2.1.1.3. Diterpenes

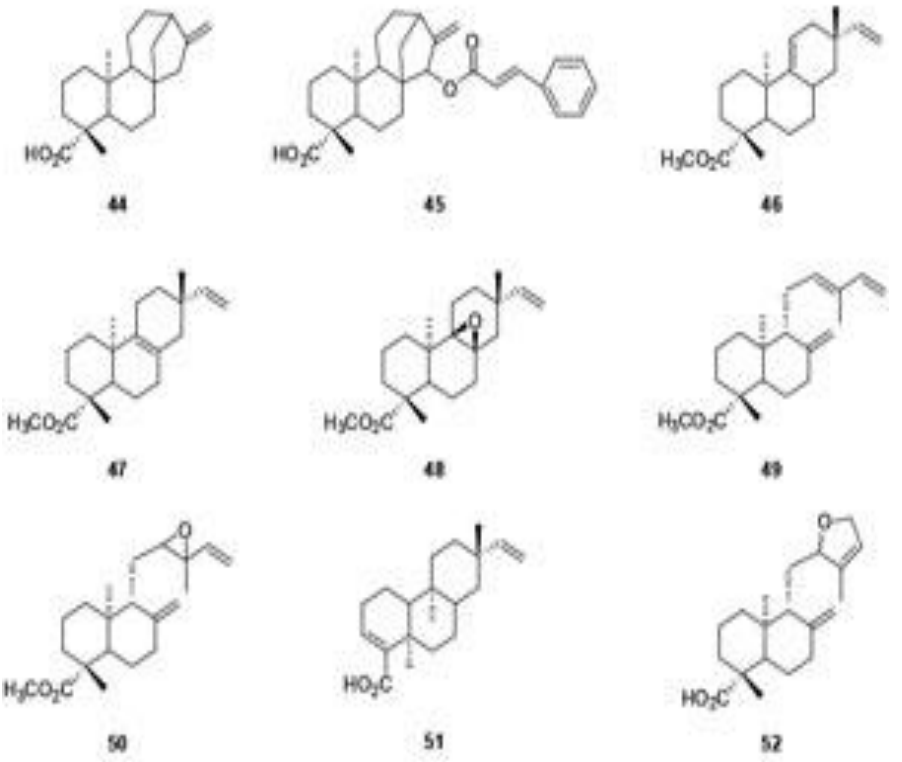
Some diterpenes are common in *Mikania* genus like kaurenoic acid (**44**), the main component of ethanolic extract in *M. obtusata* (Alves et al., 1995) and *M. glomerata* (Barbosa et al., 1994), which is characterized by its trypanocidal activity. Also, the kaurenoic acid has other important activities such as antimicrobial, antinociceptive, anti-inflammatory and smooth muscle relaxant (Costa-Lotufo et al., 2002; Wilkens et al., 2002; Cunha et al., 2003).

In *M. laevigata* the main representatives are cinnamoylgrandifloric acid (**45**), isopropiloxigrandifloric acid, isobutiloxi-grandifloric acid and kaurenol (Oliveira et al., 1984; Bighetti et al., 2005; Yatsuda et al., 2005; Santos et al., 2006; Bolina et al., 2009). Furthermore, from *M. oblongifolia* aerial parts were obtained cinnamoylgrandifloric and others terpenes (Vichnewski et al., 1977).

Cruz & Roque (1992) isolated from *M. triangularis* stems, found in the state of São Paulo, Brazil, a new diterpene acid, methyl *ent*-7-α-hidroxypimara-8,15-dien-19-oate and other diterpene acids known as methyl-*ent*-pimara-9 (11),15-dien-19-oate (**46**), methyl-*ent*-pimara-8,15-dien-19-oate (**47**), methyl-8,9α-epoxy-*ent*-pimara-15-en-19-oate (**48**), methyl-7β-hydroxy-*ent*-pimara-8,15-dien-19-oate and methyl-7α-hydroxy-*ent*-pimara-8,15-dien-19-oate. In addition, the acidic fraction of hexane extract is composed of various pimaradienes acids, which show antibacterial activity (Cruz et al., 1996).

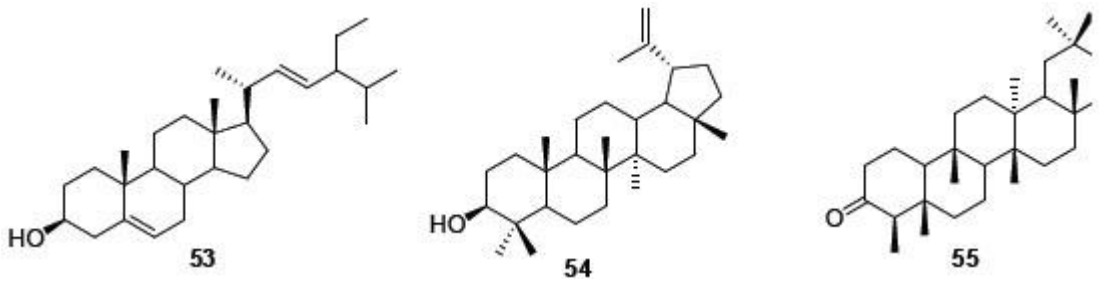
In a study performed by (Nunez et al., 2004) on leaves of *Mikania* sp. nov., found in the state of Bahia, Brazil, several diterpenes were obtained: labda-8(17),12,14-trien-19-oic methyl ester (**49**), pimara-9(11),15-dien-19-oic methyl ester, labda-8(17),13(16),14-trien-19-oic methyl ester, labda-12α-epoxy-8(17),14-dien-19-oic methyl ester, labda-12β-epoxy-8(17),14-dien-19-oic methyl ester (**50**), erythroxyla-3,15-dien-19-oic acid (**51**), labda-12,15-epoxy-8(17),13-dien-19-oic acid (**52**), and labda-12,13-dihydroxy-8(17),14-dien-19-oic methyl ester.

Phytosterols or terpenoids The most common phytosterols present in approximately 10% of species of *Mikania*, that has its chemical composition determined, are stigmasterol (**53**), lupeol (**54**) and sitosterol. These compounds have been detected in the aerial parts and are found in the species *M.micrantha* (Herz et al., 1975; Nicollier and Thompson, 1981), *M. glomerata* (Barbosa et al., 1994), *M. cordata* (Kiang et al., 1968; Aguinaldo et al., 1995)



The presence of other common phytosterols as campesterol and taraxasterol has been reported in species like *M. cordifolia* (Oliveira et al., 2006)

The terpenoids amyrin and friedelin (**54**), abundant in *Mikania* genus, were reported in *M. micrantha*, *M. cordata*, *M. cordifolia* (Oliveira et al., 2006), *M. minima* and *M. lasiandrae* (Soares et al., 2007), among others species.



Among the exotic representatives of terpenoids present in *Mikania* genus were reported τ-muurolol in *M. hookeriana* (Reis et al., 2003), stigmasta-4,22-dien-3-one in *M. microptera*, olean-9(11),13-dien-3-one in *M. rimachii* (Díaz et al., 1992) and 19,20-dihydroxy-16-oxo-geranyl from *M. luetzelburgii* (Bohlmann et al., 1981).

2.1.1.4. Flavonoids

Flavonoids are popular due to their antioxidant activity and are widely present in *Mikania* genus supporting its pharmacological activity. In *M. laevigata* flavonoids glycosides as patuletin 3-*O*-β-D-glucopyranoside (**56**), kaempferol 3-*O*-β-D-glucopyranoside (**57**), quercetin 3-*O*-β-D-glucopyranoside (**58**) and 3,3',5-trihydroxy-4',6,7-trimethoxyflavone are the representatives compounds (Ferreira & Oliveira, 2010). In *M. cordata*, flavonoids weredescribed as patuletine-3-*O*-β-D-6''-(p-coumaroyl), glucoside(6-methoxyquercetin-3-*O*-β-D-6''-(*p*-coumaroyl)glucoside), mikanin-3-*O*-sulfate (salt as Ca^{+2}) (**59**), eupalitin-3-*O*-sulphate (as salt K^{+}) (**60**), eupalitin-3-*O*-β-D-glucoside (**61**), 6-methoxykaempherol-3-*O*-β-D-glucoside (**62**), nepetin (**63**) and kaempherol-3-*O*-α-L-rhamnoside (**64**) (Aguinaldo et al., 2003). For the same species, it was reported the isolation of a flavone, mikanin-(3,5-dihydroxy-4',6,7-trimethoxyflavone) with epifriedelinol from roots and fumaric acid from leaves and stems (Kiang et al., 1965).

In *M. cordifolia* a quercetin derivative was identified as quercetin-3-*O*-glucoside. In *M. micrantha* was isolated and identified eupalitin, eupafolin, luteolin (**65**) (Wei et al., 2004), mikanin, alpinetin, mikanin-3-*O*-sulfate (**59**) (Herz et al., 1975; Nicollier & Thompson, 1981; Boeker et al., 1987; Cuenca et al., 1988; Jiang et al., 2001). In the same specie was identified 3,4',5,7-tetrahydroxy-6-methoxyflavone3-*O*-β-D-glucopyranoside by (Huang et al.,2009).

Kaempferol-3-*O*-glucoside (**57**) and quercetin-3-*O*-glucoside (**58**) were identified from *M. Parodii* (Gregorio et al., 2008). Naringenin (**66**) was identified from *M. grazielae* (Bohlmann et al., 1982b) and a specific flavone derivative called batatifolin was found in *M. batatifolia* (Herz and Santhanam, 1969).

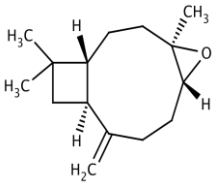
2.1.1.5. Caffeoylquinic acid and derivatives

The chemical compound 5-caffeoylquinic acid is a caffeic acid ester, also known as a chlorogenic acid, commonly found in a wide number of plants, *e.g.* coffee. It is produced in plants via an ester bond between the carboxyl group of caffeic acid and the 5-hydroxyl group of quinic acid (Clifford et al., 2006). The chlorogenic acid and caffeic acid were reported as dampening the risk of chronic diseases such as inflammation, cardiovascular diseases and cancer (Boyer & Liu, 2004; Bonita et al., 2007).

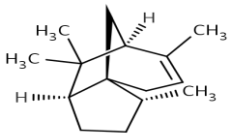
In *M. micrantha* was reported the presence of 3,5-di-*O*-caffeoylquinic acid n-butyl ester and 3,4-di-*O*-caffeoylquinic acid *n*-butyl ester (Wei et al., 2004). The same 3,5-di-*O*-caffeoylquinic acid (**67**) was reported in *M. cordifolia*, beyond others derivatives like 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid (**68**), 4,5-di-*O*-caffeoylquinic acid and 3-*O*-feruloyl,5-*O*-caffeoylquinic acid (Gregorio et al., 2008). In *M. lasiandrae* was also reported the presence of caffeoylquinic acid (Soares et al.,2007). In *M. hirsutissima* was reported the presence of 1,5-dicaffeoyl-quinic acid (Oliveira, 1972; Ohkoshi et al.,2004)

Compound	Percentage (%)
α- cubebene	21.3
Caryophyllene	10.1
α-bisabolol	6.6
ϒ-curcumene	6.3
B-pinene	4.1
copaene	4.1
α-cedrene	4.9
Spathulenol	3

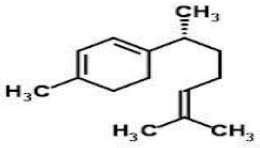
Table 01: Sesquiterpenes in *Mikania cordata* in the part of leaves



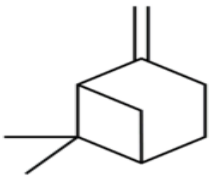
Caryophyllene oxide



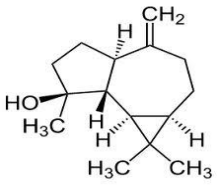
α-cedrene



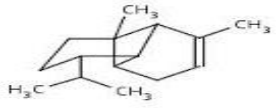
γ-curcumene



β -pinene

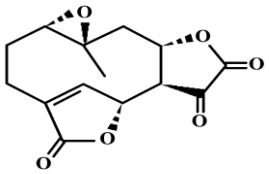


Spathulenol



Copaene

2.1.2 New-isolated constituent: Deoxymikanolide (1, 10-epoxy-4,11(13)-germac-radiene12,8;15,6-diolide.



Deoxymikanolid

2.2Pharmacological studies

2.2.1 The Antibacterial and Cytotoxic Properties

In 2011 scientist M. Sekendar and his co-worker studied on the antibacterial and cytotoxic properties of ethanol extract of leaves of *Mikania cordata* (Burm.f.) B.L. Robinson. To determine antibacterial activities, the extract was tested against four Gram positive and six Gram negative bacteria at three concentrations (500, 800, 1000 μg disc-1) through disc diffusion method. The extract showed moderate antibacterial actions and that was increased by increasing the concentration of the sample. The maximum antimicrobial potential was obtained against *Shigella flexneri* and no sen sitivity was found for *Klebsiella* sp. comparatively gram-positive bacteria demonstrated more susceptibility to the extract than gram-negative bacteria. Cytotoxic property of the sample was done using Brine shrimp lethality bioassay where it did not show noticeable toxicity. So, our present study reveals that the leaves extract of *M. cordata* possess considerable antibacterial properties along with lesser amount of cytotoxicity. (Sekendar et al., 2011)

2.2.2 Analgesic test

In 2001 scientist M. Ahmed and his co worker studied on the analgesic activity of the crude extract of *Mikania cordata* and deoxymikanolide significantly inhibited acetic acid-induced writhing in mice. Three other sesquiterpene dilactones isolated from the same plant, namely mikanolide, dihydromikanolide and scandenolide, did not show significant analgesic activity. (Ahmed et al., 2001)

2.2.3 Anti-inflammatory activity test

In 2006 scientist S. Bhattacharya and his co-worker studied on the antiinflammatory profile of *Mikania cordata* (Burm) B. L. The methanolic fraction of the root extract of Mikania cordata was found to possess an inhibitory effect on carrageenin and other mediator-induced oedema; there was a significant inhibition of protein exudation, an increase in peritoneal capillary permeability and leucocyte migration in inflammatory conditions. The extract significantly inhibited both cotton pellet and carrageenin-induced granuloma formation, was effective in experimentally induced arthritic conditions and turpentine-induced joint oedema. The extract also possessed inhibitory effects on sodium urate-induced experimental gout. A significant reduction of pyrexia was also found to occur when rats were treated with the extract. Thus it may be concluded that the methanolic fraction of M. cordata root extract possessed significant antiinflammatory effects in exudative, proliferative and chronic phases of inflammation and demonstrated an antipyretic activity (Bhattacharya et al 2006).

2.2.4 Anti carcinogenic test

In 1994 A. Bishayee, and his co-worker studied on Anti carcinogenic biological response of *Mikania cordata,* at frist the study has been evaluated through its effects on Phase 1 and 2 of

the hepatic drug-detoxifying enzyme system in rats. Although oral administration of a methanolic extract of this plant root (50, 100 or 150 mg/kg for 4, 8 or 12 weeks) has been found to have very little or no effect on hepatic microsomal cytochrome *P*-450 and cytochrome b_5 contents as well as NADPH cytochrome *c* reductase activity, it afforded a marked induction of uridine diphosphoglucuronyl transferase activities of liver microsomes. The extract also significantly increased the activities of microsomal uridine diphosphoglucose dehydrogenase, reduced nicotinamide adenine dinucleotide (phosphate): quinine reductase and cytosolic glutathione *s*-transferases with a concomittant elevation in the contents of reduced glutathione. All these effects were found to be dose-dependent and maintained during 12 weeks of the extract treatment. Results of the study clearly indicate that the intracellular contents of active intermediates of various xenobiotics including chemical carcinogens would be reduced by the specific enhancement of drug-detoxifying enzymes in the liver of rats treated with the plant extract (Bishayee et al.,1994).

2.2.5 Effect on nervous system

Mikania extracts possesses some neuropharmacological properties confirmed. The studies with methanolic fraction of *M. cordata* root extract on experimental animals caused alterations in the general behavior pattern (*e.g.* reduction in spontaneous motility, analgesia, and suppression of aggressive behaviour), suppression of conditioned avoidance response and showed antagonism to amphetamine toxicity. The observations suggest that the root of *M. cordata* possesses a potent central nervous system-depressant action (Bhattacharya, et al., 1988).

2.2.6 Anti-stress Activity test

In 1995 A. Bishayee, and his co-worker studied on the effects of *Mikania cordata* root extract where the extract were investigated on stress-induced alterations in central neurotransmitters, viz., adrenaline (Ad), noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT), and enzymatic activities of monoamine oxidase (MAO) in brain and succinic dehydrogenase (SDH) in brain and liver of mice. Both 5 h swimming and 24 h immobilization stress elicited a decrease in the levels of Ad and NA whereas they increased those of DA and 5-HT. Pretreatment with M. cordata root extract for 15 days prevented the decrement in Ad and NA and increment in 5-HT while the level of DA was further increased. There was a marked inhibition in brain MAO and stimulation in brain and liver SDH activities following both types of stress. The extract restored MAO activity towards normalization whereas it facilitated stress-induced changes in SDH activities. These dose-dependent biochemical responses may be the possible mechanism of anti-stress activity of this plant extract (Bishayee et al.,1995).

2.2.7 Hepatic protein synthesis test

In 1992 scientist Mandal and his co-worker studied on the effect of *Mikania Cordata* root extract was evaluated on the rate of hepatic protein synthesis in vivo in CCl4-induced liver damage. Pre-treatment with the root extract (100 mg/kg, once daily for successive 5 days) showed a marked enhancement in the levels of hepatic DNA, RNA and protein content that were adversely affected with CCl4 treatment in the experimental mice. Increase in the total protein mass, fractional rate of protein synthesis (% of protein synthesized/day), total rate of protein synthesis (fractional rate x protein mass), ribosomal capacity (RNA/protein), ribosomal efficiency (rate/ribosome) and high turnover rate of protein (protein/DNA) in response to the pre-treatment of the root extract in hepatic tissue indicated the tissue repair

leading to a functional improvement of the hepatocytes that were disorganised with carbon tetrachloride intoxication(Mandal et al., 1992).

2.2.8 Anti-ulcer activity

In 2006 scientist S. Pal and his co worker studied on the methanolic fraction of the root extract *M. cordata* where it was found to possess significant anti-ulcer activity in different experimental models. In preventive tests the extract demonstrated significant protective action in gastric lesions induced by acetylsalicylic acid, serotonin and indomethacin in experimental rats. Significant protection was observed with the extract in chemically-induced duodenal lesions. Significant enhancement of the healing process was also found to occur in acetic acid-induced chronic gastric lesions in experimental animals (Pal et al., 2006).

Chapter-3
Materials &Methods

3. Materials and methods

3.1 Collection of plant

The plant was collected from Foridpur district of Bangladesh. A voucher specimen (Accession number: 38479) had been deposited at the Bangladesh National Herbarium. The proper time of harvesting or collecting is particularly important because the nature and the quantity of constituents very gently in some species according to the season.

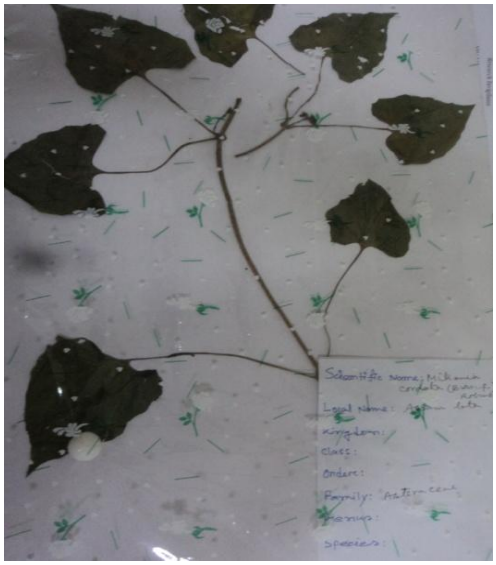


Figure 02: Herbarium sheet of *Mikania cordata*

3.2 Drying

Drying is the most common and fundamental method for post- harvest preservation of medicinal plants because it allows for the quick conservation of the medicinal qualities of the plants material in an uncomplicated manner. The plant was dried in room temperature and drying was completed within 2 to 3 weeks. By drying the plant materials one removes sufficient moisture to ensure good keeping qualities and to prevent molding the action of enzyme, the action of bacteria and chemical or other possible changes. Proper and successful

drying involved two main principles: control of temperature and regulation of air flow. The plant material can be dried by room temperature.

3.3 Cutting, Grinding and Sieving

The stem was cut into small pieces and placed in the grinding machine to make it fine powder. As the stem is too hard, very small amount of pieces were placed in the hopper with an average rotation. The grinded powder was collected very carefully.

3.4 Blending

Finally the powder was placed in blender machine in order to get more fine powder. During the entire blending process precautions were taken to avoid all kind of cross contamination and product loss.

3.5. Preparation of plant extraction

The whole part of the plant was dried in room temperature for approximately two weeks. Then the dried plants were taken into fine powder by using a grinding machine. Then the extraction process was done.

At first 2kg dried plant dust of *Mikania cordata* was soaked in 10L ethanol in four bottles. Then it was kept in room temperature for 3 days and everyday it was used to shake properly to ensure the maximum amount of constituents present in the grinded plant become soluble into ethanol. After 3 days later, the mixture was filtered. For filtration, white cotton cloth was used. After filtration two parts were obtained.

1. The residue portion over the filter
2. The filtered part

The filtrated part, which contains the substance soluble in ethanol, poured into a 1000 round bottle flask, and then the flask was placed in a rotary evaporator. The evaporation was done

at 53 degree Celsius temperature. The number of rotation per minute was selected as 125 RPM. The pressure of vacuum pump machine was 6 bars. The water flow through the distillation chamber was also provided in a satisfactory flow rate.

Figure 03: Rotary Evaporation

3.6 Crystal formation

After completing rotary crystal formation was occurred that was good in amount. These crystals are clear and stable. These crystals are not soluble in polar and not polar solvent and intermediate solvent. Further investigation will be continued to know about these crystals.

Figure 04: Formation of crystals from crude extract

3.7 Material and reagents

The materials used are:

1. Rotary Evaporator
2. Screw Cap Test Tube
3. Test Tubes
4. Vials
5. Beaker(Large, Medium And Small)
6. Round Bottomed Flask
7. Flat Bottomed Flask
8. Conical Flask
9. Volumetric Flask
10. Measuring Cylinder
11. Aluminium Foil Paper
12. Spatula And Glass Rod
13. Pipette
14. Pipette Pumper
15. Micropipettes
16. Pasteur Pipette
17. Blender Machine
18. Appendrof Test Tube
19. Micropipette
20. Micropipette Tip
21. Petri Dishes
22. Nutrient Agar
23. Agar Powder

24. Cotton Wool

25. Glass Container For Agar

26. Glass Container For Isotonic Solution

27. Gas Burner

28. Forceps And Tongs

29. Labels And Masking Tape

30. Filter Paper

31. Permanent Marking Pen

32. Glass Spreader

33. Autoclave

34. Laminar Air Flow

35. Incubator

36. Hot Air Oven

37. TLC Plate

38. TLC Tanks

39. TLC Paper Cutter

40. Scissor

41. Capillary Tubes

42. Ph Meter

43. Pencil And Pen

44. Scale

45. UV Lamp

46. Hot Plate

47. Tissue Paper

48. Oxygen Blower Device

49. Table Lamp
50. Magnifying Glass
51. Punch Machine
52. Analytical Balance
53. Glass Slides

The reagents involved are:

1. Ethanol
2. N- Hexane
3. Ethyl Acetate
4. Chloroform
5. Sulphuric Acid
6. Sodium Chloride Salt
7. Isotonic Solution (0.9% Sodium Chloride Solution)
8. Distilled Water
9. Sodium Hydroxide Solution
10. Tweed
11. Dimethyl Sulfoxide(DMSO)
12. 2,2-Diphenyl-1-Pecylhydrazyl(D PPH) Solution
13. Ascorbic Acid (Vitamin C) Tablet
14. Potassium Dichromate
15. Amoxicillin Capsule

3.8 Evaluation of antioxidant activity

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deletorious effect of free radicals in the human body and to prevent deterioration of fat and other constituents of foodstuff. In both cases, there is a preference for antioxidations from natural rather than from synthetic sources. There is therefore a parallel increase in the use of methods for methods for estimating the efficiency of such substance as antioxidant.

Oxidative stress involves a series of free radical chain reaction processes. It is associated with several types of biological damage, DNA damage, diabetes, respiratory tract disorders, carcinogenesis and cellular degeneration related to aging(Anderson et al.,2000). Continuous exposure to chemicals and contaminants may led to an increase in the amount of free radicals in the body beyond its capacity them and causes irreversible oxidative damage (Tseng et al.,1997).

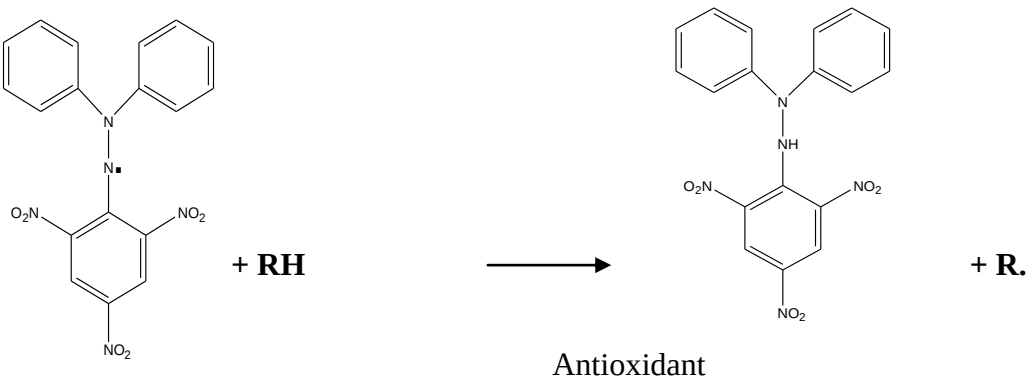
Plants (fruits, vegetables, medicinal herbs etc) may contain a wide variety of free radical scavenging molecules such as phenolic compounds (e.g. phenolic acids, flavonoids, quinines, coumarins, lignans, stilbenses, tannins), nitrogen compounds(alkaloid, amines, betalains), vitamins, terpenoids (including carotenoid) and some other endogenous metabolism which are rich in antioxidant activity. Epidemiological studies have shown that many of these antioxidant conpounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent(Owen et al.,2000).

The purpose of this study was to evaluate the antioxidant activity of *Mikania cordata* to find out a new potential source of natural antioxidant.

3.8.1. Principle of antioxidant test

The present study was aimed at evaluating the *in vitro* free radical scavenging activity of *Mikania cordata* using 1,1-diphenyl-2-picrylhydrazyl (DPPH) by the method of(Arpona *et al,2013*) 2.0 ml of a methanol solution of the extract at different concentration were mixed with 2.0 ml of a DPPH methanol solution (40µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of ascorbic acid by UV spectrophotometer.

The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorption of the DPPH radical at 517 nm reduces when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH (DPPH-H). DPPH radical scavenging activity is described as IC50 which is the concentration of samples to produce 50% reduction of the DPPH.



* DPPH = 1,1-diphenyl-2-picrylhydrazyl

Figure 05: Reaction of DPPH with reactive free radical

3.8.2. Preparation of DPPH solution

Calculation

M.W. of DPPH=394.32

So, for 0.1mmol/L amount of DPPH requires= (0.0001×394.32)gm

=0.039432gm of DPPH for 1Litre

Now,

1000ml solution requires 0.039432gm of DPPH

So, 50ml solution requires (0.039432×50)/ 1000gm of DPPH

=0.002 of DPPH or 2 mg of DPPH

Procedure

A dry 250ml conical flask was cleaned and covered with an aluminium foil protect its contents from light. Accurately weighted 1mg of DPPH placed in conical flask and 50ml methanol was added to prepare 0.1mmol/L or 40µg/ml DPPH solution.

3.8.3. Preparation of sample solution

Accurately weighted 10 mg of plant extract was taken into a vial and 2ml of methanol was added and the concentration of final solution is 5µg/µl

Seven test tube were taken and covered with an aluminium foil protect its contents from light and these test tubes were marked for different concentration by using stock solution and 2ml of methanol to each test tube.

3.8.4. Preparation of standard solution

Accurately weighted 10 mg of ascorbic acid as standard was taken into a vial and 2 ml of methanol was added and the concentration of final solution is 5 µg/µl

Seven test tube were taken and covered with an aluminium foil protect its contents from light and these test tubes were marked for different concentration by using stock solution and 2ml of methanol to each test tube.

3.8.5. Measurement of DPPH radical scavenging activity

2ml of a methanol solution of the extract at different concentration were mixed with 2ml of a DPPH methanol solution and this mixture was vigorously shaked and left at 25^{o}C for 30 minutes in the dark. After 30 minutes reaction period at room temperature in dark place the absorption was measured at 517nm of methanol as blank by UV spectrophotometer.

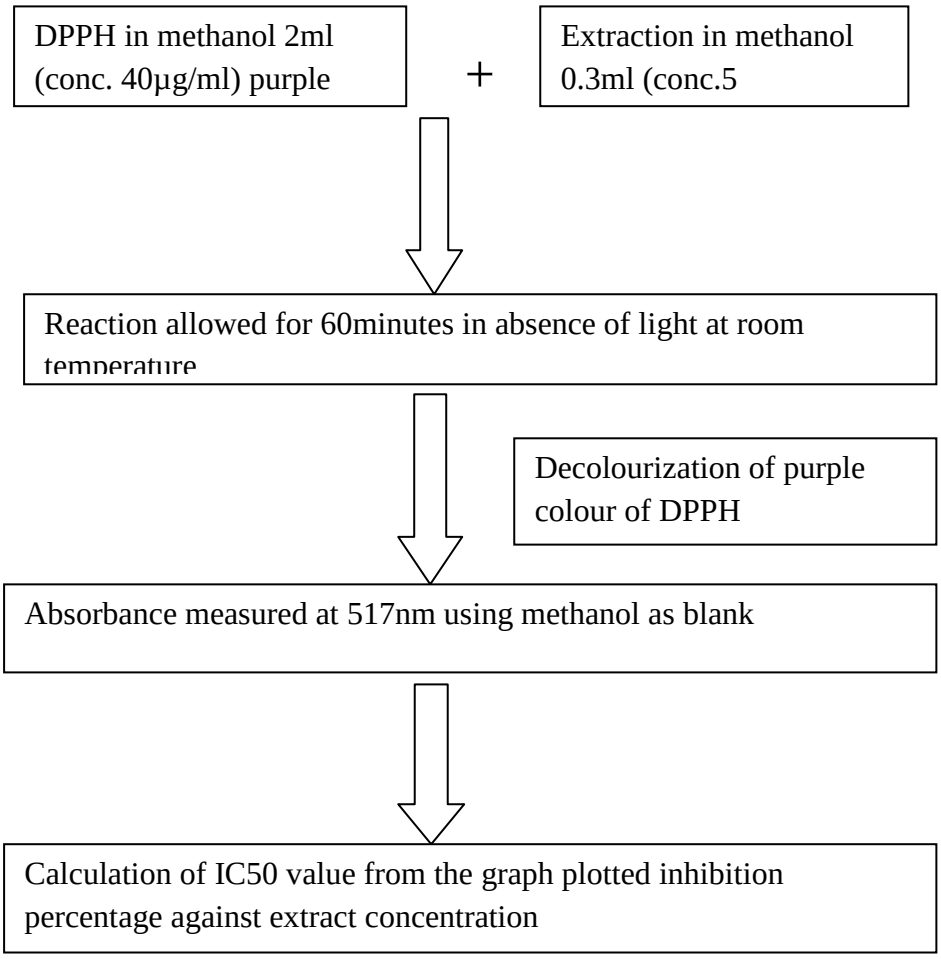


Figure 06: Schematic representation of the method of assaying free radical scavenging activity

3.9 Evaluation of cytotoxic activity

The pharmacological evaluation of substances from plants is an established method for the identification of lead compound, which can lead to the development of novel and safe medical agents. The in-vivo lethality in a sample zoological organism can be used as a convenient monitor for screening and fraction in the discovery and monitoring of bioactive natural products. *Artemia salina* lethality test such a test organism and developed a protocol for brine shrimp lethality bioassay to monitor cytotoxicity of a compound. (Meyer et al., 1982)

When screening for biological active plant constituents, the selection of the plant species to be studied is obviously a crucial factor for the ultimate success of the investigation. The utilization of bioassays for the monitoring of extracts, fractions and compounds obtained from plant, is frequently incorporated in Phytochemical research. The in vivo lethality in a simple zoological organism such as the brine shrimp (*Artemia salina)* lethality test (BST) might be used as a simple tool to guide screening and fractionation of physiologically active plant extract, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive (Meyer et al., 1982)

The importance of medicinal plants and traditional health systems in solving the heath care problems of the world is gaining increasing attention. Because of this resurgence of inerest the research on plants of medicinal importance is growing phenomenally at the international level, often to the detriment of natural habitats and mother population in the countries of origin. Bioactive compounds are almost always toxic in high doses. Thus, in vivo lethality in a simple zoological organism can be used as convenient monitor for screening and fractionation in discovery and monitoring of bioactive natural products. In order to study the

toxicity, we performed brine shrimp lethality bioassay which based on the ability to kill laboratory culture brine shrimp. The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity, detention of fungal toxins, heavy metal, pesticides and cytotoxicity testing of dental materials. It can also be extrapolated for cell-line toxicity and antitumor activity (Meyer et al., 1982).

This method is attractive because it is very simple, inexpensive, less time consuming and low toxin amounts are sufficiently to perform the test. In the present study ethanol extract of *Mikania cordata* were screened for their cytotoxic using brine shrimp lethality test.

3.9 .1 Principle of cytotoxic activity test

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in DMSO and by the addition of calculated amount of DMSO, desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in test tubes containing 5 ml of simulated sea water. Then samples of different concentrations are added to the premarket test tubes through micropipette. The assay is performed using three replicates. Survivors are counted after 24 hours. These data are processed in a simple program to estimate LC50 values with 95% confidence intervals for statistically significant comparisons of potencies.

Figure 07: Brine Shrimp

3.9.2 Preparation of sea water

38 gm sea salt (pure NaCl) was accurately weighed, dissolved in one litter of distilled water and filtered off to get clear solution. pH of the solution is adjusted to 8.5 by using 0.1N NaOH solution.

3.9.3 Hatching of brine shrimp

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. One day was allowed to hatch the shrimp and to be matured as nauplii. 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. With the help of a Pasteur pipette 10 living shrimps were added to each of the test tubes containing 5ml of seawater.

3.9.4 Preparation of test solutions with samples of experimental plant

Clean test tubes were taken. These test tubes were used for six different concentrations (one test tube for each concentration) of test samples and six test tubes were taken for standard drug Potassium dichromate for six concentrations of it and another three test tubes for control test. The test samples of 8mg were taken and dissolved in 200µl of pure dimethyl sulfoxide

(DMSO) in vials to get stock solutions. Then 100 µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 800 µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100 µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400 µg/ml,200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml after 6 dilutions.

3.9.5 Preparation of control group

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 possible factors are nullified. Usually two types of control groups are used -

i. Positive control
ii. Negative control

Preparation of the positive control group

Positive control in a cytotoxicity study is a widely accepted cytotoxicity agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, Potassium dichromate is used as the positive control. Measured amount of the Potassium dichromate is dissolved in DMSO to get an initial concentration of 20µg/ml from which serial dilutions are made using DMSO to get 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, and 0.625µg/ml. Then the positive control solutions are added to the pre-marked test tubes containing ten living brine shrimp nauplii in 5 ml simulated seawater to get the positive control groups.

Preparation of the negative control group

100 µl of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials showa rapid mortality rate, then the test are considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

3.9.6 Counting of nauplii and data analysis

After 24 hours, the test tubes were inspected using a magnifying glass and the number of survivors was counted. The percent (%) mortality was calculated for each dilution. The effectiveness of plant product is usually expressed as a median lethal concentration (LC_{50}) value. These represent the concentration of the chemical product death in half of the test subjects affect a certain exposure period.

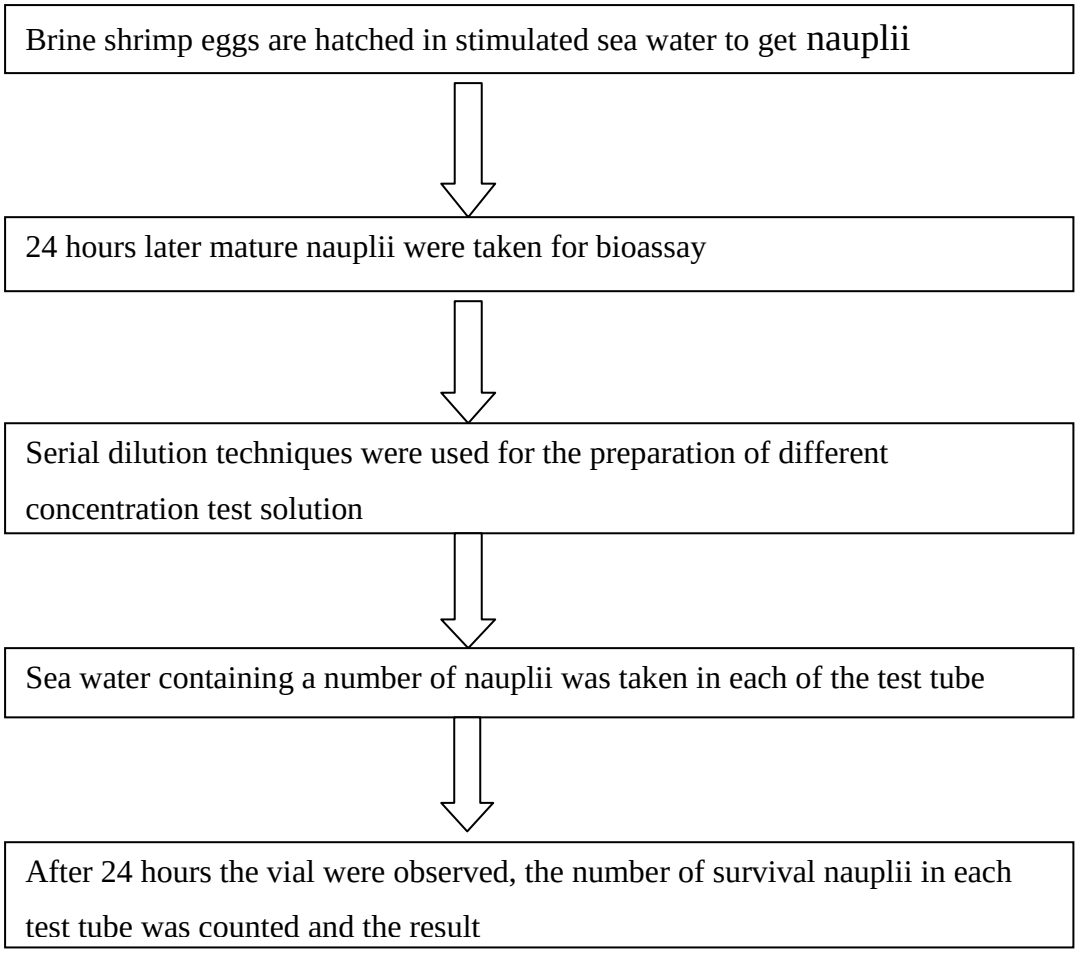


Figure 08: Schematic diagram of cytotoxic

3.10 Evaluation of antimicrobial activity

Bacteria are responsible for many infectious diseases. The increasing clinical importance of drug resistant bacteria pathogens has lent additional urgency to antibacterial research. The antibacterial screening which is the first stage of antibacterial research is performed to ascertain the susceptibility of various bacterial to any agent.

Antimicrobials are one of our most important weapons in fighting bacterial infections. However, over the past few decades, the health benefits offered by the antimicrobials are diminishing due to increased resistance by microorganisms, it is essential to investigate newer drugs with lesser resistance. Antimicrobial can be synthetic or can be isolated from the microorganism itself. But the implication of plant extract for the curing of microbial infection has been since the dark ages. And plant extract and their isolated compound are still valuable antimicrobials which are mostly used as lead compounds for the synthesis of an array of antimicrobials.

The use of herbs and medicinal plants were carried out by every culture on earth, through written or oral tradition. They relied on vast variety of natural chemicals found for their therapeutic properties. The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms.

3.10.1 Microbial operation

Microbes were cultured in nutrient agar at 37^{o}C.At first all Petri dishes, test tubes, appendrof micropipette tip, appendrof tube, spreader and other glass wares were cleaned and after that sterilized. Now for the cultivation of bacteria nutrient agar is required and to do so 2.8% agar solution is prepared and after that kept on autoclave machine and sterilized at 121^{o}C, after 1 hour all the materials were taken out and kept inside the luminary airflow. Prepared nutrient

agar was poured in each Petri dish and screw cap test tube then kept for few minutes to solidify. Agar poured in each dish such a way that each dish contain 15-20ml agar. Then from the test tube containing microbial strains, sub cultured was done in Petri dish and test tube. This was done by increasing a sterile inoculating loop inside the fresh culture then the loop containing microorganism was streaked across the surface of agar medium of both Petri dish and test tube in zigzag pattern. Before inoculation, the inoculation loop was exposed to the flame of burner to make it sterile. In this manner, one by one all the agar plates and test tubes were inoculated with microorganism was cultured in both test tube and Petri dish. If cultured was only created in petri dish then there were possibility of contamination so culture in test tube was option from where another subculture can be made in case of contamination . if only culture was done in Petri dish there was change of drying out due to large surface area of dish. After that for overnight incubation all the petri dishes and test tubes were placed in a microbiological incubator for the optimum growth of microorganism. After overnight incubation Petri dish and test tubes were taken out from incubator and stored in refrigerator for further use in in-vivo antimicrobial test.

Figure 09: Autoclave

Figure 10: Hot Air Oven

3.10.2 Preparation of culture media

First of all a bottle was taken clean then required amount (2.8% w/v) of agar was measured and poured into the bottle then distilled water was added to get required volume. Then the bottle is placed into the autoclave machine and here sterilized at 121^{o}C for 15 minutes. After that the bottle was taken out from autoclave machine and kept inside the laminar flow hood and poured on the appropriately labelled agar plate for each organism to be tested.

Ingredients	Quantity
Beef extract	3gm
peptone	5gm
Agar	15gm
water	100ml

Table 02: Formula of nutrient agar

3.10.3 Preparation of inoculum

1. Suspension of organism should be prepared and it has to make freshly prior to experiment. To get reliable result organism has to be in their log phase of growth and it is best to make subcultures of the organisms previous day of testing. Four to five isolated colonies are taken out by using a sterile inoculation loop from the culture

2. Then these colonies are suspended in 2 ml of sterile saline and then to distribute uniformly, vortexing is done.

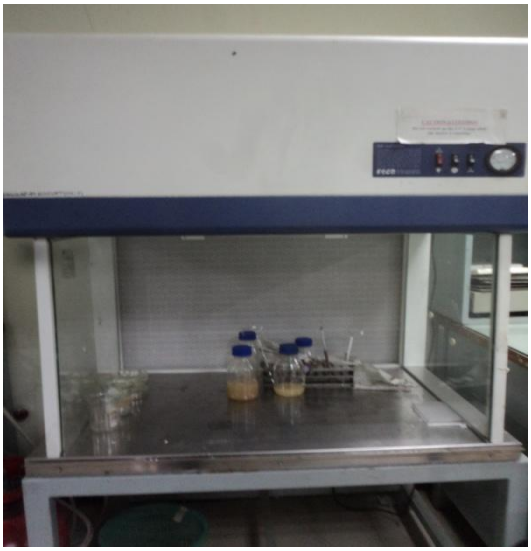


Figure11: Laminar Air Flow

3.10.4 Pouring media on plate

The way of pouring media on plate is as follows:

1. Agar is poured on the base of the petri dish and lid is kept open for a while that allows the excess water to evaporate from the surface of agar plate and make it dry.

2. Agar poured in each dish such a way that each dish contains 15-20 ml agar , and it is very crucial for the size of zone of inhibition since shallow layer of agar will produce a larger zone of inhabitation than a deeper layer.

3.10.5 Inoculation of the agar plate

1. First of all a sterile tip is placed on the micropipette and using this 100 µl suspension from the inoculums tube is taken and poured on the middle of the plate.

2. Then with the help of a spreader the drop of bacterial suspension is spread uniformly throughout the entire agar surface.

3. Then the micropipette tip is discarded.

4. The lid of the plate is kept open for a while to dry the agar surface.

5. After that antimicrobial agent is added to the plate it may be by doing hole in the agar of placing disk or other way.

Figure 12: Incubator

3.10.6 Incubation of the plates

1. Petri dishes are kept into incubator because it provides optimum environment condition ($32^{o}C+2^{O}C$) for the growth of microorganism.

2. Incubation of plates in CO_2 is not good for growth of microbes because it can decrease the P^{H} of the agar.

3. After overnight incubation (18-24hours) plates are kept outside of incubator and result is observed.

3.10.7 Measuring zone of inhibition

1. When the agar plates are kept outside the incubator the diameter of zone of inhibition is measured by using slide callipers in millimetre.

2. Usually measurement is done by naked eye and it is done by holding the plate a few inches above a black, non-reflecting surface illuminated with reflected light.

3. Result is recorded in result sheet. If the diameter of the zone cannot be read, then measurement from the centre of the disk to a point on the circumference of the zone where a district edge is present (the radius) is done and multiplied the measurement by 2 to determine the diameter.

4. Growth on the edges of the disk is considered as 0 mm.

3.10.8 Methods to detect antimicrobial activity

There are several methods to evaluate the antimicrobial test including

1. Disk diffusion method
2. Well dilution method

3. Minimum inhibitory concentration
4. Minimum microbial concentration

In our experiment we used both Disk diffusion method and well dilution method

3.10.8.1 Disk diffusion method

This method is used to determine the susceptibility or sensitivity of various microorganism agents. Here pathogenic microorganism are grown in laboratory setting and disk impregnated with antimicrobial agent are applied on the culture plate and growth of microorganism around the disk is observered, if no growth occur then the applied compound is considered as antimicrobial agent . in this method disk of approximately 6 mm in diameter is prepare from paper by using hole puncher and then put on autoclave machine for sterilization. When a 6-mm filter paper disk with placed on agar plate, upon placing the disk on the agar plate water is automatically absorbed into the disk the agar as result antimicrobial agent also diffuse into the surrounding agar. Here the concentration of antimicrobial agent remain highest near the disk due to the difference in rate of diffusion and the extraction rate of the antimicrobial agent out of the disk is higher than the rate of diffusion of antimicrobial through the agar so logarithmic reduction is concentration occurs as the distance from the increase. Solubility and molecular weight are the prime factor that influences the diffusion rate of the antimicrobial through the agar. For lager molecule diffusion rate is slower and quicker diffusion rate for smaller molecule. So that depending on those factor antimicrobial got unique breakpoint zone size that represent susceptibility of microorganism to that antimicrobial compound. Here for the preparation of inoculum McFalarland standards are used and here the turbidity of prepared bacterial suspension must be as like as 0.5 McFalarland standards. In the method prior to placing antimicrobial disk, agar plate is inoculated with a suspension of microbes to be tested, here simultaneous growth of the

microbes and diffusion of the antimicrobial agent occurs. Here growth of microbes is occurring if it has ability to resist the inhibitory effects of the antimicrobial compound. The depth of agar is important issue that can interfere with size of the zone of inhibition. Shallow layer of agar will produce a larger one of inhibition than a deeper layer.

3.10.8.1.1 Disk preparation

1. Disk is prepared from filter paper by using hole puncher, diameter of each disk is 6 mm

2. After the peroration of disk it is sterilized and then the disks are bring impregnated by the prepared solution of antimicrobial agent by the help of micropipette

3. Then these disks are placed in incubator to allow drying

3.10.8.1.2 Placement of the antibiotic disks

1. For the placement of disk few rules to be followed. Disk should not be placed to close of each other and should maintain a distance and it should be at least 24mm far away from each other, and maximum 12 disks should be placed on a 150-mm plate and 5disks on a 100-mm plate. If disk are placed too each other than measuring zone of inhibition is difficult.

2. Disk is placed on the agar plate by using a forceps which allows the disk to remain close to agar plate. Shape of the zone of inhibition should be affected by the disrupt edge of the agar plate.

3. Once all the disks are placed into plate it is kept into refrigerator for 2-3 hours so that the rapid diffusion of the antimicrobial agent can be take place.

4. After that the plates are taken out from the agar plate and placed inside the incubator for overnight incubation.

3.10.8.2 Well dilution method

In this method agar is poured on the plate allowed to dry, then freshly prepared inoculum suspension is poured on it then spread uniformly throughout the plate with help of sterile glass rod spreader. Then with the help of a sterile cork borer well of 0.5cm diameter was made in the agar plate. Depending on the plate size 9-12 wells are done and since different doses or fractions are applied so labelling is done or the wells are done by following special pattern that no need to label otherwise can be understand that which well contain what dose or fraction . Then in those well suspension or solution of antimicrobial compound is added and for the e diffusion of the applied antimicrobial compound in the agar media, plates are placed at room temperature for an hour. Then the plates are incubated for overnight. Then after 18 hours plates are taken out from incubator and zone of inhibition is measured (Kang et al., 2001).

Chapter-4
Result & discussion

4. Result and discussion

4.1 Antioxidant activity test

The ethanolic extract of *Mikania cordata* were subjected to free radical scavenging activity by the method of (Arpona et al., 2013) here, ascorbic acid was used as reference standard.

Absorbance of blank	**Concentration µg/ml**	**Absorbance of the extract**	**% of inhibition**	**IC_{50}**
0.675	5	0.665	1.48	216.62
	10	0.636	5.77	
	20	0.608	9.92	
	40	0.570	15.56	
	60	0.555	18.59	
	80	0.531	21.33	
	100	0.522	22.67	

Table 03: DPPH radical activity of plant extracts (*Mikania cordata*)

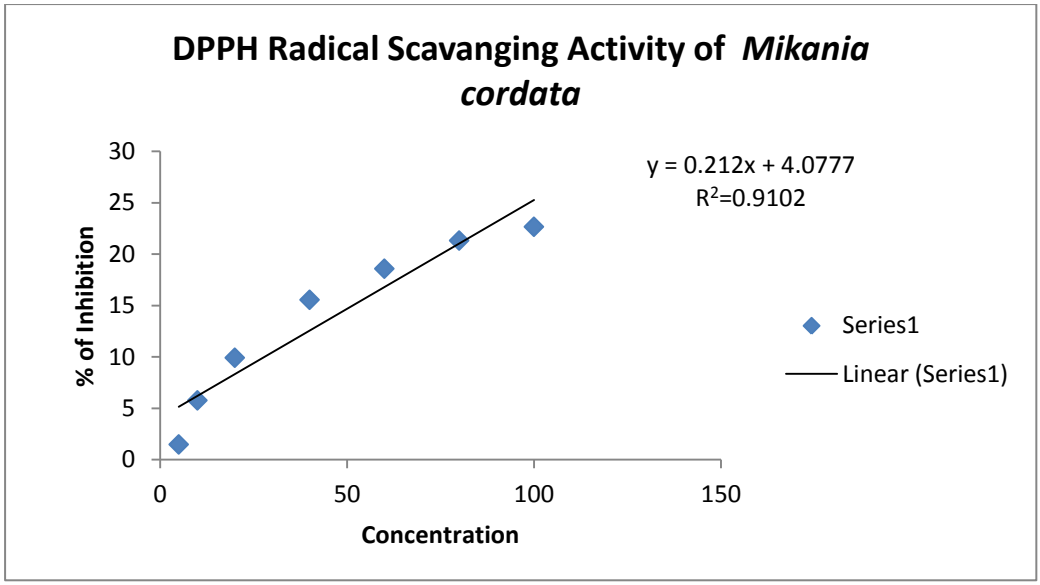


Figure 13: DPPH Radical Scavenging Activity of *Mikania cordata*

Absorbance of blank	Concentration µg/ml	Absorbance of the Ascorbic Acid	% of inhibition	IC_{50}
0.675	5	0.407	39.70	4.22
	10	0.347	48.59	
	20	0.236	65.03	
	40	0.116	82.28	
	60	0.031	95.41	
	80	0.015	97.78	
	100	0.012	98.22	

Table 04: DPPH radical activity of Standard (Ascorbic Acid)

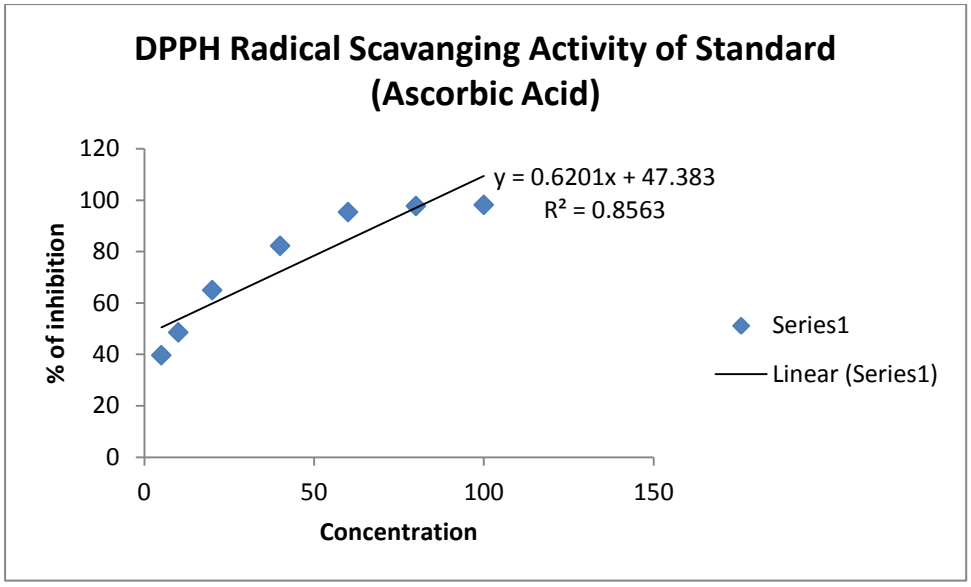


Figure 14: DPPH radical activity of Standard (Ascorbic Acid)

Discussion: The result of the tests is present in the following figure. The extract demonstrated an antioxidant activity by using DPPH Where DPPH radical activity of plant extract with an $IC_{50\ is}$ 216.62 µg/ml, so the antioxidant activity of the extract is not significant, it is mild.

4.2. Cytotoxic activity test

The concentration-mortality data were analyzed statistically by using probit analysis and linear regression using a simple PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value.

SL No	Conc. µg/ml	Log Conc.	Total No of Nauplii	No of Alive Nauplii	No of dead Nauplii	% of Mortality	LC_{50}	LC_{90}
1	20.00	1.30	10	0	10	100	1.04	9.95
2	10.00	1.00	10	1	9	90		
3	5.00	0.70	10	2	8	80		
4	2.50	0.40	10	3	7	70		
5	1.25	0.10	10	5	5	50		
6	0.625	-0.20	10	6	4	40		

Table 05: Effect of Potassium dichromate (positive control) on brine shrimp nauplii

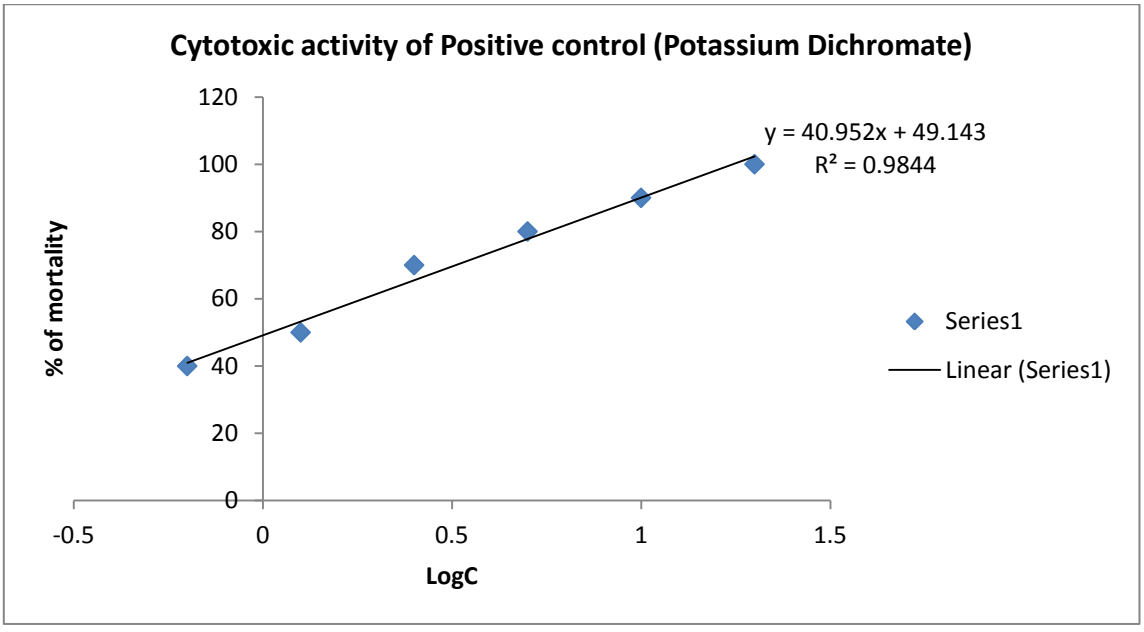


Figure15: Effect of Potassium Dichromate (positive control) on brine shrimp nauplii

SL No	Conc. µg/ml	Log Conc.	Total No of Nauplii	No of Alive Nauplii	No of dead Nauplii	% of Mortality	LC_{50}	LC_{90}
1	800	2.90	10	0	10	100	29.49	433.06
2	400	2.60	10	1	9	90		
3	200	2.30	10	2	8	80		
4	100	2.00	10	4	6	60		
5	50	1.70	10	4	6	60		
6	25	1.40	10	5	5	50		

Table 06: Effect of Ethanolic crude extract of *M. cordata* on brine shrimp nauplii

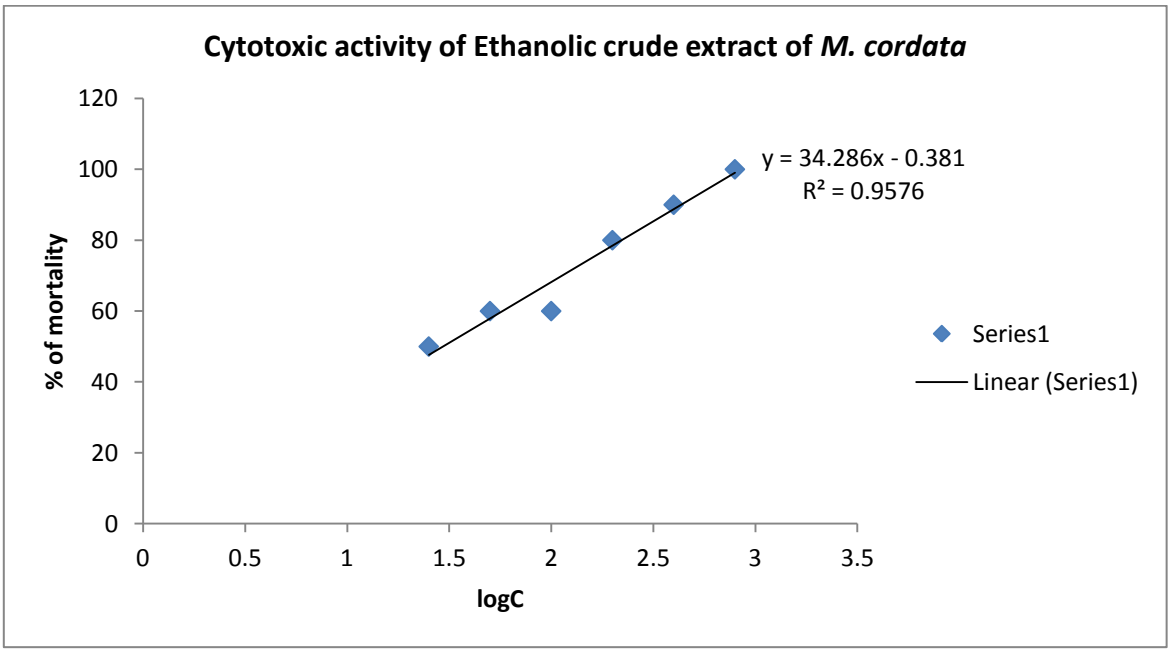


Figure16: Effect of Ethanolic crude extract of *M. cordata* on brine shrimp nauplii

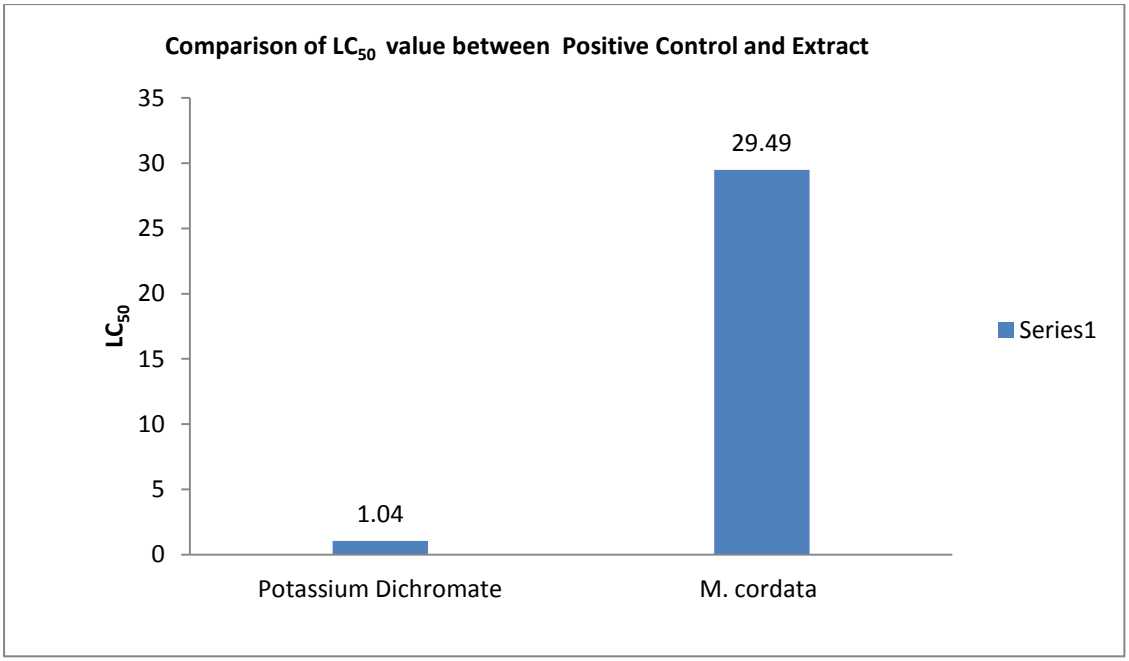


Figure17: Comparison between LC_{50} between Positive Control and Extract

Discussion

The result of the tests is present in the following figure. The extract demonstrated a cytotoxicity activity against Brine Shrimp Nauplii with an LC_{50} value of 29.49µg/ml. It shows moderate toxicity effect.Further research is needed on the determination of the cytotoxic activity of the plant.

4.3. Antimicrobial activity tests

Antimicrobial activity test against sixteen bacteria *Bacillus cereus,Bacillus megaterium, Bacillus subtilis, Salmonella paratyphi, Salmonella typhi, Vibrio parahemolyticus, Vibrio mimicus, Staphylococcus aureus ,Escherichia coli,Shigella dysenteria , Pseudomonas aeruginosa , Sarcina lutea,Shigella doydii, Saccharomyces cerevaceae, Candida albicanns, Aspergillus neiger* with plant crude extract *Mikania cordata*.

Concentration of Sample	Zone of Inhibition mm
200 µg/ml	N/A
400 µg/ml	N/A
800 µg/ml	N/A
1000 µg/ml	N/A
1200 µg/ml	N/A
1600 µg/ml	N/A
1800 µg/ml	N/A

Table 07: The concentration of sample and zone of inhibition

Discussion

Different concentration of sample of *Mikania Cordata* was used to observe antimicrobial activity test, but no significant inhibition was observed to any bacteria. Further research is needed on the determination of the antimicrobial activity of the plant.

Reference

Ahmed, M. Rahman, M.T., Alimuzzaman, M, Shilpi, J.A. (2001) Analgesic sesquiterpene dilactone from *Mikania cordata*. *Journal of Fitoterapia*, 72 (8) 919-921

Alves, T.M.A., Chaves, P.P.G., Santos, L.M.S.T., Nagem, T.J., Murta, S.M.F., Ceravolo, I.P., Romanha, A.J., Zani, C.L. (1995) A diterpene from *Mikania obtusata* active on *Trypanosoma cruzi*. *Planta Med* 61(3) 85-87.

Arpona, H., Shubhra, K. D., Ismet, A.J. (2013) Anti-inflammatory and antioxidant activities of ethanolic extract of aerial parts of *Vernonia patula* (Dryand.) Merr. *Asian Pac J Trop Biomed*, 3 (10) 798-805

Aguinaldo, A.M., Abe, F., Yamauchi, T., Padolina, W.G.(1995) Germacranolides of *Mikania cordata*. *Phytochemistry*, 38(6)1441-1443.

Bhattacharya, S. Pal and A. K. Nag Chaudhuri ((1992) Pharmacological Studies of the Antiinflammatory Profile of Mikania cordata (Burm) B. L. Robinson Root Extract in Rodents. *Journal of Phytotherapy Research*, 6(3)255-260

Bishayee A. and Chatterjee,M. (1995) Mechanism of Anti-stress Activity of Mikania cordata Root Extract in Albino Mice. *Journal of Elsevier Fitoterapia* 33 (3), 215-221.

Bishayee A.and Chatterjee, M. (1994) Anticarcinogenic biological response of *Mikania cordata*: reflections in hepatic biotransformation systems. *Journal of Elsevier Fitoterapia*, 81 (2) 193–200.

Bhattacharya, S., Pal, S., Chaudhuri, A.K.N. (1988) Neuropharmacological studies on *Mikania cordata* root extract. *Planta Medica*, 54(6)483-487

Barbosa, A.D., Ferreira, R.C.V., Valente, P. H. M. (1994) Atividade antimicrobiana de extratos fluidos de plantas medicinais brasileiras. *Lecta,* 12 (3) 153-163

Bohlmann, F., Adler, A., Schuster, A., Gupta. R.K., King, R.M., Robinson,H.(1981) Diterpenes from *Mikania species. Phytochemistry,* 20:1899-1902

Boyer, J., Liu, R.H. (2004) Apple phytochemicals and their health benefits. *Nutr J* 3:5.

Costa-Lotufo, L.V., Cunha, G.M.A., Farias, P. A. M., Viana, G.S.B., Cunha, K.M.A., Pessoa, C., Morais, M.O., Silveira, E.R., Gramosa, N.V., Rao, V. S. N.(2002)The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oleo-resin. *Toxicon,* 40(5)1231-1234.

Cunha, K.M.A., Paiva, L.A.F., Santos, F.A., Gramosa, N.V., Silveira, E.R,. Rao V.S.N. (2003) Smooth muscle relaxant effect of kaurenoic acid, a diterpene from *Copaífera langsdorffii* on rat uterus *in vivo. Phytotherapy Res,* 17(3)320-324

Facey, P.C., Peart, P.C., Porter, R.B.R (2010) The antibacterial activities of mikanolide and its derivatives. *W Indian Med J,* 59(3)249-252.

Ferreira, F.P., Oliveira, D.C.R. (2010) New constituents from *Mikania laevigata* Shultz Bip.ex Baker. *Tetrahedron Lett* , 51 (4) 6856-6859.

Gasparetto, J.C., Campos, FR., Budel, J.M., Pontarolo, R. (2010) *Mikania glomerata* Spreng. e *M. laevigata* Sch. Bip. ex Baker, Asteraceae:estudos agronômicos, genéticos, morfoanatômicos, químicos, farmacológicos, toxicológicos e uso nos programas de fitoterapia do Brasil. *Rev Bras Farmacogn,* 20(4)627-640.

Gutierrez, A.B., Herz, W. (1988) Bisabolones and other constituents of *Mikania shushunensis. Phytochemistry,* 27 (4) 3871-3874.

Ghani, A. (1998), *Medicinal plants of Bangladesh: Chemical constituents and uses.* Dhaka, Asiatic Society of Bangladesh.

Huang, H., Ye, W., Wei, X., Zhang, C. (2009) Allelopathic potential of sesquiterpene lactones and phenolic constituents from *Mikania micrantha* H.B.K. *Biochem Syst Ecol* 36(4)867-871

Herz, W., Santhanam, P.S. (1969) Isolation,structure and synthesis of 4',5,6,7-tetrahydroxy-3'-methoxyflavone (Batatifolin), a new flavone from *Mikania batatifolia* DC. *Tetrahedron Lett* ,39 (4) 3419-3421.

Kiang, A.K., Sim, K.Y., Yoong, S.W. (1968) Constituents of *Mikania cordata* (Burm. F.) B. L. Robinson (Compositae)-II. *Phytochemistry.* 7(2)1035-1037

Kiang, A.K., Sim, K.Y., Goh, J. (1965) Constituents of *Mikania cordata* (burm. f.) B. L. Robinson (compositae). Isolation of mikanin, epifriedelinol, and fumaric acid; the structure of mikanin. *J Chem Soc*: 6371-6374.

Mandal PK, Bishayee A, Chatterjee M (1992) Stimulation of hepatic protein synthesis in response to Mikania cordata root extract in carbon tetrachloride-induced hepatotoxicity in mice. *The Italian Journal of Biochemistry*, 41(6)345-351

Meyer, B., Ferrigni, R., Putnam, E., Jacobsen, B., Nichols, E., Mclaughlin,L. (1982) Brine shrimp : a convenient general bioassay for active plant constituents. *Planta Medicine,* 45(2) 31-34

Marcy J., Balunas, A., Douglas, K.(2005) Drug discovery from medicinal plants *Life Sciences* 78 (2) 431 – 441

Oliveira, F.D. (1972) Contribuição para o estudo botânico de *Mikania hirsutissima* DC. *var.hirsutissima*. II. Morfologia externa e anatomia da folha, flor, fruto e semente. *Rev Farm Bioquim,* 10(3)15-36.

Oliveira, P.A., Turatti, I.C.C., Oliveira, D.C.O.(2006).Comparative analysis of triterpenoids from *Mikania cordifolia* collected from four different locations. *Rev Bras Cienc Farm,* 42(5)547-552.

Owen W ., Giacosa, W ., Hull, E ., Haubner, R., Spiegelhalder, B., Bartsch, H.,(2000) The antioxidant /anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer,* 36(10) 1235-1247

Oliveira, F., Alvarenga, M.A., Akisue, G., Akisue, M.K. (1984) Isolamento e identificação de componentes químicos de*Mikania glomerata* Sprengel e de *Mikania laevigata* Schultz Bip. ex Baker. *Rev Farm Bioquim,* 2(2)169-183.

Pal, S., Bhattacharya, S. Chaudhuri, A. K.N. (2006) 'The effects of *Mikania cordata* (Burm) B. L. Robins. Root extract on gastro-duodenal ulcer models in rats and guinea pigs' *Journal of Phytotherapy Research*, 2 (4)180–182

Pereira, N.A., Pereira, B.M.R., Nascimento, M.C., Parente, J.P., Mors, W.B. (1994) Pharmacological screening of plants recommended by folk medicine as anti-snake venom. IV. Proction against jararaca venom by isolated constituints.*Planta Medica* ,60(4)99-100.

Soares, A.P., Nascimento, A.M., Taleb-Contini, S.H., Oliveira, D.C.R. (2007) Constituents of *Mikania lasiandrae. Chem Nat Compd* 43(4)708-709.

Sekendar A.Saiful, I., Masudur, R ., Rabiul, I . Mohammed ,A .S. and Rafi, k. I.(2011) Antibacterial and Cytotoxic Activity of Ethanol Extract of *Mikania Cordata* (Burm.F.) B. L. Robinson Leaves. *Journal of Basic and Clinical Pharmacy*, 002(002) 103-107

Tseng H., Kao S., Chu Y., Chou P., Lin W., and Wang J., (1997) Protective Effects of Dried Flower Extracts of *Hibiscus sabdariffa* L. Against Oxidative Stress in Rat Primary Hepatocytes. *Food Chemistry and Toxicology*, 35(4) 1159-1164

Vichnewski, W., Filho, H.F.L., Murari, R., Herz, W.(1977) Cinnamoylgrandifloric acid from *Mikania oblongifolia. Phytochemistry,* 16(6)2028-2029

Wei, X., Huang, H., Wu, P., Cao, H., Ye, W. (2004) Phenolic constituents from *Mikania micrantha. Biochem Syst Ecol,* 32(5)1091-1096.